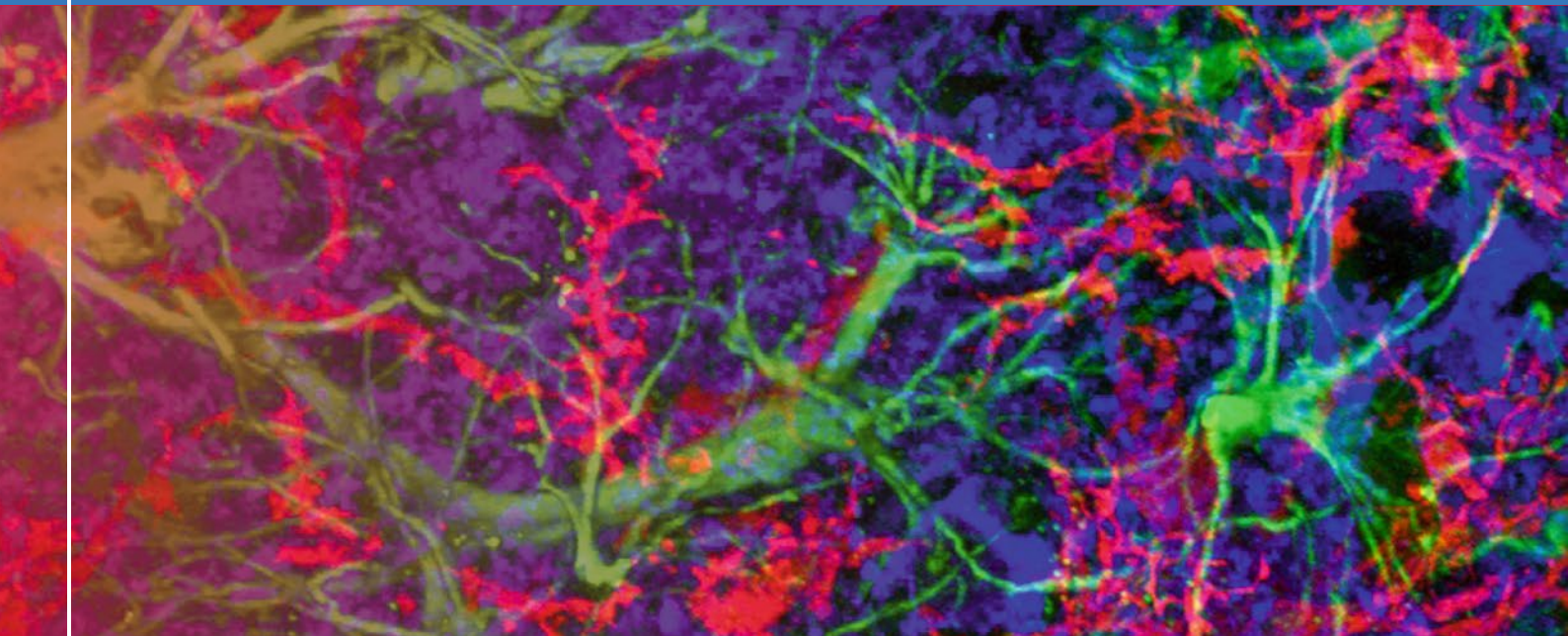


Tsuneya Ikezu
Howard E. Gendelman *Editors*



Neuroimmune Pharmacology

Second Edition

Serge Przedborski · Eliezer Masliah · Marco Cosentino
Associate Editors

 Springer

Neuroimmune Pharmacology

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Preface

In the past three decades, enormous strides have been made in our understanding of the relationships between inflammation, immune responses, and degenerative human diseases. Neuroinflammation has grown from a tiny beginning to its current status as the largest field of brain research. Its growth continues to be rapid so this dominance will continue to expand. The field commenced with the obscure identification of activated microglia in the brains of Alzheimer's disease cases. A grant application of ours to the Canadian government to explore the ramifications of this finding was turned down with the terse comment: "This hypothesis is ridiculous!"

To grasp the dimensions of growth in this field, and to gain a perspective of future opportunities, today's neuroscientists only need to read this volume from cover to cover. The developing information has mostly appeared in specialty journals that have dealt only with isolated aspects of these tightly related fields. As a result, contemporary scientists have had a difficult time finding sources, even in review articles, that provide an integrated picture. This updated volume, by assembling chapters that demonstrate the relationship between these historically separated fields, overcome that difficulty. There are 56 chapters which cover a broad spectrum of topics on immunology of the nervous system. Included are diseases that result from immunological dysfunction, current therapeutic approaches, and prospects for the future. Overall, it integrates cutting-edge neuroscience, immunology, pharmacology, neurogenetics, neurogenesis, gene therapy, adjuvant therapy, nanomedicine, pharmacogenetics, biomarkers, proteomics, and magnetic resonance imaging. It is a rich harvest and readers will gain a perspective that has not previously been so readily available. Exposure to such a wealth of ideas is bound to inspire readers to undertake new and productive research initiatives.

The modern era of research into neuroinflammation and its relationship to neurodegenerative diseases began in the 1960s with the elaboration by Ralph van Furth of the monocyte phagocytic system. He injected labeled monocytes into animals and followed their migration and maturation into resident phagocytes in all body tissues. This provided a closure between Metchnikoff's 1882 discovery of mesodermal attack cells in starfish larvae, which he named phagocytes, and del Rio Hortega's 1919 discovery of phagocytic mesodermal cells entering the brain, which he named microglia. Hortega's results had always been questioned, and for more than two further decades, the controversy continued as to whether microglia were truly phagocytes of mesodermal origin or were merely typical brain cells of epidermal origin. Resolving the controversy required development of the techniques of immunohistochemistry and monoclonal antibody production. These tools for exploring brain biochemistry at the cellular level opened new vistas for understanding brain functioning and the pathogenesis of human disease. Using these tools, our laboratory and that of Joseph Rogers in Sun City at that time demonstrated that HLA-DR was strongly expressed on activated microglia. The identification of HLA-DR, a well-known leukocyte marker displayed by antigen-presenting cells, on these cells vindicated both Hortega and van Furth. The way was paved for many productive investigations exploring the properties of microglial cells and their relationship to inflammation and immune responses. This example of a conjunction between a fundamental concept and technical advances to establish its validity has been repeated many times since, as the chapters in this volume illustrate.

For a time, the concept that the brain is immunologically privileged held sway among neuroscientists. This was based on a narrow view that only the invasion of brain by lymphocytes could be taken as evidence of an inflammatory response. But immunohistochemistry, coupled with newly developed molecular biological techniques, revealed that a spectrum of inflammatory mediators, including many known to cause tissue damage, was produced within the brain by resident brain cells. These discoveries required entirely new interpretations as to the nature of neuroinflammation and its relationship to immune responses. The innate immune system, operating at the local level in brain, has clearly proved to be the first line of defense. Indeed, the basic discoveries from studying the response of the brain in a variety of neurological diseases are causing a reevaluation of a number of peripheral degenerative disorders where innate immune responses, which had previously been ignored, have been shown to play a critical role in their pathogenesis. In other words, those studying the brain are providing immunologists with revolutionary new concepts regarding classical peripheral diseases. The insights of this volume need to be interpreted in this broader context.

Major neurologic disorders and details of their pathobiology are presented as individual chapters. They involve disorders where innate immune responses predominate, as in Alzheimer's disease, to others such as multiple sclerosis, where adaptive immune responses predominate, and others which seem to involve both. We have suggested that diseases involving self-damage generated by innate immune responses be defined as autotoxic to differentiate them from classical autoimmune diseases where self-damage is generated by adaptive immune responses. The common theme, however, is the involvement of microglia as the effector cells.

Genetics is well covered. It is a rapidly moving field. The methodology for linking familial disease to DNA mutations commenced in the late 1970s through identification of restriction fragment-linked polymorphisms. By 1983, when James Gusella and his colleagues demonstrated a linkage of the G8 fragment to Huntington disease, only about 18 markers were known. Now over 1,500,000 single-nucleotide polymorphisms have been localized so that every centimorgan of the human genome can be explored. This advance has been coupled with rapid methods for sequencing DNA. The report on genetics must be regarded as the tiny tip of a giant iceberg where much below the surface will soon be revealed.

The ultimate objective of neuroscientists studying human disease is to find more effective treatments. Part 3 covers the pharmacology of existing drugs, as well as describing approaches now in clinical evaluation, and those still at the bench level. Some of these include concepts that depart from established therapeutic approaches, giving the reader much food for thought.

There is an important chapter on the new field of biomarkers. Biomarkers have established that neurodegenerative disorders such as Alzheimer's disease commence at least a decade before clinical signs develop. A window of opportunity is opened up where early anti-inflammatory intervention can arrest disease progression and abort disease development. This can be combined with brain imaging to measure objectively the effects of therapeutic agents in diseases where progressive brain degeneration occurs.

In summary, this is a volume not to be put on the shelf as a reference text, but to be read cover to cover by aspiring neuroscientists.

Introducing Neuroimmune Pharmacology

Neuroscience, immunology, and pharmacology are each of and by themselves broad disciplines that, without argument, impact upon a large component of what we come to know as biomedical science (Elenkov et al. 2000; Gendelman 2002; Holzer et al. 2015; McGeer and McGeer 2004). Each, by themselves and even more so when put together, is multidisciplinary and require, for the student, both a broad knowledge and deep understanding of molecular and cellular biology, neuroimmunity, the functional blood–brain barrier, neurochemistry, neuroinfectious and neurodegenerative disorders, cancer, and neurodevelopment. For many, it is considered a branch of immunology, but that is a start point as the field bridges investigations of drug action and development with nervous system biology and disease pathogenesis. To those engaged in this field, linking of the disciplines is ever more challenging as when they are joined, they come interactive. The bridges between disciplines are what we now call multidisciplinary science and require another level of insight. However, we posit that each need be first understood as a single entity. To this end, we forged chapters that cover the structure, function, and biology of each of the fields independent of one another. Then step-by-step they are each combined one with the other to form the basis of our engagement with the environment, to disease and a means to restore homeostasis by protective immune and pharmacologic means. Indeed, drugs that include immune modulators can certainly influence organ function, aging, and tissue homeostasis and improve clinical outcomes.

Indeed, a special feature of “humankind” among other species is the presence of an extraordinary complex immune system that can be used to protect against a plethora of harmful microbial pathogens, including viruses, bacteria, and parasites, as well as abnormal cells and proteins (Petranyi 2002; Obermeier et al. 2016). This underlies the complexity of the human genome which encodes expansive immune-related genes not found in lower species (Hughes 2002). When the immune system is compromised, disease occurs and often does with ferocity; a wide range of clinical manifestations ensue that follows as a consequence of neurodegenerative, psychiatric, cancer, and infectious diseases, or those elicited by the immune system’s attack on itself. The latter is commonly referred to as “destructive” autoimmunity (Christen and von Herrath 2004). Interestingly enough, the immune system may sometimes be an impediment to therapy. Indeed, modulating its function is required for long-term and successful organ transplantation (Samaniego et al. 2006; Horst et al. 2016). On balance, modulation of the immune system can affect “neuroprotective” responses for certain diseases (Anderson et al. 2014).

Like the immune system, the nervous system contains surveillance functions and also possesses a number of functional roles that include mentation, movement, reasoning, sensation, vision, hearing, learning, breathing, and most behaviors. The nervous system includes defined tissue structures such as the brain, spinal cord, and peripheral nerves. On the cellular level, it includes networks of nerve cells with a variety of functional activities; complex networks and communications; supportive and regulatory cells, called glia; and a protective barrier that precludes the entry of a variety of macromolecules, cells, and proteins. It also possesses connections throughout the body that permits it function. Neuroscience is the discipline used to explore each of the nervous system regions and cells that include their networks and modes of communication in health and disease. As humans, we have ~100 billion neurons that are each

functional units contained within the nervous system. Molecular and biochemical studies along with cell and animal systems were each used alone and together to explore and define neural biology. The task of neurosciences is to better understand the brain's function in the context of ontogeny, organism development, and aberrations during disease. Neuroscience is an interdisciplinary field and evolved as such during the past quarter-century. It includes neurobiology, neurochemistry, neurophysiology, mathematics, psychology, computer neuroscience, and learning and behavior. It also included the field of immunology (Sehgal and Berger 2000). In a historical context, the brain, for a long time, was considered an immune-privileged organ, meaning that its protective shield or barrier, commonly termed the blood–brain barrier, served to protect, defend, and as a consequence exclude ingress of toxins, cells, and pathogens (Streilein 1993; Becher et al. 2000). This has undergone a reassessment of purpose (Louveau 2015). Nonetheless, this is balanced by the fact that resident inflammatory cells do exist inside the brain and are capable of producing robust immune responses. In recent years, we have come to accept that it is the mononuclear phagocytes (MP; perivascular macrophage and microglia) that are disease perpetrators, while the astrocyte serves as “supportive” and “homeostatic” in nurturing neurons and in protection against the ravages of disease (Gendelman 2002; Simard and Nedergaard 2004; Trendelenburg and Dirnagl 2005). The neuron in this neuroimmune model is the passive recipient of the battles that rage between the MP and the astrocyte. Findings that have emerged over the past half-decade have challenged this model. We now know that dependent upon environmental cues and disease, microglia, astrocytes, and other neural cell elements including endothelial cells and oligodendrocytes possess immunoregulatory functions. We also know that microglia and astrocytes dependent upon the environment and stimuli can be supportive, destructive, or both. Even more importantly, neurons can secrete immunoregulatory factors and engage directly into cell–cell–environmental cue stimulations. To make the system perhaps even more complex, local neuroimmune processes can result in the recruitment of T cells and enticement of the adaptive immune response, significantly affecting disease outcomes (Olson and Gendelman 2016). All in all, things appear more complex than once thought even in the past decade.

These three disciplines and the complexities inherent in each academic field is perhaps the most multidisciplinary, serving to bring scientists and clinicians together with knowledge of neurobiology, immunology, pharmacology, biochemistry, cellular and molecular biology, virology, genetics, gene therapy, medicinal chemistry, nanomedicine, proteomics, pathology, and physiology. Even more than immunology and neuroscience, pharmacology integrates a broad knowledge in scientific disciplines, enabling the pharmacologist a unique perspective to tackle drug-, hormone-, immune-, and chemical-related pathways as they affect human health and behavior. Drug actions and therapeutic developments form the basis of such discoveries, but the central understanding of how they act provides vision for further research to improve human well-being and health.

This textbook is unique in scope by serving to investigate the intersection of this new discipline. Neuropharmacologists study drug actions including neurochemical disorders underlying a broad range of diseases such as psychiatric disorders (e.g., schizophrenia and depression) and neurodegenerative diseases (e.g., Alzheimer's and Parkinson's diseases). Drugs can also be used to examine neurophysiological or neurobiochemical changes as they affect brain, behavior, movement, and mental status. Immunopharmacology seeks to control the immune response in the treatment and prevention of disease. Research does include immunosuppressant agents used in organ transplant as well as developing agents that affect bone marrow function and cell differentiation in cancer therapies.

What then defines the field of *neuroimmune pharmacology*? Is it simply a field that intersects the three disciplines of neuroscience, immunology, and pharmacology in seeking to better define the epidemiology, prevention, and treatment of immune disorders of the nervous systems (Fig. 1)? Are these disorders limited in their scope in affecting behavior, cognition, motor, and sensory symptoms, or do they also involve developmental and degenerative disorders? It is clear that the immune system is linked, in whole or in part, to diseases that develop

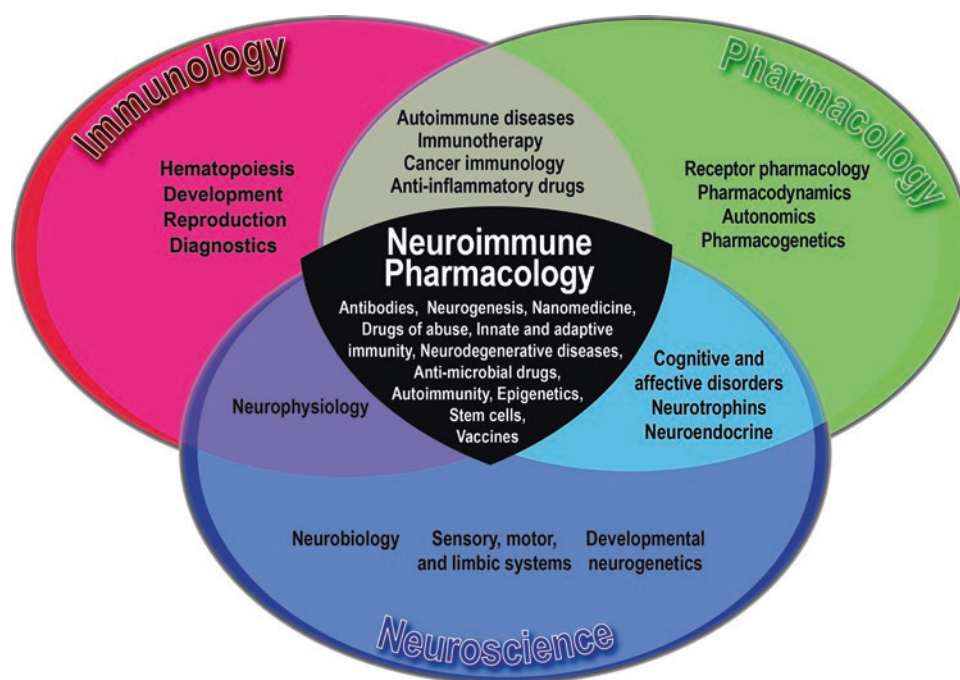


Fig. 1 This Venn diagram pictorially represents the fields of immunology, pharmacology and neuroscience with common elements overlapping into the discipline of neuroimmune pharmacology. The blackened area depicts the diseases and research areas covered in this book and integral to the field of study

as a consequence of genetic abnormalities and a broad range of environmental cues (including microbial infections and abused drugs) and to toxins. So, where does neuroimmune pharmacology find its niche? These can occur, in part, as a consequence of neuropeptides, neurotransmitters, cytokines, chemokines, and abused drugs. Like much in science, we are left with more questions than answers. In the end, we seek avenues for translational research and better understanding of disease mechanisms. Diseases are together linked to microbial agents, by inflammatory processes, by emergence of cancerous cells or tumors, by stress, by environmental cues, and by genetic disturbances. No matter the cause, harnessing the immune processes for pharmacological benefit will, at days end, provide “real” solutions to positively affect some of the most significant and feared disorders of our century.

What do we seek to accomplish by editing such a textbook? First, we would be remiss in not acknowledging the pivotal discoveries made by others when research fields intersect. These include the discovery and characterization of the guanosine triphosphate (GTP) binding and prion proteins (Gilman 1995; Rodbell 1995; Prusiner 1998, 2001), neurotransmission and memory functions (Carlsson 2001; Greengard 2001; Kandel 2001), and odorant receptors (Buck 2000; Axel 2005). We posit that new discoveries can and will be made through the intersections of neuroscience, immunology, and pharmacology and as such sought to define it for the student. The notion that inflammation contributes in significant manner to neurodegeneration and significantly beyond autoimmune diseases is brought front and center and demonstrated without ambiguity for multiple sclerosis, peripheral neuropathies, Alzheimer’s and Parkinson’s disease, and amyotrophic lateral sclerosis as well as for microglial infections of the nervous system including NeuroAIDS where microglial activation is central to disease processes (Hooten et al. 2015; Appel et al. 1995; Toyka and Gold 2003; McGeer and McGeer 2004; Ercolini and Miller 2005; Gendelman 2002; Gendelman and Mosley 2015). Perhaps most importantly, we have laid the groundwork for how the immune system can be harnessed either through its modulation, through altering blood–brain barrier integrity and function, or by drug-delivery strategies that target the brain. This second edition will add on to the success

of the first edition with additional chapters on emerging topics, including but not limited to enteric nervous system, microbiota, innate immunity signaling, exosomes, stress granules, microRNA, autism spectrum disorders, traumatic brain injury, biomarkers, macromolecular therapeutics, and “omics” pharmacology.

No doubt this textbook is an expansive read for the student and scholar alike. To this end, we are humbled by its realization and even more so during this second edition. These words lay only the beginnings to what we believe will be a significant future footprint into the integration between neuroscience, immunology, and pharmacology.

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Abbreviations

¹ H	Proton
¹ H-MRSI	Proton magnetic resonance spectroscopic imaging
2D SDS-PAGE	Two-dimensional polyacrylamide gel electrophoresis
3'UTR	3'-Untranslated region
3HK	3-Hydroxykynurenine
5-ASA	5-Aminosalicylic acid
5-HIAA	5-Hydroxyindole acetic acid
5-HT	5-Hydroxy tryptophan
5-HT	5-Hydroxytryptamine
6-OHDA	6-Hydroxydopamine
8-OHdG	8-Hydroxy-2'-deoxyguanosine
γc	Common γ-chain
AAAD	Aromatic L-amino acid decarboxylase
AAV	Adeno-associated virus
Ab	Antibody
Aβ	Amyloid-β
ABD	Adamantiades-Behçet's disease
ABP	Actin-binding protein
AC	Anterior chamber
ACAID	Anterior chamber-associated immune deviation
αCamKII	α-Calcium/calmodulin-dependent protein kinase II
ACh	Acetylcholine
AChE	Acetylcholinesterase
ACTH	Adrenocorticotrophic hormone (pro-opiomelanocortin; POMC)
AD	Alzheimer's disease
ADHD	Attention deficit hypersensitivity disorder
ADI	Acceleration/deceleration injury
ADAM	A disintegrin and metalloprotease
ADCC	Antibody-dependent cellular cytotoxicity
ADEM	Acute disseminated encephalomyelitis
AF	Activation factor
Ag	Antigens
AGE	Advanced glycation end products
AGM	Aorta-gonad mesonephros
AHSCT	Autologous hematopoietic stem cell transplantation
AICA	Anterior inferior cerebellar artery
AID	Activation-induced (cytidine) deaminase
AIDP	Acute inflammatory demyelinating polyradiculoneuropathy
AIDS	Acquired immunodeficiency syndrome
AIF	Apoptosis inducing factor
AIR	Autoimmune retinopathy
AIRE	Autoimmune regulator

AIS	Anterior chamber-associated immune deviation (ACAID)-inducing signal
ALR	AIM2-like receptors
ALS	Amyotrophic lateral sclerosis
AMAN	Acute motor axonal neuropathy
AML	Acute myeloid leukemia
AMN	Adrenomyeloneuropathy
AMPA	α -Amino-3-hydroxy-5-methylisoxazole-4-propionic acid
AMSAN	Acute motor-sensory axonal neuropathy
ANG	Angiotensin II
ANI	Asymptomatic neurocognitive impairment
ANS	Autonomic nervous system
AP	Area postrema
AP-1	Activating protein-1
APAF 1	Apoptotic peptidase activating factor 1
APC	Antigen-presenting cells
aPKC	Atypical protein kinase C
aPL	Antiphospholipid antibody
APO1	Apoptosis antigen 1 (Fas/CD95)
apoE	Apolipoprotein E
APP	Amyloid precursor protein
APPs	Acute phase proteins
AQP4	Aquaporin-4
ARE	AU-rich response element
ARMD	Age-related macular degeneration
ART	Antiretroviral therapy
ASD	Autism spectrum disorders
ASL	Arterial spin-labeled
ASTIN	Acute stroke therapy by inhibition of neutrophils
AT	Adoptive transfer
ATL	Adult T cell leukemia
ATON	Atacicept in optic neuritis
ATP	Adenosine triphosphate
AVE	Anterior visceral endoderm
AVG	Anti-viral granule
AVP	Arginine vasopressin
AV3V	Anteroventral third ventricular
AZT	3'-Azidothymidine/zidovudine
BACE	Beta-site amyloid precursor protein cleaving enzyme
BBB	Blood-brain barrier
BBMEC	Bovine brain microvessel endothelial cells
BCR	B-cell receptor
BCRP	Breast cancer resistance protein
BCSFB	Blood-CSF barrier
BD	Bipolar disorder
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor (FGF2)
bHLH	Basic helix-loop-helix
BHV-1	Bovine herpesvirus-1
BLBP	Brain lipid binding protein
BLIMP-1	B lymphocyte-induced maturation protein-1
BLV	Bovine leukemia virus
BM	Bone marrow
BMDM	Bone marrow-derived macrophages

BMP	Bone morphogenetic protein
BMVEC	Brain microvascular endothelial cell
BP	Biological processes
BrdU	Bromodeoxyuridine
BRMs	Biological response modifiers
BSE	Bovine spongiform encephalopathy
BTLA	B and T lymphocyte attenuator
C/EBP	CCAAT box/enhancer binding protein
C3d	Complement C3 fragment d
C4d	Complement C4 fragment d
CA	Cornu ammonis
CA II	Carbonic anhydrase II
CaMKII	Calcium/calmodulin-dependent protein kinase II
CAMs	Cell adhesion molecules
CAPS	Cryopyrin-associated periodic syndromes
CAR	Cancer-associated retinopathy
CARD	Caspase recruitment domain
CARD15	NOD2/caspase recruitment domain 15
CB	Cannabinoid
CB1	Cannabinoid receptor 1
CB2	Cannabinoid receptor 2
CBA	Cytokine bead arrays
CBF	Cerebral blood flow
CBF1	C promoter binding factor 1
CBP	cAMP-response element binding protein (CREB)-binding protein
CBV	Cerebral blood volume
CCI	Controlled cortical impact
CCK	Cholecystokinin
CCT	Central corneal thickness
CD	Cluster of differentiation
CD11b	Complement component 3 receptor 3 subunit (integrin alpha M; ITGAM)
CD40L	CD40 ligand (TNFSF5)
CDP	Common DC progenitor
CDR	Complementarity-determining region
CDV	Canine distemper virus
CEP	Carboxyethylpyrrole
CFT	2- β -Carbomethoxy-3 β -(4-fluorophenyl) tropane
cGMP	Cyclic guanosine 5'-monophosphate
CGRP	Calcitonin gene-related peptide
CHAT	Choline acetyltransferase
CHN	Congenital hypomyelinating neuropathy
Cho	Choline
CIDP	Chronic inflammatory demyelinating polyradiculoneuropathy
CINC1	Cytokine-induced neutrophil chemoattractant-1
CIS	Clinically isolated syndrome
CJD	Creutzfeldt-Jakob disease
CLA	Cutaneous leukocyte antigen
CLL	Chronic lymphocytic leukemia
CLP	Common lymphoid progenitor
CMAP	Compound motor action potential
CMC	Critical micelle concentration
CMP	Common myeloid precursor
CMT	Charcot-Marie-Tooth (disease)

CMV	Cytomegalovirus
CNG	cGMP-gated
CNPase	Cyclic nucleotide 3' phosphohydrolase
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CO	Cytochrome oxidase
COMT	Catechol- <i>O</i> -methyltransferase
Con A	Concanavalin A
COP-1	Copolymer-1
COX	Cyclooxygenase (prostaglandin-endoperoxide synthase; PTGS)
CP	Choroid plexus
CR	Complement receptor
CRaBP	Cellular retinal binding protein (retinaldehyde binding protein 1; RLBP1)
CRD	Carbohydrate-recognition domains
CRE	cAMP-responsive element
Cre	Creatine
CREB	cAMP-response element binding protein
CRH	Corticotrophin-releasing hormone
CRID	Cytokine release inhibitory drugs
CRP	C-reactive protein
CRPM	Collapsing response mediator protein
CRVO	Central retinal vein occlusion
CSF	Cerebrospinal fluid
CSF-1R	Colony-stimulating factor receptor
CSPG	Chondroitin sulfate proteoglycans
CT	Computed tomography
CTA	Computed tomographic angiography
CTE	Chronic traumatic encephalopathy
cTEC	Cortical thymic epithelial cell (TEC)
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen-4
CTP	Circulating T-cell progenitors
CVF	Cobra venom factor
CVO	Circumventricular organ
Cx	Connexin
cyto c	Cytochrome c
D1R	Type-1 family of dopamine receptors
D9-THC	D9-tetrahydrocannabinol
DA	Daniel's strain of Theiler's virus
DA	Dopamine
DAF	Decay-accelerating factor
DAG	Diacylglycerol
DAMP	Danger-associated molecular patterns
DAT	Dopamine transporter (solute carrier family 6A3; SLC6A3)
D β H	Dopamine- β -hydroxylase
DC	Dendritic cells
DCX	Doublecortin
ddC	Dideoxycytidine
ddI	Dideoxyinosine
DG	Dentate gyrus
dGTP	Deoxyguanosine triphosphate
DHA	Docosahexaenoic acid
Dhh	Desert hedgehog

DHP	Dihydropyridine
DIGE	Difference gel electrophoresis
DIRA	Deficiency of IL-1 receptor antagonist
DISC	Death-inducing signaling complex
DM	Diabetes mellitus
DN	Double/dominant negative
DNA	Deoxyribonucleic acids
Doc2	Double C2 protein
DOR	Delta-opioid receptor
Dox	Doxorubicin
Dox	Doxycycline
DR	Dopamine receptors
DRPLA	Dentatorubral-pallidoluysian atrophy
DSI	Depolarization induced suppression of inhibition
DSPN	Distal sensory peripheral neuropathy
DSS	Dejerine-Sottas syndrome
DTH	Delayed-type hypersensitivity
DTI	Diffusion tensor imaging
DTR	Diphtheria toxin receptor
DWI	Diffusion weighted imaging
E2F	Early-region-2 transcription factor
EAE	Experimental allergic/autoimmune encephalomyelitis
EAN	Experimental allergic/autoimmune neuritis
EAU	Experimental autoimmune uveitis
EBV	Epstein-Barr virus
ECT	Electroconvulsive therapy
EDSS	Expanded disability status scale
EEG	Electroencephalography
EGC	Embryonic germ cell
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EIAV	Equine infectious anemia virus
ELAVL4	Embryonic lethal, abnormal vision, Drosophila-like 4
ELP	Early lymphoid progenitors
eLTP	Early long-term potentiation
ELVIS	Extravasation through Leaky Vasculature and subsequent Inflammatory cell-mediated Sequestration
EMG	Electromyography
EMP	Erythromyeloid progenitors
EndoG	Endonuclease G
EOAD	Early-onset Alzheimer's disease
EPI	Echo-planar imaging
EPR	Enhanced permeability and retention
EPS	Extrapyramidal symptoms
EPSC	Excitatory postsynaptic currents
EPSP	Excitatory postsynaptic potential
ER	Endoplasmic reticulum
ERK2	Extracellular signal-regulated kinase 2
ESC	Embryonic stem cell
ESCRT	Endosomal-sorting complex required for transport
ESI-MS/MS	Electrospray ionization-mass spectrometry/mass spectrometry
ET	Endothelin
ETP	Early T lineage progenitor

EV	Extracellular vesicles
FA	Fractional anisotropy
FAD	Familial Alzheimer's disease
fALS	Familial amyotrophic lateral sclerosis
FasL	Fas ligand (FASLG)
FcγR-1	Fc receptor IgG, high affinity-1
fCJD	Familial Creutzfeldt-Jakob disease
FcR	Fc receptor
FDA	Food and Drug Administration
FDC	Follicular dendritic cell
FDG	Fluorodeoxyglucose
FDOPA	6-[(18)F]fluoro-L-dopa
FFI	Fatal familial insomnia
FGF	Fibroblast growth factor
FID	Free induction decay
FIRE	Febrile infection-related epilepsy syndrome
FIV	Feline immunodeficiency virus
FR	Fractalkine (CX3CL1)
fMRI	Functional magnetic resonance imaging
Foxp3	Forkhead box P3 transcription factor
FPI	Fluid percussion injury
FR	Folate receptor
FRC	Fibroblastic reticular cell
FRS2	Fibroblast growth factor receptor substrate 2
FS	Fisher syndrome
FSH	Follicle-stimulating hormone
FTD	Frontotemporal dementia
FT-ICR	Fourier transformed ion cyclotron resonance mass spectrometry
FTLD	Frontotemporal lobar dementia
FUS	Fused in sarcoma
Fz/PCP	Frizzled/planar cell polarity
GA	Glatiramer acetate
GABA	Gamma-aminobutyric acid
GAD	Glutamate decarboxylase
GalC	Galactocerebroside
GALT	Gut-associated lymphoid tissue
GAP	GTPase activating protein
GAPDH	Glyceraldehyde 3 phosphate dehydrogenase
GBM	Glioblastoma multiforme
GBS	Guillain-Barré syndrome
GC	Germinal center
GCDC	Germinal center dendritic cell
GCL	Ganglion cell layer
GEF	Guanine nucleotide exchange factor
GL	Granular cell layer
GC-MS	Gas chromatography combined with mass spectrometry
G-CSF	Granulocyte colony-stimulating factor
Gd	Gadolinium
GDF	Growth and differentiation factor
GDNF	Glial-derived neurotrophic factor
GDP	Guanosine diphosphate
GEF	GDP-GTP exchange factor
GFAP	Glial fibrillary acidic protein

GFP	Green fluorescent protein
GI	Gastrointestinal
GKAP	Guanylate kinase-associated protein
GLC1A	Chromosome 1 open-angle glaucoma gene
Gln	Glutamine
GLP-1	Glucagon-like peptide-1
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Granulocyte monocyte precursor
GnRH	Gonadotropin-releasing hormone
GO	Gene ontology
GO	Graves' ophthalmopathy
GPCR	G-protein-coupled receptor
GPI	Glycosylphosphatidylinositol
GR	Glucocorticoid receptor
GRIP	Glucocorticoid receptor-interacting protein
GRO- α	Growth-related oncogene alpha
GSH	Glutathione
GSS	Gerstmann-Straussler-Scheinker disease
GSTO1	Glutathione <i>s</i> -transferase omega-1
GT	G-protein transducing
GTP	Guanosine triphosphate
GUCY	Guanylate cyclase
GWAS	Meta-genome-wide association study
HAART	Highly active antiretroviral therapy
HAD	HIV-associated dementia
HAM/TSP	HTLV-I associated myelopathy/tropical spastic paraparesis
HAND	HIV-associated neurocognitive disorder
HAT	Histone acetyltransferase
HAV	Hepatitis A virus
HBsAg	Hepatitis B surface antigen
HCV	Hepatitis C virus
HCMV	Human cytomegalovirus
HD	Huntington's disease
HDAC	Histone deacetylase
HDLS	Hereditary diffuse leukoencephalopathy with spheroids
HES	Hairy and enhancer of split homolog
HEV	High endothelial venule
HFS	High frequency stimulation
Hh	Hedgehog
HHH	Hypervolemic-hemodilution and hypertensive
HHV-6	Human herpes virus-6
HIV-1	Human immunodeficiency virus type 1
HIVE	Human immunodeficiency virus encephalitis
HLA	Human leukocyte antigen
HLH	Helix-loop-helix
HMG-CoA	3-Hydroxy-3-methylglutaryl coenzyme A
HNE	4-Hydroxy-2-nonenal
hnRNP-A1	Heterogeneous nuclear ribonuclear protein-A1
HPA	Hypothalamic-pituitary-adrenal
HPMA	<i>N</i> -(2-Hydroxypropyl) methacrylamide
HRP	Horseradish peroxidase
HSC	Hematopoietic stem cell
HSC70	Heat shock cognate protein 70

Hsp	Heat shock protein
HSV	Herpes simplex virus
HSVE	Herpes simplex virus-mediated encephalitis
HT	Huntington's disease
HTLV	Human T-cell lymphotropic virus type
HTRA2	High temperature requirement serine protease 2
Htt	Huntingtin
HUVEC	Human umbilical vein endothelial cells
HveA	Herpesvirus entry mediator A
I-1	Regulatory protein inhibitor-1
IBS	Inflammatory bowel syndrome
ICAM	Intracellular adhesion molecule
ICAT	Isotope-coded affinity tags
ICE	IL-1 β -converting enzyme
ICGA	Indocyanine green angiography
ICH	Intracerebral hemorrhage
iCJD	Iatrogenic Creutzfeldt-Jakob disease
ICOS	Inducible co-stimulatory molecule
ICV	Intra-cerebro-ventricular
Id	Inhibitor of differentiation
IDE	Insulin degrading enzyme
IDO	Indoleamine 2,3-dioxygenase
IE	Immediate early
IF	Intermediate filament
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IgG	Immunoglobulin G
IGIV	Immunoglobulin intravenous therapy
Ihh	Indian hedgehog
IIDD	Idiopathic inflammatory demyelinating disease
I κ B	Inhibitory kappa B
IKK	I κ B kinase
IL	Interleukin
IL1RA	IL-1 receptor antagonist
ILBD	Incidental Lewy body disease
ILK	Integrin-linked kinase
ILM	Inner limiting membrane
ILV	Intraluminal vesicles
IM	Intramuscular
IMAC	Immobilized metal affinity chromatography
IMPDH	Inosine monophosphate dehydrogenase
iNKR	Inhibitory natural killer cell receptor
INL	Inner nuclear layer
INO	Internuclear ophthalmoplegia
iNOS	Inducible nitric oxide synthase (NOS2A)
IOP	Intraocular pressure
IP	Interferon-inducible protein
IPC	Intermediate progenitor cells
InsP3R	Inositol 1,4,5-triphosphate receptor
IPL	Inner plexiform layer
IRAK	IL-1 receptor-associated protein kinase
IRBP	Interphotoreceptor retinoid-binding protein

IRF	Interferon regulatory factor
ISCOM	Immunostimulating complexes
ISH	In situ hybridization
IT15	Interesting transcript 15
ITAM	Immunoreceptor tyrosine-based activation motif
ITGAM	Integrin, alpha M
ITR	Inverted terminal repeat
IVDU	Intravenous drug use
IVIg	Intravenous immunoglobulin
JAK	Janus kinase
JEV	Japanese encephalitis virus
JNK	c-Jun N-terminal kinase
KLH	Keyhole limpet hemocyanin
KO	Knockout
KOR	Kappa opioid peptide receptor
KYN	Kynurenine
KYNA	Kynurenic acid
LAK	Lymphokine-activated killer (cell)
L-AP4	L-2-Amino-4-phosphonobutyric acid
LAT	Latency-associated transcript
LAMP	Lysosome-associated membrane protein
LB	Lewy bodies
LC	Locus coeruleus
LCA	Leukocyte common antigen
LC-FTICR MS	Liquid chromatography Fourier transform ion cyclotron resonance mass
LC-MS	Liquid chromatography combined with mass spectrometry
LC-UV-SPE-NMR	Liquid chromatography, UV detection, solid phase extraction, and nuclear magnetic resonance
LD	Linkage disequilibrium
LDL	Low density lipoprotein
LFA-1	Leukocyte function-associated antigen-1 (integrin beta 2; ITGB2)
LGN	Lateral geniculate nucleus
LH	Luteinizing hormone
LIF	Leukemia inhibitory factor
Lingo-1	Leucine-rich repeat and Ig domain containing Nogo receptor-interacting protein-1
LMN	Lower motor neuron
LOAD	Late-onset Alzheimer's disease
LOS	Lipooligosaccharide
LPA	Lysophosphatidic acid
LPBN	Lateral parabrachial nucleus of the pons
LPS	Lipopolysaccharide
LRP-1	Lipoprotein receptor-related protein-1
LRR	Leucine-rich repeat
LRRK2	Leucine-rich repeat kinase 2
LT	Lymphotoxins
LTD	Long-term depression
LTNP	Long-term nonprogressors
LTP	Long-term potentiation
LTR	Long-terminal repeat
LT-bR	Lymphotoxin beta receptor
MCAO	Middle cerebral arterial occlusion
MCI	Mild cognitive impairment

MG	Myasthenia gravis
MHV	Mouse hepatitis virus
mI	Myoinositol
MIF	Migration inhibitory factor
Mint1	Munc-18 interacting protein 1
MIP	Macrophage inflammatory protein
MJO	Machado-Joseph disease
MME	Membrane metalloendopeptidase (neprilysin)
MMP	Matrix metalloproteinase
MMSE	Mini-Mental State Examination
MNGC	Multinucleated giant cell
MnPO	Median preoptic nucleus
Mn-SOD	Manganese superoxide dismutase
MOAT	Multispecific organic anion transporter
MOBP	Myelin-associated/oligodendrocyte basic protein
MOG	Myelin oligodendrocyte glycoprotein
MOI	Multiplicity of infection
MOR	mu opioid receptor
MOSP	Myelin/oligodendrocyte-specific protein
MP	Mononuclear phagocytes
MPA	Mycophenolic acid
MPL	Monophosphoryl lipid A
MPO	Myeloperoxidase
MPO	Medial preoptic area
MAC	Membrane attack complex
MPP	Multipotent progenitors
MPP+	1-Methyl-4-phenylpyridinium
MAdCAM-1	Mucosal addressin cell adhesion molecule-1
MAG	Myelin-associated glycoprotein
MAML	Mammalian mastermind-like
MAO	Monoamino-oxidase
MAP	Microtubule-associated protein
MAPK	Mitogen-activated protein kinases
Mash1	Mammalian achaete-scute homologue 1
MBGI	Myelin-based growth inhibitor
MBP	Myelin basic protein
MC	Mast cell
MC-1R	Melanocortin-1 receptor
MCMD	Minor cognitive motor disorder
MCP	Membrane cofactor protein (CD46)
MCP-1	Monocyte chemoattractant protein-1 (CCL2)
M-CSF	Macrophage colony-stimulating factor (CSF1)
MD	Major depression
MDA	Malondialdehyde
MDD	Major depressive disorder
MDM	Monocyte-derived macrophages
MDP	Muramyl-dipeptide
MDP	Monocytes and dendritic cell progenitor
MDR	Multidrug resistant
MDSC	Myeloid-derived suppressor cells
ME	Median eminence
MEG	Magnetoencephalography
MEPP	Miniature end-plate potential

MFS	Miller Fisher syndrome
MHC	Major histocompatibility complex
MHC-II	Class II major histocompatibility complex
MHPG	Methoxy-hydroxy-phenylethanolamine
MLR	Mixed lymphocyte reaction
MLV	Murine leukemia virus
MND	Mild neurocognitive disorder
MP	Mononuclear phagocyte
MPMV	Mason-Pfizer monkey virus
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MR	Mineralocorticoid receptors
MRA	Magnetic resonance angiography
MRI	Magnetic resonance imaging
MRP	Multidrug resistance protein
MRS	Magnetic resonance spectroscopy
MRSI	Magnetic resonance spectroscopic imaging
MS	Multiple sclerosis
MSA	Multisystem atrophy
MSCs	Myelinating Schwann cells
MSH	Melanocyte-stimulating hormone
MSN	Medium spiny neuron
mSOD1	Mutant Cu ²⁺ /Zn ²⁺ superoxide dismutase 1
MSRV	Multiple sclerosis retrovirus
MT	Magnetization transfer
Mtb	Mycobacterium tuberculosis
MTI	Magnetization transfer imaging
mTEC	Medullary thymic epithelial cell
mTOR	Mammalian target of rapamycin
MTR	Magnetization transfer ratio
MUC1	Mucin type 1 glycoprotein
MuLV	Murine leukemia virus
Munc-18	Mammalian homologue of unc-18
MV	Microvesicles
MVB	Multivesicular bodies
MVE	Murray Valley encephalitis virus
MW	Molecular weight
MZ	Marginal zone
NAA	<i>N</i> -Acetyl-aspartate
NAC	<i>N</i> -Acetyl cysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NB	Nucleotide-binding domain
NCAM	Neural cell adhesion molecule
NCC	Neural crest cell
NE	Norepinephrine
NEP	Neutral endopeptidase metalloendopeptidase
NET	Norepinephrine transporter
NeuN	Neuronal nuclei
NF	Neurofilament
NF-kB	Nuclear factor-k-B
NFAT	Nuclear factor of activated T lymphocytes
NFL	Nerve fiber layer
NFT	Neurofibrillary tangles
Ng-CAM	Neuronal-glial cell adhesion molecule (L1/NILE)

NGF	Nerve growth factor
NgR	Nogo-66 receptor
NICD	Notch intracellular domain
NK	Natural killer (cells)
NKT	Natural killer T (cells)
NLR	Nod-like receptors
NMDA	<i>N</i> -methyl-D-aspartate
NMDAR	<i>N</i> -methyl-D-aspartate receptors
NMJ	Neuromuscular junction
NMO	Neuromyelitis optica
NMR	Nuclear magnetic resonance
NMSCs	Nonmyelinating Schwann cells
nNOS	Neuronal nitric oxide synthase
NNRTIs	Nonnucleoside analogue reverse transcriptase inhibitors
NO	Nitric oxide
NOD	Nucleotide oligomerization domain
NOD	Non-obese diabetic mice
NOS	Nitric oxide synthase
NOT	Nucleus of the optic tract
NP	Nanoparticle
NPC	Neural progenitor cell
NPY	Neuropeptide Y
NPZ-8	Neuropsychological Z score for 8 tests
NR	Nuclear receptor
NRG	Neuregulin
NRL	Nuclear receptor ligand
NRTI	Nucleoside analogue reverse transcriptase inhibitors
NSAID	Nonsteroidal anti-inflammatory drug
NSC	Neural stem cell
NSE	Neuron specific enolase
NSF	<i>N</i> -ethylmaleimide sensitive factor
NT	3-Nitrotyrosine
NTF	Neurotrophin
NVU	Neurovascular unit
OB	Olfactory bulb
OCB	Oligoclonal band
OCD	Obsessive compulsive disorder
ODN	Oligonucleotides
OE	Olfactory epithelium
OHT	Ocular hypertension
OL	Oligodendrocyte
OLM	Outer limiting membrane
OMgp	Oligodendrocyte-myelin glycoprotein
OMP	Olfactory marker protein
ONH	Optic nerve head
ONL	Outer nuclear layer
OP	Oligodendrocyte progenitors
OPC	Oligodendrocyte progenitor cell
OPCA	Olivopontocerebellar atrophy
OPL	Outer plexiform layer
ORF	Open reading frame
ORN	Olfactory response neuron
OSP	Oligodendrocyte-specific protein

OVA	Ovalbumin
OVLT	Organum vasculosum of the lamina terminalis
P0	Myelin protein zero
p75NTR	p75 neurotrophin receptor (nerve growth factor receptor; NGFR)
PACAP	Pituitary adenylate cyclase-activating polypeptide
PACT	Protein activator of the interferon-induced protein kinase
PAF	Platelet-activating factor
PAG	Periaqueductal gray
PAMP	Pathogen-associated molecular pattern
PANDAS	Pediatric autoimmune neuropsychiatric disorders associated with Streptococcus
PANSS	Positive and negative syndrome scale
PARP	Poly(ADP-ribose) polymerase
PASAT	Paced Auditory Serial Addition Test
PBBS	Peripheral benzodiazepine binding sites
PBL	Peripheral blood lymphocyte
PBMC	Peripheral blood mononuclear cell
PBR	Peripheral benzodiazepine receptor
PCP	Phencyclidine
PCR	Polymerase chain reaction
PD	Parkinson's disease
pDC	Plasmacytoid dendritic cells
PD1	Program death-1
PDE	Phosphodiesterase
PDGF	Platelet-derived growth factor
PDTC	Pyrrolidine dithiocarbamate
PE	Plasma exchange
Pe	Periventricular
PEG	Polyethylene glycol
PEI	Polyethyleneimine
PENK	Proenkephalin
PERG	Pattern electroretinogram
PET	Positron emission tomography
PFS	Periodic fever syndromes
PG	Prostaglandin
Pgp	P-glycoprotein
PHF	Paired helical filament
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol-3-kinase
PICA	Posterior inferior cerebellar artery
PICK1	Protein interacting with C kinase 1
PKA	cAMP-dependent protein kinase
PKG	Protein kinase G
PLGA	Poly(D,L-lactide-co-glycolide)
PLOSL	Polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy
PLP	Proteolipid protein
PMCA	Plasma membrane bound Ca ²⁺ -ATPase
PMD	Pelizaeus-Merzbacher disease
PML	Progressive multifocal leukoencephalopathy
PMN	Polymorphonuclear (leukocyte)
PMP22	Peripheral myelin protein 22
PNS	Peripheral nervous system
POAG	Primary open-angle glaucoma
polyQ	Polyglutamine

POMC	Pro-opiomelanocortin
POU3F2	POU class 3 homeobox 2
PP	Protein phosphatase
PPAR	Peroxisome proliferator activated receptor
PPF	Paired-pulse facilitation
PPG	Poly(propylene glycol)
PP-MS	Primary progressive multiple sclerosis
PR	Photoreceptor
PrPc	Cellular prion protein
PrPres	Protease resistance prion
PrPsc	Disease-associated prion protein
PRR	Pattern recognition receptor
PS	Presenilin (PSEN)
PSA-NCAM	Poly-sialylated form of the neural cell adhesion molecule
PSCs	Perisynaptic Schwann cells
PSD	Postsynaptic density
PSP	Progressive supranuclear palsy
PSW	Periodic sharp wave
Ptc	Patched, a hedgehog receptor
PTM	Post-translational modifications
PTP	Post-tetanic potentiation
PTSD	Post-traumatic stress disorder
PTZ	Pentylene-tetrazol
PVL	Periventricular leukomalacia
PVM	Perivascular macrophages
PVN	Paraventricular nucleus
PYD	Pyrin domain
PYY	Peptide YY
RA	Rheumatoid arthritis
Rag	Recombination-activating gene
RAGE	Receptor for advanced glycation end product
RANTES	Regulated upon activation normal T-cell expressed and secreted (CCL5)
RAS	Renin-angiotensin system
Rb	Retinoblastoma
RBP	RNA-binding proteins
REM	Rapid eye movement
RER	Rough endoplasmic reticulum
RF	Radiofrequency
RFLPs	Restriction fragment length polymorphisms
RGC	Retinal ganglion cell
RISC	RNA-induced silencing complex
RIG1	Retinoic acid-inducible gene 1
RIM	Rab3-interacting molecule
RIP	Receptor-interacting protein
RMS	Rostral migratory stream
RNAi	RNA interference
RNI	Reactive nitrogen intermediates
RNP	Ribonucleoprotein
RNS	Reactive nitrogen species
ROCK	Rho kinase
ROHHAD	Rapid-onset obesity, hypoventilation, hypothalamic dysfunction, and autonomic dysregulation
ROI	Reactive oxygen intermediate

ROR γ	Retinoic-acid-receptor-related orphan receptor- γ
ROS	Reactive oxygen species
RP	Relapsing polychondritis
RPE	Retinal pigment epithelial (cells)
RRM	RNA recognition motifs
RRMS	Relapsing and remitting multiple sclerosis
RSV	Rous sarcoma virus
RTK	Receptor tyrosine kinase
rt-PA	Recombinant tissue plasminogen activator
RT-PCR	Reverse transcription polymerase chain reaction
RyR	Ryanodine receptor
sALS	Sporadic amyotrophic lateral sclerosis
SAP	Synapse-associated protein
SAPAP	SAP-associated protein (discs, large homolog-associated protein-1; DLGAP1)
SAPK	Stress-activated protein kinase (JNK, MAPK8)
sAPP	Secreted β -amyloid precursor protein
SBMA	Spinobulbar muscular atrophy
SCs	Schwann cells
SC	Superior colliculus
SCA-3	Spinocerebellar ataxia-3
scFv	Single-chain Fv antibodies
SCI	Spinal cord injury
SCID	Severe combined immunodeficiency
sCJD	Sporadic Creutzfeldt-Jakob disease
SCPs	Schwann cell precursor
sCrry	Soluble complement receptor-related protein y
SDF-1	Stromal cell-derived factor 1 (CXCL12)
SEC	Sinus endothelial cell
SELDI-TOF	Surface enhanced laser desorption ionization time-of-flight
SER	Smooth endoplasmic reticulum
SERCA	Sarco(endo)plasmic reticulum Ca^{2+} -ATPase
SERT	Serotonin transporter
sFI	Sporadic fatal insomnia
SFO	Subfornical organ
SG	Stress granule
SGLPG	Sulfated glucuronyl lactosaminyl paragloboside
SGZ	Subgranular zone
Shh	Sonic hedgehog
sIg	Surface immunoglobulin
sIL-2R	Soluble IL-2 receptor
SITA	Swedish interactive thresholding algorithm
SIV	Simian immunodeficiency virus
SIVE	Simian immunodeficiency virus encephalitis
SLE	Systemic lupus erythematosus
SMA	Spinal muscular atrophy
SMAC	Second mitochondrial-derived activator of caspase
SMase	Sphingomyelinase
SMN	Survival motor neuron gene
SN	Substantia nigra
SNAP	Sensory nerve action potential
SNAP-25	Synaptosome-associated protein of 25,000 daltons
SNARE	NSF attachment receptor
SNpc	Substantia nigra pars compacta

SNPs	Single-nucleotide polymorphisms
SNS	Sympathetic nervous system
SOCS	Suppressors of cytokine signaling
SOD1	Superoxide dismutase 1
SON	Supraoptic nuclei
SP1	Specificity protein 1
SPARC	Secreted protein acidic and rich in cysteine
SPECT	Single photon emission computed tomography
SPG-II	Spastic paraplegia type II
SR-A	Scavenger receptor type A
SRBCs	Sheep red blood cells
SREBP	Sterol regulatory element-binding protein
SRF	Serum response factor
SSRI	Selective serotonin reuptake inhibitors
STAT	Signal transducers and activators of transcription
STP	Short-term potentiation
SV5	Simian virus 5
SVZ	Subventricular zone
SWAP	Short wavelength automated perimetry
SWATH	Sequential window acquisition of all theoretical mass spectra
SWI	Susceptibility weighted imaging
SYN	α -Synuclein
SZ	Schizophrenia
T	Tesla
Tat	Trans-activator of transcription
TBE	Tick-borne encephalitis virus
TBI	Traumatic brain injury
TBP	TATA-binding protein
TCA	Tricarboxylic acid
TCR	T-cell receptor
TCV	T-cell vaccination
TDO	Tryptophan 2,3-dioxygenase
TE	Echo time
TEC	Thymic epithelial cell
TES	Traumatic encephalopathy syndrome
Teff	T effector cells
TF	Transcription factor
TG	Trigeminal ganglia
TGF	Transforming growth factor
TH	Thyroid hormone
TH	Tyrosine hydroxylase
Th1	T helper type 1 cell
Th2	T helper type 2 cell
TIA	Transient ischemic attack
TIR	Toll/IL-1 receptor
TJ	Tight junction
TLR	Toll-like receptor
TMEV	Theiler's mouse encephalomyelitis virus
TMT	Trimethyltin
TMZ	Temozolomide
TN	Terminal nuclei
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor

TOR1	Target of rapamycin 1
TRAF	TNF-receptor mediated factor
TRAIL	TNF-related apoptosis-inducing ligand (TNFSF10)
TRANCE	TNF-related activation-induced cytokine (TNFSF11)
TRANCER	TRANCE receptor (TNFRSF11A)
TRE	Tax responsive element
Treg	T regulatory cells
TREM	Triggering receptor expressed on myeloid cells
Trk	Receptor tyrosine kinase
TRP	Transient receptor potential
TRPC	Transient receptor potential canonical channels
TSA	Tissue-specific antigen
TSE	Transmissible spongiform encephalopathies
TSP	Thrombospondins
TULP-1	Tubby-lie protein 1
Tyk2	Protein tyrosine kinase 2
UACA	Uveal autoantigen with coiled domains and ankyrin repeats
UMN	Upper motor neuron
V1	Primary visual cortex
VAMP	Vesicle-associated membrane protein
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VEP	Visual evoked potential
VIP	Vasoactive intestinal peptide
VMAT-2	Vesicular monoamine transporter-2 (solute carrier family 18; SLC18A2)
VZV	Varicella-zoster virus
WDM	Welander distal myopathy
WKAH	Wistar-King-Aptekman-Hokudai
WNV	West Nile virus
X-ALD	X-adrenoleukodystrophy
XIAP	X-linked inhibitor of apoptosis protein
ZO-1	Zonula occludens-1 (TJP1)

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Part I

Immunology of the Nervous System

Howard E. Gendelman and Eliezer Masliah

Abstract

Neuroinflammatory processes play a significant role in health and disease of the nervous system. These regulate development, maintenance and sustenance of brain cells and their connections. Linked to aging, epidemiologic, animal, human, and therapeutic studies all support the presence of a neuroinflammatory cascade in disease. This is highlighted by the neurotoxic potential of microglia. In steady state, microglia serve to protect the nervous system by acting as debris scavengers, killers of microbial pathogens, and regulators of innate and adaptive immune responses. In neurodegenerative diseases, activated microglia affect neuronal injury and death through production of glutamate, pro-inflammatory factors, reactive oxygen species, quinolinic acid amongst others and by mobilization of adaptive immune responses and cell chemotaxis leading to transendothelial migration of immunocytes across the blood-brain barrier and perpetuation of neural damage. As disease progresses, inflammatory secretions engage neighboring glial cells, including astrocytes and endothelial cells, resulting in a vicious cycle of autocrine and paracrine amplification of inflammation perpetuating tissue injury. Such pathogenic processes contribute to neurodegeneration. Research from others and our own laboratories seek to harness such inflammatory processes with the singular goal of developing therapeutic interventions that positively affect the tempo and progression of human disease.

Key words

Alzheimer's disease • Microglia • Neurodegenerative disorders • Neuroinflammatory processes • Parkinson's disease

Neuroinflammatory processes play a significant role in health and disease of the nervous system. These regulate development, maintenance and sustenance of brain cells and their connections. Linked to aging, epidemiologic, animal, human, and therapeutic studies all support the presence of a neuroin-

flammatory cascade in disease. This is highlighted by the neurotoxic potential of microglia. In steady state, microglia serve to protect the nervous system by acting as debris scavengers, killers of microbial pathogens, and regulators of innate and adaptive immune responses. In neurodegenerative diseases, activated microglia affect neuronal injury and death through production of glutamate, pro-inflammatory factors, reactive oxygen species, quinolinic acid amongst others and by mobilization of adaptive immune responses and cell chemotaxis leading to transendothelial migration of immunocytes across the blood-brain barrier and perpetuation of neural damage. As disease progresses, inflammatory secretions engage neighboring glial cells, including astrocytes and endothelial cells, resulting in a vicious cycle of autocrine and

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paracrine amplification of inflammation perpetuating tissue injury. Such pathogenic processes contribute to neurodegeneration. Research from others and our own laboratories seek to harness such inflammatory processes with the singular goal of developing therapeutic interventions that positively affect the tempo and progression of human disease.

As the life expectancy of the human population continues to increase, the possibility of developing neuroinflammatory and neurodegenerative diseases have increased considerably during the past 50 years. Of the neurodegenerative disorders Alzheimer's disease continued to be the leading cause of dementia in the aging population. Traditionally, neurodegenerative disorders have been defined as conditions where there is selective loss of neurons within specific region of the brain accompanied by astrogliosis. However, in the past 20 years, we have learned that the pathological process leading to the dysfunction of selected circuitries in the brain initiates with damage to the synapses rather than with the loss of neurons. In fact, neuronal loss is a late event that is probably preceded by damage to axons and dendrites followed by shrinkage of the neuronal cell body and abnormal accumulation of filamentous proteins.

Therefore, the revised concept of neurodegeneration suggests that neuronal injury initiates at the synaptic junction and propagates throughout selected circuitries leading to neuronal dysfunction which resolves in the classical clinical symptoms characteristic to each of the neurodegenerative disorders (Hashimoto and Masliah 2003). So for example in Alzheimer's disease early damage to the synapses between the entorhinal cortex and the molecular layer of the dentate gyrus (perforant pathway) resolves in the short term memory deficits characteristic of this dementing disorder. Later on disconnection of the cortico-cortico fibers in the frontal, parietal, and temporal cortex resolved in more severe memory deficits, and alterations in executive functions and abstraction. Degeneration of connections between the nucleus basalis of Meynert and the neocortex resolves in attention and memory deficits that usually associated with loss of cholinergic neurons. Other circuitries and neuronal populations are also affected in Alzheimer's disease illustrating the complexity of these disorders and the fact

that the concept of single population is affected is limited. That is the case with several other disorders including Parkinson's disease where degeneration is not limited to the dopaminergic system, but also involves the limbic system, the raphe nucleus, the insula, and other systems.

In response to the injury neurons produce adhesions molecules and trophic factors that recruit astroglial and microglial cells to participate in the process of repair of the damage. In addition the microvasculature and other glial systems might also participate in the process. Thus, neurodegeneration is accompanied by astrogliosis, microgliosis, and microvascular remodeling. While astroglial cells initially produce trophic factors and cytokines that aid in the tissue repair, eventually these factors could amplify the inflammatory response, increase vascular permeability and result in microglial activation, which in turn might lead to the production of more proinflammatory cytokines and chemokines. A critical balance between the repair and proinflammatory factors often determines the future rate and progression of the degenerative process.

The understanding of the mechanisms of neurodegeneration and inflammatory response in these neurological conditions has seen a tremendous progress in the past 10 years. It is now recognized that probably small soluble misfolded protein aggregates denominated oligomers are responsible for the injury. So for example in Alzheimer's disease A β protein oligomers might damage the synapses in neocortical regions and the limbic system while in Parkinson's disease α -synuclein oligomers may damage the axons in the striatum, brainstem and cortical regions. While significant progress has been made in understanding the fundamental mechanisms for the neuronal injury, less is known about the reasons for the selective neuronal vulnerability characteristic to these neurological conditions and the role of the innate immune system in this process.

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William A. Banks

Abstract

The blood-brain barriers (BBBs) are mainly located at the levels of the vasculature, choroid plexus, and the circumventricular organs and play multiple roles in neuroimmunology. The ability of the BBBs to separate the blood and its contents from the central nervous system (CNS) is largely responsible for the CNS being an immune-privileged region. However, the BBB then revises this separation in a regulated way by a variety of mechanisms, including the ability to transport cytokines, regulate the entry of immune cells into the brain, and to itself secrete into the blood and into the CNS immunoactive substances. The BBB thereby participates in a number of neuroimmune axes that allow communication between the CNS and the peripheral immune cells. Failure of the highly regulated activities of the BBBs can be both a cause and consequence of immune diseases.

Keywords

Active transport • Adsorptive endocytosis • Adsorptive transcytosis • AIDS • Blood-brain barrier • Brain • Brain endothelial cell • Central nervous system • Cytokine • Endothelin • Facilitated diffusion • Immune cell • Interleukin • Neuroimmune • Neurovascular unit • Opiate • Transmembrane diffusion • Transport • Tumor necrosis factor • Virus

2.1 Introduction

The roles played by the blood-brain barrier (BBB) in neuroimmunology are diverse and ever expanding. Conceptually, the BBB is those processes that restrict, control, or otherwise influence the exchange of substances between the peripheral circulation and the brain interstitial fluid and cerebrospinal fluid (CSF). More concretely, there are multiple BBBs: the barrier formed by monolayers of endothelial cells (the vascular BBB), the barrier formed by ependymal/epithelial cells (the blood-CSF barrier) and a barrier formed by tanycytes interposed between the circumventricular organs and the

adjacent brain tissue. It is the restrictive properties of the BBB that limit and control the trafficking of immune cells and prevent the unrestricted leakage of immune active substances from the blood into the brain that renders the central nervous system (CNS) an immune-privileged area. But the BBB also transports immunoactive substances between the blood and CNS, responds to immunoactive substances secreted into the blood or CNS fluids, and secretes substances into those compartments. These last three processes of transport, responsiveness, and secretion allow the BBB to be in constant cross talk with other cells in both the CNS and periphery in a formation referred to as the neurovascular unit (NVU). The BBB both responds to and influences the CNS and peripheral microenvironment and does so through reacting to immunoactive substances, including cytokines, chemokines, prostaglandins, and nitric oxide. Several consequences arise from this cross talk, including that under physiological conditions the metabolic needs of the brain are met by the BBB. The BBB helps to keep the CNS informed of

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peripheral events including immune events and is central to the formation and functioning of neuroimmune axes. When any of these physiological consequences are violated, disease can arise; and the BBB can be a target, a cause, or a conduit to treatment of those diseases.

2.1.1 Structures and Functions of the BBB

Evidence for an interface between the circulation and the CNS dates back to the end of the nineteenth century (Bradbury 1979). The best known of those early studies were done by a young Paul Erlich who found that some dyes did not stain the brain after their peripheral injection. Erlich concluded erroneously that the lack of staining was because these dyes did not bind to brain tissue. Several decades later, Goldmann, a student of Erlich's, found that these dyes could stain the brain when injected intravenously. Thus, these studies were reinterpreted as evidence in favor of some sort of barrier between the CNS and blood.

The location and nature of that barrier was controversial through much of the twentieth century. Elegant studies by Davson and colleagues identified the barrier at the vascular level. However, alternative opinions were held until Reese and coworkers conducted classic studies with the electron microscope in the late 1960s (Brightman and Reese 1969; Reese and Karnovsky 1967). Previous work had shown no difference between vascular beds of peripheral tissues and the CNS when studied grossly or at the light microscope level. However, Reese and coworkers found numerous differences at the ultrastructural level. These included a much-reduced rate of pinocytosis and an absence of intracellular fenestrations. Currently, the most widely discussed finding is the presence of tight junctions between adjacent endothelial cells. The tight junctions, low rate of pinocytosis, and low number of intracellular fenestrations effectively eliminate intercellular gaps and pores. This, in turn, essentially eliminates the production of a plasma-derived ultrafiltrate and hence the leakage of serum proteins into the brain.

From this single change, the lack of a production of an ultrafiltrate, evolves a large number of consequences for CNS function. Obviously, it is the basis of the restriction of protein access, which first defined the BBB in late nineteenth century. The need for an efficient lymphatic system is eliminated, but the lack of a classic lymphatic system means that the CNS needs other methods to rid itself of the free water and wastes produced by metabolism and the secretions of the choroid plexus. Without production of an ultrafiltrate, the CNS depends on other methods to extract nourishment from the blood. The BBB addresses this need with a large number of selective transporters for substances from electrolytes to regulatory proteins (Davson and Segal 1996b, c). Because the CNS is not equipped to handle an ultrafiltrate, the reintroduction of a

leaky BBB, as with hypertensive crisis, can result in increased intracranial pressure and encephalopathy (Al-Sarraf and Phillip 2003; Johansson 1989; Mayhan and Heistad 1985).

2.1.2 The Various BBBs

The BBB is not a single barrier but several barriers, which are in parallel. This contrasts with the testis-blood barrier, which consists of several barriers in series (Holash et al. 1993; Neaves 1977). The most studied of these barriers are the vascular barrier and the choroid plexus. Often, the terms BBB and blood-cerebrospinal (CSF) fluid barrier are used to refer specifically to the vascular barrier and the barrier formed at the choroid plexus, respectively. The least studied barriers are the barriers formed by tanycytes at the circumventricular organs (CVO) and other specialized neural barriers, such as the blood-retinal barrier (Neuwelt et al. 2008).

2.1.2.1 Vascular BBB

The vascular BBB occurs because of the modifications, noted by Reese and co-workers, in the endothelial cells that comprise the capillary bed and line the venules and arterioles of the CNS (upper panel Fig. 2.1). It is likely that these three regions are highly specialized. For example, immune cells primarily cross at the venules, and most of the classic transporters are located at the capillaries (Engelhardt and Wolburg 2004). No CNS cell is more than about 40 μm from a capillary. This means that a substance that can cross the vascular BBB can immediately access the entire CNS. Substances that cross the vascular BBB can be either flow-dependent or not dependent on flow rate. A flow-dependent substance is one in which the BBB extracts from the blood nearly the maximal amount possible (Kety 1987). The only way to increase the amount of a flow-dependent substance entering the brain is to increase the flow rate to the brain. Glucose is an example of a flow-dependent substance (Rapoport et al. 1981). A brain region that is particularly active has its increased demand for glucose met by an increase in regional blood flow. In contrast, transport of a cytokine such as tumor necrosis factor- α (TNF- α) is not flow dependent. Only a small percent of the TNF- α in blood is extracted by the brain via the saturable transporter for TNF- α located at the BBB (Gutierrez et al. 1993). Alterations of blood flow within physiological limits do not alter the uptake of TNF- α from blood by brain. However, extreme changes in the rate of blood flow or capillary tortuosity can result in rheological changes, such as the loss of laminar flow. Such alterations likely occur in stroke, AIDS, and Alzheimer's disease (de la Torre and Mussivand 1993; Nelson et al. 1999). This may result in impaired permeation of flow-dependent and non-flow dependent substances.

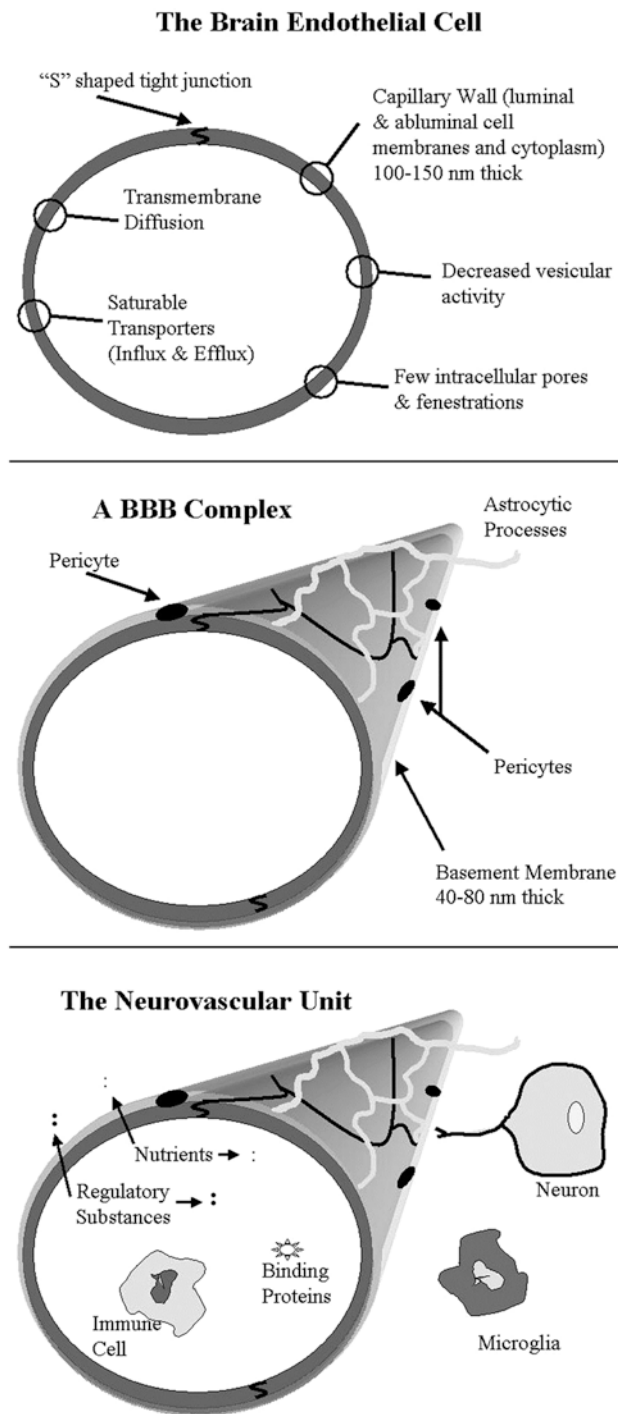


Fig. 2.1 The vascular blood-brain barrier: the *upper panel* illustrates the brain endothelial cell. This is the functional and anatomical site of both barrier function and of saturable and non-saturable mechanisms of passage. The major modifications allowing both barrier function and selective penetration of substances are indicated. The *middle panel* illustrates other cell types and structures important in BBB function. Pericytes are embedded in a basement membrane and astrocytes form a structure over the capillary bed above the pericytes and basement membrane. Both cell types are in paracellular communication with the brain endothelial cells. The *lower panel* illustrates the neurovascular unit, a concept that emphasizes integration of peripheral, BBB, and central interactions

The vascular BBB has regional variations in terms of function and susceptibilities to disease. Huber et al. noted that some regions of the brain suffer larger and earlier disruptions to their barriers during diabetes mellitus than others (Huber 2008; Huber et al. 2006). Many peptides and regulatory substances have unique regional variations in their transport rates across the BBB (Banks et al. 1996; Banks and Kastin 1998). It is assumed that these reflect on brain function. For example, the brain region with the highest rate of transport of leptin is the region of the arcuate nucleus, an area important in leptin-mediated control of feeding (Banks et al. 1996; Schwartz et al. 2000).

2.1.2.2 Choroid Plexus

The choroid plexus are bags composed of monolayers of epithelial cells that project into the ventricles and contain a capillary plexus (Johanson 1988). The capillaries do not have barrier function and so produce an ultrafiltrate that fills the bag. The epithelial cells have tight junctions and so prevent the ultrafiltrate from entering the ventricular space. Unlike the capillaries, the epithelial cells of the choroid plexus have a high rate of vesicular turnover that is responsible for the production of the CSF. However, the CSF is not an ultrafiltrate but a secreted substance. The choroid plexus also has many selective transport systems, some of which are specific to it or are enriched in comparison to the vascular BBB. In some cases, the choroid plexus seems not to be a system complementary to the vascular BBB, but one that is contrary to it. For example, efflux of amyloid beta peptide by the vascular BBB is impaired with aging but is increased with aging at the choroid plexus (Pascale et al. 2011). Thus, the choroid plexus should not be thought of as a secondary barrier but as an independent system with its own unique characteristics.

2.1.2.3 Barriers at Circumventricular Organs

The CNS of mammals contains seven regions of the brain where the vasculature does not fully participate in a BBB (Gross et al. 1987). These regions have at least one side that faces a ventricle and so are termed circumventricular organs (CVOs). Together, they comprise about 0.5 % of the brain by weight. Their capillaries allow the production of an ultrafiltrate and so their cells are in more intimate contact with the circulation. They are known to play vital roles as sensing organs for critical peripheral events; for example, they act as emetic centers and are important in blood pressure modulation (Johnson and Deckwerth 1993; Ferguson 1991). They can relay their signals to the rest of the brain by neurons that project from them to distant brain regions or project to them from other brain regions. However, the mixing of their interstitial fluids with that of adjacent brain tissue or CSF has been shown to be limited in most studies (Peruzzo et al. 2000; Plotkin et al. 1996; Rethelyi 1984). Diffusion through brain tissue is poor, and this alone would tend to produce a

limit to mixing within a few hundred microns of the CVO (Cserr and Berman 1978; de Lange et al. 1995). However, the major factor preventing leakage of substances from the CVO into the adjacent CSF and brain tissue is a physical barrier to diffusion. The epithelial cells that line the ventricles form tight junctions when they are next to CVOs, thus limiting CVO-to-CSF diffusion. A functional barrier formed by bands of tanycytes also exists for the diffusion of substances from the CVO to the adjacent brain region (Peruzzo et al. 2000; Plotkin et al. 1996; Rethelyi 1984). These tanycytes themselves can project to the cells and vessels within the CVO and can respond dynamically to relay information to adjacent regions of the brain that have BBBs (Langlet et al. 2013). A recent review by Rodriguez (Rodriguez et al. 2010) has explored the various aspects of the CVO barriers.

2.1.2.4 Other Specialized Neural Barriers

These barriers include the blood-retinal barrier, blood-spinal cord barrier, and blood-nerve barriers (for a full listing, see Neuwelt et al. (2008)). These barriers generally include tight junctions participating in a vascular barrier but exhibit varying degrees of leakiness. They can also vary markedly in transporter activity from the vascular BBB during both health and in response to disease (Pan et al. 2008a; Pan and Kastin 2003; Prockop et al. 1996).

2.1.3 Concept of the Neurovascular Unit and Comparison to Peripheral Vascular Beds

The endothelial cell is the anatomical location of the barrier aspect of the vascular BBB and of its various saturable transporters (Fig. 2.1). Capillary beds from peripheral tissues have numerous intracellular and intercellular pores and fenestrations and high rates of pinocytosis that account for their leakiness. The brain endothelial cell engages in comparatively little macropinocytosis, has few intracellular pores or fenestrations, and intercellular pores or gaps are eliminated because of tight junctions.

However, the brain endothelial cell does not function in isolation. The abluminal (brain side) of the capillary is encased in a basement membrane 40–80 nm thick. This membrane does not act as a barrier to molecules but may restrict viral-sized particles (Muldoon et al. 1999). It also holds pericytes in close approximation to the endothelial cell (Balabanov and Dore-Duffy 1998). The pericyte is a pluripotent cell and a modulator of BBB function (Dore-Duffy et al. 2000; Deli et al. 2005; Dore-Duffy 2008). It, like astrocytes and microglia, secretes cytokines and other immune active substances both constitutively and when induced (Kovac et al. 2011). In the rat hippocampus, astrocytes project endfeet that surround the capillary in what looks at the ultrastructural level like a

complete covering, albeit without intercellular tight junctions (Mathiisen et al. 2010). Astrocytes and pericytes both secrete substances that induce tight junction formation in endothelial cells (Deli et al. 2005; Daneman et al. 2010). All the major cell types of the NVU (pericytes, astrocytes, microglia, and endothelial cells) secrete a variety of substances, including cytokines, into their local environment (Fabry et al. 1993; Nath et al. 1999; Banks 2014). Pericytes and astrocytes play interrelated but distinct roles in BBB function. For example, pericytes are the primary protectors of the blood-retinal and blood-brain barriers during glycemic stress (Romeo et al. 2002; Nakaoke et al. 2007).

It is clear that immune cell trafficking occurs across the normal BBB (Greenwood et al. 2011). One study found that about one in 5000 intravenously injected lymphocytes resided in the brain at any given time and that uptake was affected by strain and immune activation (Banks et al. 2012). The microglia may be at equilibrium with circulating macrophages/monocytes, although whether monocytes cross the BBB of the adult healthy animal to become microglia seems to still be unresolved (Williams and Hickey 1995). Other immune cells also enter and exit the CNS at unknown rates and frequencies as influenced by yet to be determined factors. Clearly, secretions of prostaglandins, nitric oxide, and cytokines from each of these cells are important for intercellular communication and can influence endothelial cell permeability (Chao et al. 1994; Nath et al. 1999; Shafer and Murphy 1997).

The concept of the neurovascular unit (NVU) emphasizes the interactive role that cells and events within the CNS and in the circulation play on BBB permeability as well as the consequences of the permeability itself. The NVU includes other factors long known to influence the penetration of substances across the BBB, such as degradation, sequestration, and serum protein binding. The encompassing concept of the NVU is particularly useful when considering the next section, the mechanisms of transport across the BBB.

2.2 Mechanisms of Transport Across the BBB

Substances can enter or exit the CNS by a variety of mechanisms. Some of these mechanisms are operational in both the blood-to-brain (influx) and the brain-to-blood (efflux) directions; whereas, others are unidirectional.

2.2.1 Blood to CNS

Saturable and nonsaturable modes predominate influx. Within each of these categories are a diverse number of mechanisms. These different mechanisms tend to favor certain groups or types of substances.

2.2.1.1 Nonsaturable Passage

A hallmark of nonsaturable passage is that the percent of material crossing into the CNS is not affected by the amount of material available for transport. The two main mechanisms of nonsaturable passage are transmembrane or transcellular diffusion and the extracellular pathways. The former is much better studied and its principles are widely applied by industry for the development of CNS drugs; the latter has received much less attention.

Transcellular Diffusion

The most studied nonsaturable mechanism by which small molecules cross the BBB is by transmembrane or transcellular diffusion (Rapoport 1976). The major determinant of passage is the degree to which the substance is lipid soluble. A substance that is too lipid soluble will be unable to repartition into the brain's interstitial fluid and so will become trapped in the cell membranes of the BBB. A ratio of about 10:1 in favor of lipid versus aqueous solubility is near ideal for maximal passage across the BBB. The second most important determinant is molecular weight with passage being favored for smaller molecules. Other physicochemical determinants, such as charge, can occasionally become dominant for specific compounds. Work by Lipinski and colleagues in Caco-2 cells, an immortalized cell line derived from a gastrointestinal cancer, clearly shows that smaller, less charged, more lipid soluble drugs are favored in transmembrane diffusion (Lipinski et al. 1997). Many exogenous substances, including many drugs with CNS activity, enter the brain predominantly by way of transmembrane diffusion. Morphine and ethanol are prime examples of common substances that cross the BBB by this mechanism (Oldendorf 1974).

Although higher molecular weight (MW) is an impedance to transmembrane diffusion at the BBB, there seems to be no absolute molecular weight cut-off. A previous study which had thought to define such an absolute limit had discovered, in retrospect, early evidence for an efflux system (Levin 1980). The largest substance to date noted to have a measurable uptake by brain by transcellular diffusion is cytokine-induced neutrophil chemoattractant-1 (CINC1), with a MW of about 7.8 kDa (Pan and Kastin 2001a). A surprisingly large number of small, lipid soluble compounds cross the BBB at a rate considerably greater or lesser than that predicted by their physicochemical characteristics (Oldendorf 1971, 1974). Binding to serum proteins and efflux systems are major factors decreasing influx, and the presence of a saturable blood-to-brain transporter is a major factor increasing influx.

Extracellular Pathways

Albumin derived from serum is present in small amounts in the CSF, showing that the BBB is not absolute. The amount of protein in CSF, however, is very small, being about 0.5%, or 1/200th, of that in plasma. The CSF is not an ultrafiltrate but a

secreted fluid. This means that the relative and absolute concentrations of proteins, electrolytes, minerals, and other substances can differ tremendously to that of plasma. The extracellular pathways are another avenue by which substances can enter the CNS (Balin et al. 1986; Broadwell 1993). These represent what have sometimes been termed “functional leaks” at discreet areas of the brain, including the large vessels of the pial surface and subarachnoid space, the circumventricular organs, the nasal epithelium, the sensory ganglia of spinal and cranial nerves, and some deep brain regions, such as the nucleus tractus solitarius (Broadwell and Banks 1993).

The amount of a substance that enters the brain by the extracellular pathways is small. However, this route may be therapeutically relevant for compounds which have favorable peripheral pharmacokinetics, such as a long serum half-life and a small volume of distribution (Banks 2004). Antibodies, erythropoietin, and enzymes can access the brain by way of the extracellular pathways (Banks et al. 2004a, 2005a, 2007; Kozłowski et al. 1992; Grubb et al. 2008), and this may underlie their therapeutic benefits when given in high doses (Grubb et al. 2008; Alafaci et al. 2000; Ehrenreich et al. 2002; Erbyraktar et al. 2003; Morgan et al. 2000; Janus et al. 2000; Hock et al. 2003; Farr et al. 2003).

2.2.1.2 Receptor-Mediated and Saturable Transports

Saturable processes represent a diverse group of mechanisms. Included in this group are diapedesis and adsorptive endocytosis/transcytosis that share characteristics with the saturable systems.

Active Transport Versus Facilitated Diffusion

Saturable transporters (Yeagle 1987) can be divided into those which require energy (active transport) and those which do not (facilitated diffusion). Both are dependent on a protein which acts as the transporter, may have co-factors, and be modulated by physiologic and disease processes. Energy requiring systems can be unidirectional; that is, they may have only an influx or efflux component. Non-energy requiring saturable transport (facilitated diffusion) is bidirectional; that is, it transports substances in both directions with net flux being from the side of higher concentration to the side of lower concentration.

Most of the classic saturable transporters at the BBB are facilitated diffusion systems (Kaur et al. 1992). For example, GLUT-1, the transporter for glucose, is a facilitated diffusion transporter. If the level of glucose is artificially raised above that of serum (or if radioactive glucose is introduced into the CNS, but not the serum), efflux of glucose can be shown.

Transcytotic Versus Transmembrane Transport

Saturable transporters can also be categorized based on whether they use pores/channels or vesicles to transport their ligands across the BBB. In the pore system, the molecule

crosses from one side of the cell membrane to the other by passing through a cavity in the transporter protein. The substance is thus transported either into or out of the cytoplasm of the BBB cell; a second set of transporters on the opposing cell membrane completes the transfer across the BBB or the substance can rely on transmembrane diffusion. With vesicular transport, the transported substance adheres to a binding site, usually a glycoprotein. Invagination then produces a vesicle that is then routed to the opposite membrane, and the contents of the vesicle are released from the cell surface. A specificity of transport distinguishes these vesicles from the macropinocytosis whose reduction is a defining characteristic of the BBB (Reese and Karnovsky 1967).

Most small molecules, such as glucose, electrolytes, and amino acids, use pores or channels. Pore systems may be either active or facilitated diffusion systems. Vesicular transporters, on the other hand, are energy requiring and so are characterized by unidirectional transport. The best described of these vesicular dependent systems is receptor-mediated transcytosis and is characterized by clathrin- and transglutaminase-dependence (Davies et al. 1980). However, non-clathrin dependent vesicles, such as podocytes, are also likely active at the BBB.

It is reasonable to assume that very large molecules would be required to use vesicles rather than pores and channels to cross, but the molecular weight at which vesicles would be requisite is not known. It has been proposed that interleukin-2 (IL-2) is transported (Drach et al. 1996) by p-glycoprotein (P-gp). As P-gp is a pore system (Begley 2004), IL-2 would be the largest substance currently known to be transported by a pore system. Peptides much smaller than IL-2 are known to cross by vesicular dependent pathways (Shimura et al. 1991; Terasaki et al. 1992). It is clear, then, that the size of the ligand alone does not dictate the need for vesicular transport.

Diapedesis of Immune Cells

A major shift in thinking about the relation of immune cells to the CNS and BBB has occurred over the last few decades. The CNS was once viewed as separate from the immune system and sterile in terms of immune cell occupancy except under conditions of brain infection. As reviewed above, it is now clear that immune cells patrol the normal CNS, although many important questions remain. For example, a major type of brain cell, the microglia, is known to be derived from peripheral macrophages, although the extent to which the pools of peripheral macrophages and microglia mix in the normal postnatal condition is unknown.

Adsorptive Endo- and Trans-cytosis

Adsorptive endocytosis occurs when a glycoprotein on the brain's endothelial surface binds another glycoprotein in ligand like fashion (Broadwell et al. 1988; Broadwell 1989). This second glycoprotein (the ligand) may be free or attached

to the surface of a virus or immune cell (Mellman et al. 1986). The binding can initiate endocytosis with the subsequent vesicle having several potential fates (Banks and Broadwell 1994). In some cases, the vesicle is routed to lysosomes, the glycoprotein destroyed, and the vesicle rerouted to the endothelial cell surface for discharge of contents. In other cases, the vesicle can be routed to the Golgi complex and endoplasmic reticulum. In other cases still, the vesicle can be discharged at the endothelial cell surface opposite to that of uptake. In this case, the vesicle has crossed the width of the endothelial cell, and hence crossed the BBB, in a transcytotic event. What determines the fate of these vesicles is largely unknown, but at least some vesicles can engage in more than one fate (Broadwell 1993). It may be that binding of a large amount of glycoprotein to the endothelial cell can overwhelm the lysosomal pathway and result in the vesicles being routed to the transcytotic or Golgi complex pathways.

Several principles of adsorptive endocytosis and transcytosis (also termed adsorptive-mediated transcytosis) are clear. Many of the glycoprotein ligands are toxic, and endocytosis may represent a mechanism to rejuvenate or repair the membrane (Raub and Audus 1990; Vorbrodt 1994; Westergren and Johansson 1993). Viruses and other pathogens that can infect or cross the BBB have often co-opted adsorptive endocytosis/transcytosis mechanisms (Marsh 1984; Chou and Dix 1989; Schweighardt and Atwood 2001). These processes may also be related to diapedesis as many of the events of immune cell passage across the BBB resemble these endocytic mechanisms. For example, both LFA-1 (leukocyte function-associated antigen-1) and ICAM (intercellular adhesion molecule), important to immune cell passage across the BBB, are glycoproteins. Although adsorptive endocytosis is in some sense saturable because of a finite amount of any single glycoprotein on a cell surface, it is not easy to demonstrate classical saturable kinetics for this process. In fact, excess glycoprotein can sometimes further stimulate endocytosis and so lead to a paradoxical increase, rather than decrease, in the rate of passage across the BBB (Banks et al. 1997). Glycoprotein distribution on brain endothelial cells is polarized; that is, a glycoprotein may be enriched on either the luminal or abluminal membranes (Vorbrodt 1994; Zambenedetti et al. 1996). The tight junctions act as a "fence" to keep the glycoproteins confined to their respective sides of the endothelial cell (Deli et al. 2005). This means that the movement of a glycoprotein molecule (or a virus whose coat displays that glycoprotein) can be unidirectional as its transcytosis can only be initiated from the side of the brain endothelial cell that contains the ligand's complementary glycoprotein (Villegas and Broadwell 1993; Broadwell 1989). The possession and distribution of glycoproteins similarly dictate which viruses can invade the brain; neurovirulent viruses that invade the brain as free virus (as opposed to entering in Trojan horse fashion inside an infected

immune cell) can do so because they possess a glycoprotein ligand capable of binding to the BBB.

Other molecules besides glycoproteins can also induce adsorptive endocytosis/transcytosis type mechanisms. A classic example is polycationic molecules such as the poly-L-lysines and the protamines. Protamines are peptides of about 30 amino acids that contain an abundance of arginine molecules. They can induce adsorptive transcytosis so vigorously as to result in BBB disruption (Vorbodt et al. 1995; Hardebo and Kahrstrom 1985). One of the proofs that viruses co-opt adsorptive transcytosis like mechanisms is that they, like protamine, bind to heparins and heparans; indeed, protamine sulfate blocks viruses such as HIV-1 from binding to the BBB (Banks et al. 2004c; Ramos-Kuri et al. 1996; Bobardt et al. 2004). Many of the highly charged penetrating peptides, such as those derived from Tat, and many of the antibodies that target receptor-mediated transporters likely are taken up by adsorptive endocytosis/transcytosis related mechanisms (Niewoehner et al. 2014; Weissmann 1976; Herve et al. 2008).

2.2.2 CNS to Blood

Traditionally, passage in the brain-to-blood direction (efflux) has been neglected. However, efflux often accounts for the inability of otherwise effective drugs to accumulate in the CNS. Pharmacogenomic studies have suggested that the individual variation in efflux mechanisms may explain why some individuals are less sensitive to the CNS effects of drugs or more sensitive to their toxicities (Loscher and Potschka 2002). Efflux mechanisms are important to the homeostasis of the CNS, ridding the brain of toxins (Taylor 2002). The rate of efflux can be, in addition to synthesis and degradation, an important determinant of the level of a substance produced within the CNS (Chen et al. 1997; Chen and Reichlin 1998; Maness et al. 1998).

2.2.2.1 Nonsaturable

Efflux, like influx, has both saturable and non-saturable mechanisms of entry. Transmembrane diffusion occurs for both influx and efflux. Other mechanisms, such as bulk flow, are unique for efflux.

Transmembrane Diffusion

Many of the principles that govern influx by transmembrane diffusion are also important in efflux. The dramatic role that efflux by transmembrane diffusion can play can be illustrated by comparing the fate of small, lipid soluble molecules to that of a protein after intrathecal administration. Intrathecal application of small, lipid soluble molecules, such as anesthetics, can have a local effect on spinal cord function but have little or no effect on the brain (Bernards 1999). These substances readily cross the brain endothelial

cell by transmembrane diffusion and do this as easily in the brain-to-blood direction as in the blood-to-brain direction. Therefore, they are cleared from the CSF before they are able to reach the brain (McQuay et al. 1989). In contrast, proteins such as leptin and lysosomal enzymes are too large and water soluble to undergo significant transmembrane diffusion (McCarthy et al. 2002; LeBel et al. 1999). Leptin, tetanus antitoxin, and the lysosomal enzyme idursulfase can reach the brain after intrathecal administration in amounts sufficient to produce CNS effects (Calias et al. 2012; McCarthy et al. 2002; Kabura et al. 2006; LeBel et al. 1999).

Efflux by transmembrane diffusion can also contribute to the poor diffusion of substances within brain parenchyma. Diffusion within the interstitial space of the brain is dependent on Brownian motion and the production of metabolic free water as driving forces and so is very slow (Cserr 1984; Cserr and Berman 1978). However, efflux by non-saturable (and saturable) mechanisms can further reduce the distance a substance will ultimately diffuse. For example, the less lipid soluble drug atenolol can diffuse about three times further into brain tissue than can the more lipid soluble drug acetaminophen (de Lange et al. 1993).

Bulk Flow and the Glymphatic System

Bulk flow refers to the reabsorption of CSF into the blood, which occurs at the level of the arachnoid villi (Davson and Segal 1996a) and cribriform plate (Widner et al. 1987; Yamada et al. 1991). Any substance dissolved in CSF will enter the blood by this mechanism (Pollay and Davson 1963; Jones and Robinson 1982). The glymphatic system provides an important mechanism for the mixing of the CSF and brain interstitial fluid, with aquaporin-dependent fluid production at the astrocytes providing the circulant and arteriole pulsations providing the directionality (Iliff et al. 2012). Thus, the glymphatics are important not only for bulk flow, but also for the extracellular pathways, and possibly for the movement of CSF from the spinal cord into the cranium.

The characteristics of this system result in several surprising but important phenomena. For example, CSF drained at the cribriform plate, which is likely the dominant route for CSF drainage at normal CSF pressures (Boulton et al. 1999), can enter into the cervical lymphatic system. This can provide a direct route from the CNS to the cervical lymphatics (Oehmichen et al. 1979), as has been illustrated for gp120, the glycoprotein of the human immunodeficiency virus, HIV-1 (Cashion et al. 1999). This route to the lymphatics may explain why substances injected into the brain can produce a different immune response than when the substance is injected peripherally (Knopf et al. 1995; Cserr and Knopf 1992). Another example is that in some cases, the levels of a substance in blood achieved after injection into the CSF can be sustained longer and at higher levels than after an intravenous bolus (Maness et al. 1998; Chen

et al. 1997; Chen and Reichlin 1998) This is because the central injection acts similarly to an intravenous infusion, slowly delivering drug to the blood. Impairment in glymphatic circulation can result in decreased bulk flow and so may contribute to increased levels of protein and toxins in the CSF (Iliff et al. 2013).

2.2.2.2 Saturable Transport

The last decade has seen a huge increase in the interest of efflux by saturable mechanisms. Just as efflux by transmembrane diffusion can limit diffusion of a substance within the CNS, so can the presence of a saturable efflux transporter (Blasberg 1977). Much of this interest centers on the multi-drug efflux transport systems (Begley 2004), most notably p-glycoprotein (P-gp). However, other efflux transporters for peptides, proteins, endogenous substances, and drugs are known to play important roles in physiology and disease (Martins et al. 1997; Taylor 2002; Drion et al. 1996; Mealey et al. 2001). For example, peptide transport system-1 is a major regulator of brain levels of methionine enkephalin, an endogenous opiate which suppresses voluntary ethanol drinking (Plotkin et al. 1998). Depression and recovery of peptide transport systems-1 with ethanol drinking may relate to alcohol withdrawal seizures (Banks and Kastin 1989, 1994). IL-2 is currently the only cytokine known to be transported by a saturable efflux system (Banks et al. 2004b); some have postulated this transporter may be P-gp. Poor accumulation of protease inhibitors, antibiotics, AZT, anti-cancer drugs, and many other substances occurs because of efflux systems (Glynn and Yazdanian 1998; King et al. 2001; Lee et al. 1998; Loscher and Potschka 2002; Masereeuw et al. 1994; Spector and Lorenzo 1974). P-gp plays a major role in the efflux of intrathecally administered opiate analgesics (Thompson et al. 2000). Brain-to-blood transport of a corticotropin-releasing hormone is sufficient to influence splenic levels of beta-endorphin (Martins et al. 1997). Impaired efflux of amyloid β peptide, the peptide believed to cause Alzheimer's disease, develops with aging in mice which overexpress amyloid precursor protein, thus promoting further accumulation within brain of amyloid β protein (Gherzi-Egea et al. 1996; Banks et al. 2003; Deane et al. 2004). Evidence suggests that impaired transport develops in humans as well and so may be a major mechanism for induction of Alzheimer's disease (Tanzi et al. 2004; Shibata et al. 2000).

2.3 Neuroimmune Interactions

The above discussion of BBB fundamentals is tailored towards understanding the role of the BBB in neuroimmune interactions. Below are specific examples of how the BBB is involved in neuroimmune interactions.

2.3.1 Binding Sites at the BBB: Receptors and Transporters

An important distinction for understanding the function of the BBB is that of receptors vs transporters. The term "receptor" has undergone a transformation of its usage since its introduction in the late nineteenth century when it was first used to denote some physiological function. Eventually, the term receptor was used to denote a physical binding site through which a drug or hormone could exert its effects on a cell. In the 1980s, a distinction was made between "receptor" and "binding site", the former being coupled to intracellular machinery that translated its binding into a cellular effect. Binding sites on the brain endothelial cell can represent transporters, but they can also represent traditional receptors, that is, binding sites coupled to intracellular machinery. For example, brain endothelial cells have both insulin receptors and transporters. As a result, insulin is transported across the BBB to exert effects inside the CNS, but insulin also alters a number of functions of the brain endothelial cell. As examples of the latter, insulin alters the BBB transport of zidovudine (AZT (Ayre et al. 1989), tryptophan (Cangiano et al. 1983), and leptin (Kastin and Akerstrom 2001) and alters brain endothelial cell alkaline phosphatase activity (Catalan et al. 1988). BBB studies have assumed that a binding site represents transporter function and are so designed as to not consider whether receptors as well as transporters may exist at the BBB. However, a great deal of indirect evidence and some direct evidence indicates that the vascular BBB and the choroid plexus probably possess a large variety of receptors that can alter BBB functions. Besides insulin, substances which bind to and alter the function of brain endothelial cells include mu opiate receptor ligands (Baba et al. 1988; Vidal et al. 1998; Chang et al. 2001), cytokines (Ban et al. 1991; Cunningham et al. 1992; van Dam et al. 1996; Vidal et al. 1998; Moser et al. 2004; Khan et al. 2003), leptin (Kastin et al. 2000; Bjorbaek et al. 1998; Hsueh et al. 2013a), acetylcholine (Grammas and Caspers 1991), adrenergics (Walsh et al. 1987; Kalaria and Harik 1989), glutamate (Koenig et al. 1992; Krizbai et al. 1998), and chemokines (Sanders et al. 1998).

2.3.2 Permeability to Cytokines and Related Substances

The BBB is known to transport several cytokines in the blood-to-brain direction. For example, the BBB transports the IL-1s, IL-6, and TNF- α by three separate transport systems. Additionally, nerve growth factor, brain derived neurotrophic factor, interferons, neurotrophins, eotaxin, fibroblast factor 19, and leukemia inhibitory factor (Poduslo

and Curran 1996; Erickson et al. 2014; Hsueh et al. 2013b; Pan et al. 1997b, 1998a, b) are also transported across the BBB. In some cases, the same gene which gives rise to a cytokine's receptor also produces the cytokine's transporter; whereas in other cases, the receptor and transporter are different proteins (Pan and Kastin 2002; Banks et al. 2002). Recently, a BBB transporter for pituitary adenylate cyclase activating polypeptide (PACAP) was found to be the same protein which acts as a neuronal receptor for enterostatin, but not for PACAP, and acts as a lipid transporter in the liver (Martinez et al. 2003; Park et al. 2004; Dogrukol-Ak et al. 2009). In general, BBB transporters occur throughout the CNS, including the spinal cord, although the transport rate across the BBB can vary greatly among CNS regions (McLay et al. 1997; Pan et al. 1997b, 1998b; Banks et al. 1994). Enough cytokine is transported into the brain to affect CNS function. For example, IL-1 α crosses the BBB at the posterior division of the septum where it mediates cognitive impairments (Banks et al. 2001). Similarly, serum TNF- α crosses the BBB to induce CNS release of TNF- α , which in turn can induce apoptosis in the substantia nigra (Qin et al. 2007).

The cytokine transporters are not static but respond to physiological and pathological events. The transport rates of IL-1 and TNF- α each show diurnal variations (Pan et al. 2002; Banks et al. 1998b). The transport rate of TNF- α is altered in animals with experimental allergic encephalomyelitis (EAE), spinal cord injury, or blunt trauma to the brain (Pan et al. 1996, 1997a, 2003b; Pan and Kastin 2001b; Pearce et al. 2003).

2.3.3 Permeability to Other Neuroimmune Substances

Other substances with neuroimmune actions are handled by the BBB in a variety of ways. Monoamines are largely excluded by the BBB (Hardebo and Owman 1990; Kalaria et al. 1987), and opiates and opiate peptides as a rule enter the brain by transmembrane diffusion but are transported by saturable systems in the brain-to-blood direction (King et al. 2001; Banks and Kastin 1990; Elferink and Zadina 2001). Pituitary adenylate cyclase activating peptide, a member of the VIP/secretin/PACAP family, has immune functions (Arimura 1992). Transport of its two major forms across the BBB is complex, involving both brain-to-blood and blood-to-brain components (Banks et al. 1993). Its blood-to-brain transport is altered with CNS injury (Somogyvari-Vigh et al. 2000). Some of the other immune active substances whose passage across the BBB has been investigated are melanocyte stimulating hormone (Martins et al. 1996; Wilson et al. 1984), corticotrophin releasing hormone (Martins et al. 1996), and enkephalins (Banks et al. 1986; Elferink and Zadina 2001).

2.3.4 Permeability to Immune Cells

As discussed above, immune cells cross the BBB by the highly regulated process of diapedesis. The mechanism by which immune cells cross the BBB has also been greatly clarified by recent work. Two major assumptions about how immune cells would enter the CNS has not withstood investigation. The first assumption was that immune cells would enter the CNS by leaking across a disrupted BBB. However, disruptions to the BBB are usually mediated by increased vesicular activity in the endothelial cells (Vorbodt et al. 1995; Lossinsky et al. 1983; Mayhan and Heistad 1985). These vesicles of 100 nm or so could not accommodate the passage of an immune cell 10,000 nm in diameter. Even in diseases where there is both increased immune cell trafficking into the CNS and a disrupted BBB, there is often a mismatch between the site of immune cell entry and BBB disruption (Engelhardt and Wolburg 2004).

The second major assumption is that immune cells would cross between opposing endothelial cells taking the "paracellular route." However, evidence suggests that many immune cells favor a transcellular route and that cells can cross both the vascular BBB and the choroid plexus (Kivisakk et al. 2003; Wolburg et al. 2005). In brief, immune cells tunnel through venular endothelial cells leaving the intercellular tight junctions intact (Engelhardt 2008; Wolburg et al. 2005). This tunneling process is complex and is initiated when LFA-1 on an immune cell binds to ICAM on the brain endothelial cell. Other paracellular messengers, which likely include cytokines, are then released (Male 1995; Persidsky et al. 1997). Protrusions and invaginations of the endothelial cell and protrusions of the immune cell occur, with the immune cell possibly using the tight junction as an initial anchoring site (Lossinsky et al. 1991). Other ligands which have been postulated to play a role in this transcytotic process include PECAM, VE-cadherin, members of the JAM family and CD99 (Engelhardt and Wolburg 2004). Some plasma inevitably accompanies the passage of the immune cells, which can give the appearance of a disrupted BBB (Greenwood et al. 1995; Avison et al. 2004; Persidsky et al. 2000).

The immune processes that induce an immune cell to cross the BBB are complex. Quan has shown that injection of interleukin-1 into brain tissue induces immune cell trafficking, but that such induction can be blocked by injection of lipopolysaccharide (LPS) into the periphery (Ching et al. 2005, 2006; Quan et al. 1994). Although trafficking is a product of a complex interaction between the immune cell and the BBB, the degree to which trafficking occurs can reside primarily with the immune cell or the BBB and genetics or immune events can shift that dominance (Banks et al. 2012). Such events may underlie eufinflammation, the phenomenon by which subclinical activation of the innate immune system renders it increasingly resistant to such activation by increasingly stronger stimuli (Tarr et al. 2014).

2.3.5 Permeability to Viruses

Whether a virus is neurovirulent or not depends largely on its ability to cross the BBB (Chou and Dix 1989). However, viruses can induce neurotoxicity without themselves crossing the BBB by several mechanisms. For example, shed viral proteins might cross the BBB as could circulating cytokines whose release from peripheral sources is induced by the virus. However, most neurovirulent viruses seem to do their major damage directly after entering and replicating within the CNS. Some viruses can replicate within brain endothelial cells and are subsequently shed into the CNS (Cosby and Brankin 1995). Other viruses invade the CNS by crossing the BBB (Nakaoka et al. 2005). Initial uptake by either route involves events reminiscent of adsorptive endocytosis as discussed above. Viral glycoproteins bind to brain endothelial cell (or choroid plexus) glycoproteins to initiate adsorptive endocytosis. As with adsorptive endocytosis, the virus-containing vesicle is subsequently routed to various membrane systems that can include discharge to the original side of uptake or transcytosis. Sialic acid and heparan sulfate are common components of the glycoproteins involved in viral uptake by the BBB (Schweighardt and Atwood 2001; Banks et al. 2004c). In some cases, the functional glycoprotein is known. For example, rabies can bind to acetylcholine and nerve growth factor receptors (Schweighardt and Atwood 2001), and HIV-1 can bind to the mannose-6 phosphate receptor. Without an appropriate luminal or basal glycoprotein with which to bind to the BBB cell, the virus is largely excluded from the CNS.

Other principals reviewed above can play key roles in viral uptake by the brain. Activation of the immune system increases the transport of HIV-1 and other viruses across the BBB (Dohgu and Banks 2008; Chou and Dix 1989; Cosby and Brankin 1995). In the case of HIV-1, this involves inducing secretion of GM-CSF (granulocyte-macrophage colony-stimulating factor) and IL-6 from brain endothelial cells with the cytokines acting in an autocrine fashion to promote the transcytosis of the virus (Dohgu et al. 2011). This process is not dependent on pericytes but is enhanced by it (Dohgu and Banks 2013). Eventually, the immune mechanisms combine to produce the cognitive and behavioral problems seen in AIDS (Hong and Banks 2014).

2.3.6 Secretion of Neuroimmune-Active Substances

The brain endothelial cells and the epithelial cells of the choroid plexus are capable of secreting a large number of neuroimmune active substances. These include interleukins (Fabry et al. 1993; Hofman et al. 1999; Reyes et al. 1999), TNF- α (Lee et al. 2001), nerve growth factor, (Moser et al.

2004), endothelin (Didier et al. 2002), monocyte chemoattractant peptide (Chen et al. 2001), nitric oxide (Mandi et al. 1998), RANTES (Simpson et al. 1998), and prostacyclin (Faraci and Heistad 1998). Some of these substances are secreted spontaneously, and many of them can be stimulated with immunoactive substances such as LPS, bacteria, or viral proteins (Reyes et al. 1999; Hofman et al. 1999; Vadeboncoeur et al. 2003). The unique architecture of the BBB allows it to receive input from one of its surfaces and to secrete substances into the other. For example, LPS applied to the abluminal surface of brain endothelial cells in monolayer cultures will enhance release of IL-6 from the luminal surface (Verma et al. 2006).

2.3.7 Modulation of BBB Function by Neuroimmune Substances

Traditionally, neuroimmune modulation has been thought of in terms of disruption of the BBB. However, as the review above indicates, transporter functions are also vulnerable to manipulation by neuroimmune elements. Alterations in transport function are likely to be more common events than disruption, as the latter is likely seen only with extreme pathology; whereas, the former is likely a physiological, as well as a pathological, aspect of neuroimmune regulation. Other functions of the BBB, such as brain endothelial cell secretions are also clearly affected by neuroimmune events.

2.3.7.1 Agents That Increase Permeability of the BBB

Disruption was the first BBB function noted to be perturbed in neuroimmune disease. However, the review above makes it clear that an increase in BBB permeability can be induced for specific agents by increasing their blood-to-brain transport rate or inhibiting their brain-to-blood efflux rate. An additional observation is that the BBB does not respond to immune activation through a single pathway nor even responds uniformly to an activated pathway. For example, indomethacin blocks the BBB disruption induced by LPS, suggesting that disruption involves prostaglandins (Minami et al. 1998a). In contrast, indomethacin enhances the LPS-induced increase in HIV-1 penetration across the BBB and has no effect on the LPS-induced increase in insulin transport (Xaio et al. 2001; Banks et al. 1999).

2.3.7.2 Regulation of BBB Integrity and Tight Junction Function

The classic example of BBB disruption is that seen in multiple sclerosis and the animal model of that disease, EAE (Pozzilli et al. 1988; Butter et al. 1991; Juhler et al. 1984). LPS and treatment with cytokines such as TNF- α have also been shown to induce BBB disruption (Megyeri et al. 1992). As discussed

above, paracellular (through tight junctions) and transcellular mechanisms of transport exist. Although either can underlie BBB disruption, classic studies have shown that the major cause of increased protein leakage across the BBB for almost every kind of insult to the BBB or to the CNS is mediated by transcytotic mechanisms (Lossinsky et al. 1983; Vorbrodt et al. 1995). Nevertheless, recent advances in understanding tight junction assembly and regulation have encouraged many to investigate paracellular mechanisms. Both tight junction function and transcytosis are regulated events, although it is unclear to what extent protein leakage into the brain may be altered under physiological conditions. To some extent, paracellular and transcytotic routes likely involve some of the same cellular machinery, such as the cytoskeleton. TNF- α is known to induce rearrangements in cytoskeletal architecture. Additionally, cerebral ischemia, diabetes mellitus, and even intense pain are associated with alterations in the expression and cellular distribution of tight junction proteins and opening of the BBB (Brown and Davis 2002; Chehade et al. 2002; Huber et al. 2002). The importance of regulatory processes in BBB disruption is vividly illustrated by the paradoxical finding that maximal disruption does not occur at the time of the CNS injury, even when the event is traumatic but hours or days later (Baldwin et al. 1996). It is thought that it is the peripheral and central responses, such as cytokine release, to CNS injury rather than the CNS injury itself that results in BBB disruption.

2.3.7.3 Regulation of Saturable Transporters

Regulation of both influx and efflux transporters are influenced by neuroimmune events. Additionally, cytokine transporters are also affected by various CNS events.

LPS and Blood-to-Brain Transporters

LPS increases the transport across the BBB of cisplatin, insulin, leukemia inhibitory factor, and the HIV-1 viral coat glycoprotein gp120, but not of TNF- α or pituitary adenylate cyclase activating polypeptide (Banks et al. 1999; Pan et al. 2008b; Minami et al. 1998b; Nonaka et al. 2005; Osburg et al. 2002; Xaio et al. 2001). LPS affects leptin transport (Nonaka et al. 2004) through peripheral mechanisms and increases pituitary adenylate cyclase activating polypeptide binding to receptors on the BBB but does not alter transport. CNS injuries such as ischemia or trauma to the spinal cord induce a cascade of events that can affect the transport of neuroimmune substances across the BBB as discussed below.

LPS, Cytokines, and Brain-to-Blood (Efflux) Transporters

Immune modulators also alter efflux systems. In vitro studies show that LPS, TNF- α , and interferon gamma regulate P-gp (Salkeni et al. 2009; Theron et al. 2003; Hartz et al. 2006; Yu et al. 2007a, b; Stein et al. 1996; Bauer et al. 2007). In vivo studies have shown that LPS down regulates P-gp function at the

BBB (Salkeni et al. 2009). Interestingly, IL-2 appears to be both a substrate for P-gp and a modulator of its activity (Bonhomme-Faivre et al. 2002; Castagne et al. 2004; Drach et al. 1996). Because P-gp regulates the brain concentration of so many drugs and endogenous substances, immunomodulation could affect many other responses. For example, brain levels of exogenous opiate drugs such as morphine (King et al. 2001), endogenous opiates such as β -endorphin (Kastin et al. 2002), and neurotoxins such as cyclosporine (Sakata et al. 1994) would all be expected to be increased in patients given IL-2. Immunomodulation of efflux systems, therefore, could have a major effect on CNS metabolism and the response to drugs.

Activity of low-density lipoprotein receptor-related protein-1 (LRP-1) is also modulated by LPS. LRP-1 at the BBB acts as an efflux pump to amyloid beta protein (Deane et al. 2008), the substance associated causally with Alzheimer's disease. The neurovascular hypothesis states that decreases in LRP-1 activity at the BBB contributes to loss of clearance of amyloid beta protein from brain and so promotes Alzheimer's disease (Zlokovic 2005). Inhibition of LRP-1 activity leads to decreased efflux of amyloid beta protein, increases in levels of amyloid beta protein in the brain, and cognitive impairment (Jaeger et al. 2009b). Mice treated with LPS have decreased efflux of amyloid beta protein, providing a mechanism for connection between inflammatory processes and Alzheimer's disease (Jaeger et al. 2009a).

2.4 Role of the BBB in Neuroimmune Diseases

The above review has emphasized BBB/neuroimmune interactions under normal physiological conditions. However, the BBB is intimately involved in neuroimmune diseases as well. The BBB can be a target of such disease, its functions may be adaptive to disease, or it can be a contributor to the disease process. Below are some examples of the ways in which the BBB is altered in diseases with neuroimmune processes.

2.4.1 TNF- α Transport and EAE

TNF- α has a biphasic effect on many neuroimmune processes, with too little or too much producing harmful effects (Pan et al. 1997c). TNF- α mediates many of its pathological effects through its central receptors, and transport of circulating TNF- α is one source of CNS TNF- α (Pan and Kastin 2002; Gutierrez et al. 1993; Osburg et al. 2002). Induction of EAE is partially dependent on TNF- α and IL-1 (Schiffenbauer et al. 2000). Immune cell invasion in general and during EAE in particular is dependent on TNF-modulated expression of ICAM and VCAM on brain endothelial cells and of LFA-1 on immunocytes (Male 1995; Barten and Ruddle

1994). Finally, the saturable transport across the BBB of TNF- α itself is greatly increased in EAE (Pan et al. 1996).

2.4.2 CNS Injuries and Cytokine Transport

As noted above, CNS injuries can produce a disruption of the BBB, but this disruption is temporally dissociated from the injury (Pan et al. 1997a; Banks et al. 1998a; Baldwin et al. 1996). This dissociation is because the disruption is the consequence of the reactions to injury rather than to injury itself. Not surprisingly, then, CNS injuries can also produce complex alterations in the BBB transport of cytokines. Besides the example of TNF- α in EAE given above, TNF- α transport is also increased in spinal cord injury (Pan et al. 1997a, 2003b; Pan and Kastin 2001b) and after blunt trauma to the brain (Pan et al. 2003a). This increase is neither confined to the site of injury, homogeneous throughout the CNS, nor related to the disruption pattern of the BBB (Pan et al. 1997a; Pan and Kastin 2001b; Xiang et al. 2005). It is also temporally and regionally independent of the changes in BBB transport rates of other cytokines and immunoactive substances whose transport rates are also altered with CNS injury (Banks et al. 1998a; Pan et al. 1998b). The pattern also is dependent on the type of CNS injury (Pan and Kastin 2001b).

2.4.3 Anti-retrovirals and the BBB

A major problem in treating viruses which can invade the CNS, such as HIV-1, is that antiretrovirals often cross the BBB poorly (Thomas 2004). The major problem, however, is not that these substances are especially limited by their rate of transcellular diffusion, but that they are nearly all substrates for efflux systems. For example, AZT is 16 times more lipid soluble than sucrose and so should cross much more rapidly, but actually crosses at the same rate (Wu et al. 1998). AZT is a ligand for at least two efflux systems (Masereeuw et al. 1994; Wang and Sawchuck 1995; Takasawa et al. 1997), and the protease inhibitors are all substrates for P-gp (Lee et al. 1998). P-gp is expressed by immune and other cells as well with three major phenotypic clusters in humans. Those with higher expression of P-gp, and therefore less able to accumulate protease inhibitors in tissues, are more resistant to treatment for HIV-1.

Conditions associated with AIDS further complicate the interaction between efflux systems and the uptake of anti-retrovirals by the brain. P-gp is modulated by inflammation (Yu et al. 2007b, 2008) and by gp120 (Ronaldson and Bendayan 2006). Many of the protease inhibitors induce P-gp expression and function, thus likely further reducing their ability to cross the BBB (Chan et al. 2013).

2.4.4 Immune Cell Invasion

Immune cell trafficking into the CNS is important in mediating neuroimmune diseases. Immune cell invasion is an early event in multiple sclerosis and EAE (Wolburg et al. 2005). Infected immune cells are a mechanism by which HIV-1 (Koyanagi et al. 1997; Nottet et al. 1996) and perhaps prions (Klein et al. 1997) invade the CNS.

Immune cell passage across the BBB is, in turn, affected by immune modulators. LPS and the HIV-1 immunoactive protein Tat increase expression by brain endothelial cells of ICAM and VCAM (Pu et al. 2003; Nottet et al. 1996), and monocytes treated with LPS have an increased rate of passage across the BBB (Persidsky et al. 1997). In vitro studies suggest that these events may be mediated through IL-1 β and IL-6 (De Vries et al. 1994).

2.4.5 Efflux of HIV-1 Proteins and Cytokines

Because the BBB prevents the effective accumulation of many of the antivirals, the CNS can act as a reservoir of virus. This reservoir could potentially reinfect the peripheral tissues. The CNS-to-blood movement of HIV-1 has not been investigated, but movement of two of its proteins has. The coat glycoprotein HIV-1gp120 is cleared through bulk flow (Cashion et al. 1999), being reabsorbed predominately at the cribriform. As a result, it drains by way of lymphatic vessels directly to the cervical lymphatic nodes. If whole virus also takes this route, then that means that lymph nodes could be directly reinfected without the virus having to enter the circulation where it could be exposed to antiviral agents.

A CNS reservoir of virus could affect the peripheral immune system by a mechanism that does not involve reinfection of peripheral tissues. HIV-1 Tat, like HIV-1gp120, is also reabsorbed with the CSF into the blood by a nonsaturable mechanism (Banks et al. 2005b). Proteins which are enzymatically resistant in blood, such as HIV-1 Tat, HIV-1gp120, and cytokines, can achieve high levels in blood even when their only source is the CSF. Production of these proteins within the CNS with subsequent reabsorption with the CSF into blood could be a way in which CNS virus produces toxic effects at peripheral tissues.

To date, IL-2 is the only cytokine known to be transported from the brain to the blood by a saturable transporter (Banks et al. 2004b). This transporter, along with binding to plasma proteins and robust degradation by the BBB or CNS, effectively prevents much IL-2 from entering the brain. Evidence suggests that this transporter is likely P-gp. P-gp activity is decreased with HIV encephalitis (Persidsky et al. 2000), and this could lead to blood-borne IL-2 entering the CNS. Chronically, however, the binding of IL-2 to plasma proteins extends its half-life, allowing time for it to accumulate in

brain by way of the extracellular pathways. Chronic IL-2 administration induces stereotypic behaviors and is used in an animal model of schizophrenia (Zalcman 2001, 2002). Therefore, an enhanced entry of IL-2 is one mechanism by which HIV-1 could induce behavioral changes.

2.5 Summary

The BBB intimately interacts with cells and their secretions that are in both the CNS and periphery. Some neuroimmune substances, exemplified by cytokines, can cross the BBB directly and also have direct effects on the BBB. The BBB is itself a source of neuroimmune substances and can receive signals from one side, for example the brain side, and release substances in response to that signal from its other side. The passage of immune cells across the BBB is a highly regulated event as is the passage of viruses and viral particles. Overall, the BBB is an important component of the neuroimmune axis and the only component that is simultaneously physically in both the peripheral and central compartments of the neuroimmune system.

2.6 Review Questions

- What are components of the blood-brain barriers:
 - vasculature
 - choroid plexus
 - Sertoli cells
 - tanycytes
 - a and c
 - a, b, and d
 - a, c, and d
- Which statement is true about the BBB transport systems for cytokines:
 - The IL-1s and IL-6 share a transport system
 - TNF is not transported across the BBB
 - IL-6 and TNF share a transport system
 - The IL-1s, IL-6, and TNF each have their own transport system*
 - IL-1 is only transported in the brain-to-blood direction
- The circumventricular organs are:
 - Regions of the brain that do express a robust BBB*
 - Are regions outside the brain but inside the skull
 - Completely encircle the ventricles and so prevent cerebrospinal fluid from entering the blood stream
 - Only occur in people who have circumnavigated the earth
- Transcellular diffusion refers to:
 - The mechanism by which immune cells cross the BBB
 - The mechanism by which small lipid soluble substances cross the BBB*
 - The process by which molecules diffuse from one neuron to another within brain interstitial fluid
 - The process by which brain endothelial cells containing two X chromosomes can express testosterone receptors
 - None of the above
- Which statement is true:
 - Receptors on brain endothelial cells never act as BBB transporters
 - Receptors on brain endothelial cells are always the same protein as that which acts as a substance's BBB transporter
 - The brain endothelial cell receptor and the BBB transporter are the same for some substrates and different for others*
 - The brain endothelial cells do not have receptors.
 - BBB transporters are never located on brain endothelial cells
- Immune cells cross the BBB:
 - By leaking across a disrupted BBB
 - By being engulfed by macropinocytotic vesicles
 - By interacting with brain endothelial cells in a complex way*
 - Only in disease states
 - By first binding to astrocytes
- The following statements are true about brain-to-blood transport (efflux):
 - Efflux only occurs for drugs
 - P-glycoprotein is an efflux system
 - All efflux systems require energy
 - A saturable transport system can have an efflux and an influx component
 - A saturable transport system can have only an efflux component
 - b, d, e
 - b, c, e

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Regulation of Nervous System Function by Circumventricular Organs

3

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Abstract

In this chapter, we highlight the specialized features of the sensory circumventricular organs (CVO) as central nervous system (CNS) structures located at the blood-brain interface. These structures appear to play critical roles in sensing and integrating information regarding autonomic status derived from circulating signals that do not readily cross the BBB. Intriguingly, while the majority of the original literature highlighting such roles attributed primarily fluid balance and cardiovascular functions to the subfornical organ (SFO) and metabolic function to the area postrema (AP), more recent work as highlighted in this chapter has clearly demonstrated, not only overlap in these physiological roles in SFO and AP, but also additional roles for these CVOs in reproductive and of primary importance to this chapter immune signaling from the circulation to the CNS. Within not only SFO and AP, but also the organum vasculosum of the lamina terminalis, the emerging literature supports the conclusion that single neurons in these CVOs sense, and presumably integrate, signals related to all of these separately classified autonomic functions. In recognizing the potential for such integration in the sensory CVOs, it becomes important to also understand that optimal health is associated with the ability of our physiological systems to regulate these functions in an integrated rather than separate manner.

Keywords

Area postrema • Blood brain barrier • Central autonomic control • Circulating signals • Circumventricular organ • Subfornical organ

3.1 Introduction

It is well accepted that the central nervous system (CNS) is the “command center” of the body, being the system that not only collects and integrates critical autonomic information coming from either the internal or external environment of the organism, but also initiates appropriate physiological responses. Information regarding these autonomic systems comes from a variety of sensors including thermoreceptors,

baroreceptors, chemoreceptors, as well as those monitoring fluid volume, metabolic state, and immune status. It is primarily conveyed in the form of signaling molecules released from one cell to influence the function of another, such as amino acids, peptides, gases, and larger macromolecules, delivered to their site of action in target cells by: the blood (hormones), synaptic terminals (neurotransmitters) or local mechanisms (paracrine). Neuronal systems (autonomic control centers) within the CNS detect these molecules and adjust physiological systems of the body accordingly in order to maintain the critical “milieu interieur” of a well regulated homeostatic system.

Importantly, many of these signaling molecules which originate in the periphery and play important roles in the central regulation of immune function (e.g. IL-1, IL-6,

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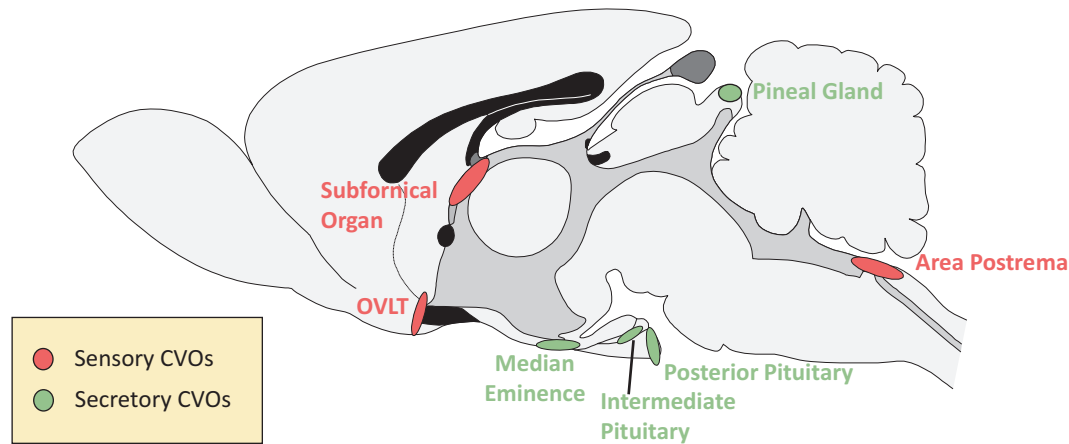


Fig. 3.1 Anatomical locations of the sensory and secretory circumventricular organs of the rat brain. Midsagittal section of the rat brain. CVOs circumventricular organs, OVLT organum vasculosum of the lamina terminalis

TNF α) are large and/or lipophobic and thus do not readily cross the blood-brain barrier (BBB), an endothelial/glial cell barrier separating the CNS from the periphery. The BBB is comprised of three layers of cells: the endothelial cells of the capillaries (this layer “faces” the interstitial fluid), the epithelial cells of the choroid plexus, and the arachnoid epithelium (an avascular layer of cells surrounding the CNS). The processes of the astroglial cells are also an important component. The cells forming these layers are connected via tight junctions and express transporters for specific molecules, both of which allow the BBB to fulfill its function of selectively excluding macromolecules and polar solutes (Abbott et al. 2006).

3.2 The Circumventricular Organs

In view of this lack of permeability, the BBB would make it difficult for the CNS structures protected by the barrier to detect critical signaling molecules in the general circulation, and thus nearly impossible for these structures to appropriately respond to these signals. How does the CNS then manage to interpret the information provided by the peripheral systems of the body? A few mechanisms of communication between the periphery and CNS exist, such as diffusion and active transport. However, the number of substances that are able to cross the BBB using either method is limited since diffusion only allows small, non-polar molecules to cross this CNS barrier. In contrast, larger lipophobic substances require a suitable transporter to cross the BBB and to date, few specific transporters have been identified.

A solution to this apparent dilemma presents itself in the form of the circumventricular organs (CVOs) of the brain. These specialized structures are highly vascularized areas of the brain whose capillaries do not contain the tight junctions

of the BBB but are instead fenestrated, meaning that gaps exist between the endothelial cells leading to the formation of Virchow-Robin Spaces (Price et al. 2008) that allows circulating blood containing large lipophobic signals to enter and pool, facilitating free exchange between the blood and nervous tissue of the body. The CVOs can be primarily classified as either secretory (neurohypophysis, median eminence, intermediate lobe, and pineal), or sensory (area postrema, subformical organ and organum vasculosum of the lamina terminalis) (Fig. 3.1), while the subcommisural organ does not fit readily into either of the categories and will not be discussed further here.

3.3 The Secretory CVOs

The primary focus of this chapter will be on the sensory CVOs, but we will first very briefly highlight roles of the secretory CVOs in the release of centrally synthesized lipophobic hormones directly into the circulation or cerebrospinal fluid. The secreted products are synthesized in parts of the brain that are protected by the BBB such as the supraoptic, paraventricular, and arcuate nuclei; and thus, these specialized areas of fenestrated capillaries permit the release of synthesized lipophobic products into general circulation. The neurohypophysis is the posterior lobe of the pituitary gland and secretes oxytocin and vasopressin, hormones involved in reproduction and fluid balance, directly into general circulation (Sofroniew 1983; Sofroniew et al. 1981). The intermediate lobe of the pituitary gland, which separates the anterior and posterior lobes of this endocrine gland in humans, synthesizes and secretes melanocyte-stimulating hormone (MSH). The median eminence (ME), perhaps the most important of the secretory CVOs, consists of neuronal terminals which “synapse” on the hypophyseal portal capillaries, the entry point for hypothalamic trophic

hormones controlling the extensive endocrine roles of the anterior pituitary. The hormones secreted by the ME include gonadotropin-releasing hormone, growth hormone-releasing hormone, dopamine, thyrotropin-releasing hormone, and corticotropin-releasing hormone. The pineal gland is the final secretory CVO which produces melatonin, a critical component in the regulation of circadian rhythms. It secretes this hormone not only into the circulation but also into the cerebrospinal fluid (Cassone et al. 1993).

3.4 Sensory CVOs

The sensory CVOs are, in many ways, contrasting structures to the secretory CVOs in that their primary function is to detect, rather than release, signals at the blood-brain interface. The three sensory CVOs are characterized by high expression levels of a variety of receptors and their resulting ability to integrate a multitude of signals. These are: the area postrema (AP), the subfornical organ (SFO), and the organum vasculosum of the lamina terminalis (OVLT). Another defining characteristic of these areas is the minimal afferent input they seem to receive relative to their extensive neural outputs. Additionally, most of the afferent input received by these three CVOs seems to come from the very structures onto which they project, indicating the probability of reciprocal communication between the sensory CVOs and the other structures. They are primarily known for their involvement in cardiovascular regulation and fluid balance; however, these structures have also been shown to be involved in the regulation of other processes such as energy metabolism, reproduction, and immune response.

3.4.1 Neuroanatomy and Connectivity of Sensory CVOs

The anatomy and connectivity of the CVOs provide valuable insight into their roles in the regulation of nervous system function. This section will outline the anatomical location and connections associated with the various sensory CVOs in order to provide a framework for the understanding of their central roles in immune function and regulation of autonomic state.

3.4.1.1 Subfornical Organ

The SFO is a highly vascularized, translucent midline structure protruding from the rostral wall of the third ventricle in the dorsal region of the lamina terminalis. It lies between the columns of the fornix, and its dorsal end is attached to the hippocampal commissure. It consists of a ventral stalk and dorsal crest, which connects to the median preoptic nucleus and the tela choroidea of the third ventricle, respectively (McKinley et al. 2003). The rich capillary network that sur-

rounds the SFO is formed by an anastomosis between branches of the anterior cerebral artery and the posterior choroidea artery. It is so dense that it must be peeled away during microdissection in order to see the SFO. Upon histological assessment, the SFO can be divided into three distinct areas based on morphology: The core region is the largest and is exclusively composed of neuronal cell bodies and glial cells. The rostral and caudal regions surround the core, and contain very few neurons and glial cells, consisting mainly of nerve fibers (Dellmann and Simpson 1979).

The major contributions to the current understanding of the SFO's neurocircuitry can be attributed to the comprehensive studies by the labs of Hernesniemi et al. (1972), Miselis (1981, 1982), and Lind et al. (1982). The earlier study used lesion techniques and Golgi staining methods (Hernesniemi et al. 1972), and the latter investigations used horseradish peroxidase injections to follow anterograde and retrograde transport of labeled proteins through axons (Miselis 1981, 1982; Lind et al. 1982). Evidence for these connections is supported in studies using electrophysiological stimulation (Ferguson and Bains 1996; Bains and Ferguson 1995).

A summary of the major and minor afferent and efferent neuronal connections to and from the SFO are outlined in Fig. 3.2. The major efferents can be grouped into two general areas:

3.4.1.2 The Neuroendocrine and Autonomic Control Centers of the Hypothalamus

Direct (monosynaptic) and indirect (polysynaptic) efferent projections terminate in the anterior and tuberal supraoptic nuclei (SONa and SONt), and the paraventricular nucleus (PVN) including its rostral accessory cluster, respectively. Electrophysiology studies demonstrated excitatory SFO projections to vasopressin- and oxytocin-secreting magnocellular neurons in the SON and PVN, as well as in parvocellular areas of the PVN, which, in turn, project either to the median eminence, the medulla, or the spinal cord. Many efferent fibers to the hypothalamus emerge from the rostral SFO and enter the columns of the fornix, diverge with the ventral stria medullaris to disperse medially and laterally over the columns of the fornix and along their dorsal border at the anterior dorsal level of the columns trajectory through the hypothalamus.

3.4.1.3 The Anteroventral Third Ventricular (AV3V) Area

This includes the median preoptic nucleus (MnPO), the anterior periventricular (Pe) area of the hypothalamus, and the organum vasculosum of the lamina terminalis (OVLT)—another sensory CVO to be discussed later. Many efferent fibers to this area emerge from the rostral SFO, pass anteriorly over the anterior commissure in the midline and either descend along the anterior border of the MnPO or enter the Pe dorsally just beneath the anterior commissure.

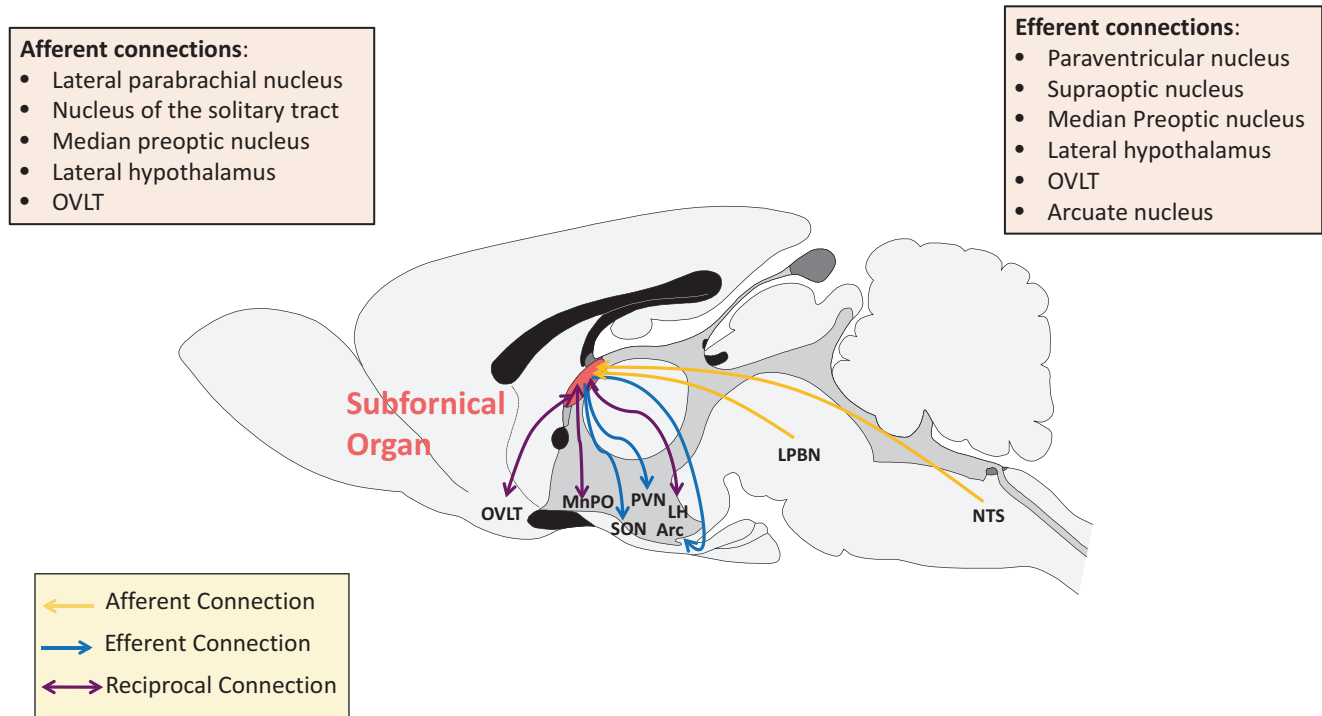


Fig. 3.2 Primary neuronal connections of the subfornical organ. A midsagittal section of rat brain representing major afferent and efferent projections of subfornical organ. Afferent pathways are represented by yellow arrows, efferent pathways are represented by blue arrows, and reciprocal projections are represented by purple arrows. *OVLT* orga-

num vasculosum of the lamina terminalis; Hypothalamic structures: *MnPO* median preoptic nucleus, *SON* supraoptic nucleus, *PVN* paraventricular nucleus, *LH* lateral hypothalamus, *Arc* arcuate nucleus; Hindbrain structures: *LPBN* lateral parabrachial nucleus, *NTS* nucleus of the solitary tract

It is important to note that dendritic trees of SFO neurons are relatively compact, and the extent of afferent connectivity is not nearly as elaborate as the efferents (Dellmann and Simpson 1979). These neuroanatomical findings suggest that there is reciprocal communication occurring between these brain regions and that perhaps the SFO's primary afferent information is received from circulating signals in the peripheral circulation as opposed to signals from other brain regions.

3.4.1.4 Organum Vasculosum of the Lamina Terminalis

The organum vasculosum of the lamina terminalis (OVLT) is a midline structure in the anterior wall of the third cerebral ventricle, located ventral to the MnPO and immediately dorsal to the optic chiasm. The OVLT, like the other sensory CVOs, contains a rich arterial blood supply and aggregation of neuronal cell bodies. In 1969, Duvernoy et al. published a detailed study illustrating the four major arterial sources of the human OVLT—a superior median source branching from the anterior communicating artery, two lateral sources from arteries which branch off from each anterior cerebral artery below the anterior communicating artery, and an inferior median source ascending from below the optic chiasm (Duvernoy et al. 1969).

The OVLT can be grouped into two major regions, the dorsal cap and the lateral regions, which each display unique projection

patterns. As is the case for the SFO, much of what is known about the neuronal connections to and from the OVLT can be attributed to a few key tracer studies conducted in the late 1970s and early 1980s that utilized the axonal transport of marker molecules, such as tritiated amino acids and horseradish peroxidase (HRP), in experimental models such as rats and sheep (Camacho and Phillips 1981; Phillips and Camacho 1987; Oldfield et al. 1991; ter Horst and Luiten 1986). The OVLT's main afferent and efferent projections are summarized in Fig. 3.3. Much like the SFO, the OVLT's dominant efferents project to magnocellular neurons of the PVN and SON (either directly or indirectly through the MnPO); and afferents are derived from the MnPO, SFO, and a variety of hypothalamic regions. Efferent connections have also been found to corticotrophin releasing factor-rich neurons in the PVN (Saper and Levisohn 1983). As in the case of SFO, outlined above, there is again a pattern of small dendritic trees of OVLT neurons, minimal afferent neural inputs, and reciprocal communication between afferent and efferent projection sites.

3.4.1.5 Area Postrema

The AP is a midline hindbrain structure located on the dorsal surface of the medulla oblongata, immediately adjacent to the NTS seen protruding into the fourth ventricle (Wislocki and Putnam 1920). The three regions of the AP include the *ventral zone*, which contains mostly glia (McKinley et al. 2003), and

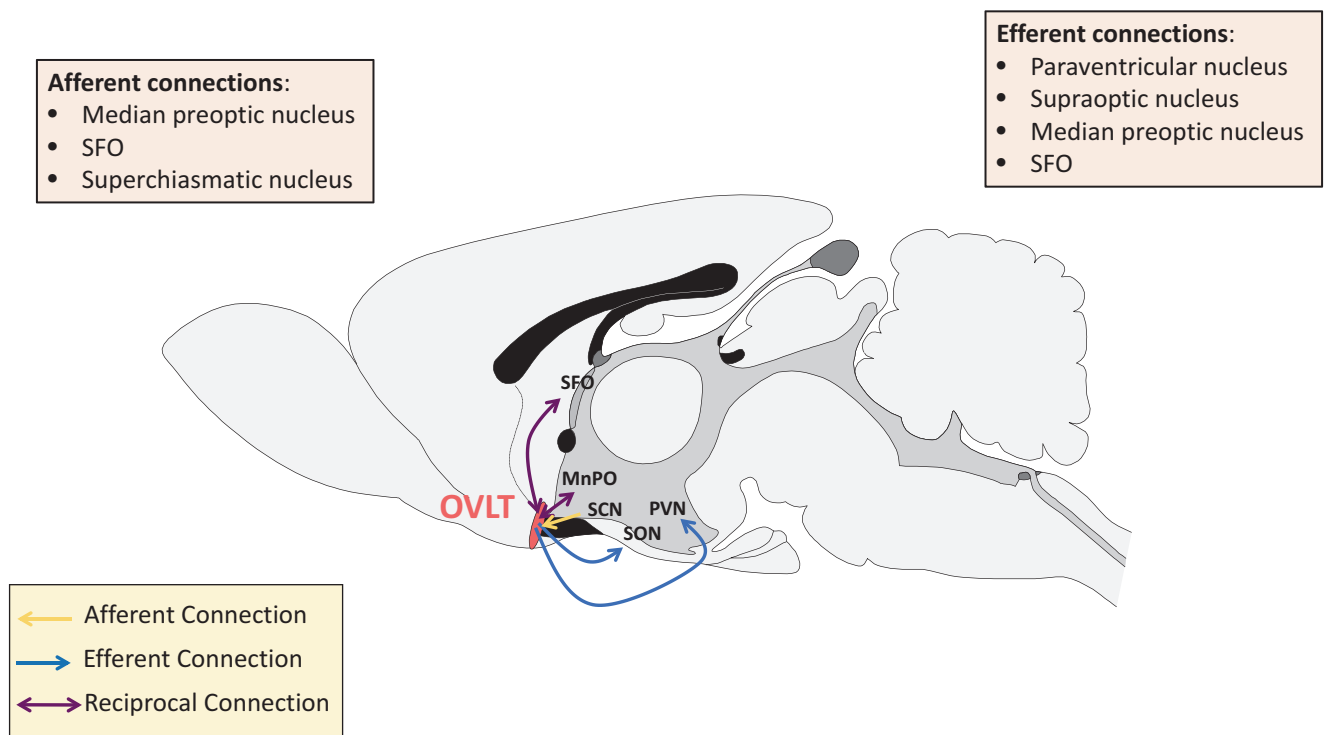


Fig. 3.3 Primary neuronal connections of the organum vasculosum of the lamina terminalis. A midsagittal section of rat brain showing major afferent and efferent projections of the organum vasculosum of the lamina terminalis (OVLT). Afferent pathways are represented by yellow

arrows, efferent pathways are represented by blue arrows, and reciprocal projections are represented by purple arrows. *SFO* subfornical organ; Hypothalamic structures: *MnPO* median preoptic nucleus, *SCN* suprachiasmatic nucleus, *SON* supraoptic nucleus, *PVN* paraventricular nucleus

the mantle zone and central zone, which are rich with neuronal cell bodies and axons situated next to ependymal cells. Separating the AP and NTS is a layer of tanycytes called the *funiculus separans* that function much like the BBB. Like the other two sensory CVOs, the AP is one of the most highly vascularized regions in the entire mammalian brain, showing a 150-fold increase in the surface area to permeability ratio when compared with the adjacent regions of the dorsomedial medulla (Gross 1991).

The AP was for decades known as the “chemoreceptor trigger zone” where noxious chemicals in the circulation acted to induce an emetic reflex (Borison and Brizzee 1951; Miller and Leslie 1994). However, despite this well studied functional role, more recent evidence suggests that the AP, like the other sensory CVOs, is also involved in various autonomic functions (to be discussed below).

Much of what is known about the anatomical connections of the AP is derived from retrograde tracing studies performed in the early- to mid-1980s. Using wheat germ agglutinin HRP (van der Kooy and Koda 1983) and cholera-toxin HRP (Shapiro and Miselis 1985), the studies illustrate the connections of the AP to and from various autonomic control centers in the medulla, pons, and hypothalamus. The AP has strong reciprocal connections to and from the lateral parabrachial nucleus of the pons (LPBN) and the NTS—two multifunctional integrative brainstem structures. It also receives

substantial input from the parvocellular regions of the paraventricular and dorsomedial nuclei of the hypothalamus, in addition to peripheral information from vagal and carotid sinus nerve afferents originating from the respiratory, gastrointestinal, and cardiovascular systems (Fig. 3.4).

Despite each sensory CVOs distinct neuroanatomy and afferent and efferent projection sites, the SFO, OVLT, and AP do share various similarities that provide us with a framework for understanding and further investigating their functional roles. Each lack a BBB, are highly vascularized, efficaciously located at an interface between the brain and the circulation, display elaborate efferent connections with few afferents, and express receptors for numerous circulating signals. Additionally, the main projection sites are reciprocated and target various secretory and integrative nuclei in the hypothalamus and brainstem that are involved in the regulation of broad ranging autonomic functions including cardiovascular control, body fluid homeostasis, reproductive function, energy homeostasis, and immune regulation. This neuroanatomical data suggests that the sensory CVOs are integrative structures involved in communicating various autonomic signals from the circulation to the central nervous system.

This concept has developed over the last few decades as a consequence of data derived from various experimental methods including electrophysiological whole-cell recordings from individual neurons both in vitro and in vivo

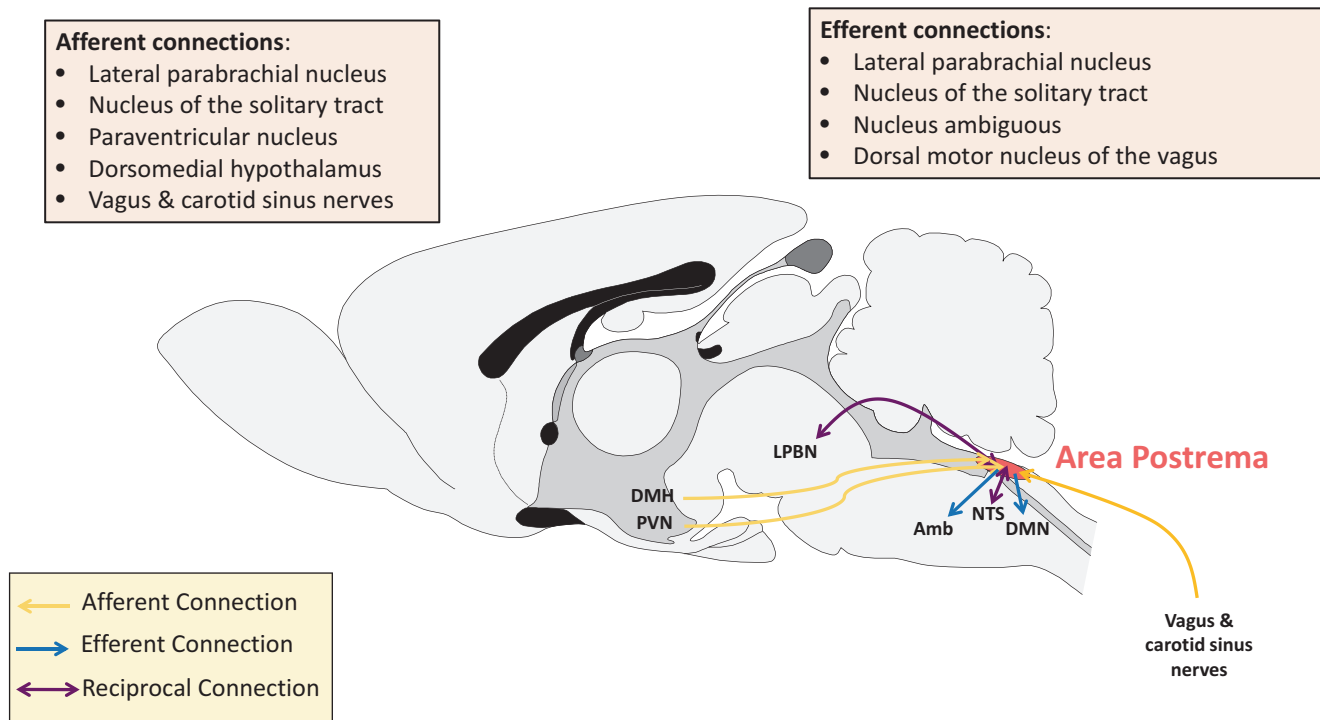


Fig. 3.4 Primary neuronal connections of the area postrema. A midsagittal section of a rat brain showing major afferent and efferent projections of the area postrema. Afferent pathways are represented by yellow arrows, efferent pathways are represented by blue arrows, and reciprocal projec-

tions are represented by purple arrows. Hypothalamic structures: *DMH* dorsal medial hypothalamus, *PVN* paraventricular nucleus; Hindbrain structures: *LPBN* lateral parabrachial nucleus, *Amb* nucleus ambiguus, *NTS* nucleus of the solitary tract, *DMN* dorsal motor nucleus of the vagus

recordings, behavioral studies using intraperitoneal injections of various circulatory signaling peptides or immune modulators, as well as electrical stimulation or lesion/ablation techniques to manipulate or disrupt signaling pathways originating in these CVOs. The following section will highlight these data to emphasize the critical role CVOs play in the regulation of autonomic and immune function.

3.5 CVO Functional Roles

Autonomic state is a term we have used to describe the combined physiological status of an organism, which results from the collective integrated homeostatic processes regulating multiple controlled variables at any one point in time. This incorporates metabolic rate, fluid levels, cardiac regulation, glucose levels, and immune function, to name a few, as our physiological systems work to maintain the aforementioned parameters at set values that are ideal for optimal physiological function. As variables deviate from ideal values, there is a systemic response, which activates or inhibits the appropriate functions, returning those variables toward the homeostatic value. In order to correctly respond to a deviation from regulated set points, systems for detection of such changes are clearly essential, and it is here that the importance of the sensory CVOs becomes evident. Many circulating signaling

molecules provide continuously updated information indicating the state of the peripheral systems. Leptin levels in circulation, for instance, will increase or decrease according to the metabolic status, angiotensin II (ANG) with fluid balance, and cytokines with immune function of the body. As previously discussed in this chapter, the majority of the CNS is protected by the BBB and therefore cannot detect changing levels of any of these signals that do not readily cross the BBB. However, neurons in the specialized sensory CVOs are able to detect such fluctuations and transmit this information to autonomic control centers protected by the BBB by CVO efferents. Of course, there are numerous signaling molecules circulating in the periphery at any given time, and the ability of the three sensory CVOs to each detect many of these signals allows them to collect and integrate such complex information; thus, they are ideally positioned as critical enablers for communication between the periphery and the CNS.

3.5.1 Cardiovascular Regulation and Fluid Balance

Cardiovascular function and fluid balance are two systems; the homeostatic regulation of which are so clearly integrated that it perhaps serves no function to attempt to separate them. They are also the functions in which the sensory CVOs are

primarily known to be involved, and thus it is in this area that many of the initial discoveries regarding sensory CVO functions were made. It is now well established that activation of SFO neurons causes rapid increases in blood pressure (Ferguson and Renaud 1984) as well as drinking (Smith et al. 1995), while activation of AP neurons also rapidly changes blood pressure (Ferguson et al. 1988), and also modulates baroreflex sensitivity (Bishop and Sanderford 2000). These two systems are dependent on appropriate balance of osmolarity, fluid volume, and electrolytes in combination with cardiac output and vascular tone, all of which can be regulated by a combination of AP, OVLT and SFO efferents. Many circulating signals provide information regarding the integrated cardiovascular and fluid balance status of an individual, and a number of these are known to be monitored by specific neurons in the sensory CVOs, some of which will be discussed below.

ANG, a peptide hormone, the concentrations of which in the circulation are regulated by BP and $[Na^+]$ does not cross the BBB. Despite this fact, circulating ANG very clearly exerts effects on autonomic output through action of the CNS, causing vasoconstriction, water intake, vasopressin, and ACTH secretion all of which contribute to an increase in BP. The SFO and AP in particular are known to express very high levels of the ANG receptor AT_1 (Gehlert et al. 1990), and single neuron recordings have clearly demonstrated that ANG excites the majority of neurons in these areas (Ferguson and Bains 1996). *In vivo* experiments performed by directly injecting ANG into the SFO result in increases in BP. What is even more convincing are the experiments in which effects of circulating ANG on drinking, BP, and hormone release are abolished by lesion of the SFO, while effects on baroreflex sensitivity are lost following AP destruction (Liu et al. 1999; Ferguson and Bains 1997). Finally, it should also be emphasized that ANG receptors have also been demonstrated in many CNS sites protected by the BBB, and it has been suggested that ANG may even be synthesized in certain areas of the CNS. Such observations clearly suggest that the CNS also uses ANG as a neurotransmitter in specific ANGergic pathways. Such a scenario only remains functionally viable where the BBB protects these areas of the CNS from circulating ANG, which would otherwise continuously activate such ANGergic synapses. Many other circulating peptides known to play critical roles in the regulation of blood pressure and body fluids have also been shown to act in the SFO and/or (Hasser and Bishop 1990) AP including vasopressin (Washburn et al. 1999b; Bishop and Hay 1993), atrial natriuretic peptides (Saavedra et al. 1987; Brown and Czarnecki 1990), endothelin (Wall et al. 1992; Ferguson and Smith 1991), and obestatin (Samson et al. 2007).

SFO and OVLT neurons have also been demonstrated to be intrinsically osmosensitive; and thus, both represent sites at which changes in circulating osmolarity can act to regulate the complex circuitry controlling integrated cardiovascular

and fluid regulation. SFO neurons have also been shown to sense calcium through the calcium sensing receptor (Washburn et al. 1999a) and sodium through modulation of the Na_x channel (Hiyama et al. 2004).

As reviewed in this section of the chapter, the three sensory CVOs are essential in the homeostatic regulation of cardiovascular function and fluid balance. Their unique ability to detect multiple peripheral signals describing the body's fluid and vascular status allows them to be a critical first step in the integrated regulation of cardiovascular function and fluid balance.

3.5.2 Reproduction

The sensory CVOs have also been shown to play roles in the regulation of reproductive function, presumably again as a consequence of their ability to detect reproductive hormones in the circulation, thus closing the loops in critical feedback control circuitry. Hamster food deprivation suppresses the normal progression of the estrous cycle, and lesion of the AP has been shown to abolish this effect (Panicker et al. 1998). Similarly, destruction of both the SFO or OVLT cause a disruption in the secretion of reproductive hormones, with OVLT lesion reducing the luteinizing hormone (LH) surge which normally induces ovulation (Piva et al. 1982), while SFO lesions have been shown to influence the proestrous follicle stimulating hormone (FSH) surge, and perhaps more importantly block normal estrous cyclicity in 50 % of animals (Limonta et al. 1981).

All three sensory CVOs have been found to express multiple signaling molecules and receptors associated with reproductive function including gonadotropin-releasing hormone (GnRH) and oxytocin, as well as estrogen, prolactin and relaxin receptors (Summerlee and Wilson 1994), further supporting their involvement in reproductive function. Activation of estrogen (Pamidimukkala and Hay 2003), prolactin (Black et al. 2014), and relaxin (Sunn et al. 2002) receptors have all also been shown to influence the excitability of neurons in these CVOs. Collectively, these data support important roles for the sensory CVOs in the regulation of reproduction and the potential integration of reproductive function with other homeostatic variables.

3.5.3 Feeding and Metabolism

Before, after, and between meals exists a complex system of hormonal signaling that aids in the digestion and regulation of food intake and metabolism. Production and secretion of various blood-borne peptides are stimulated by specific physiological events or conditions, each of which has the potential to be influenced by physiological state. For example, ghrelin is a peptide produced by ghrelin cells in the gas-

trointestinal (GI) tract when the stomach is empty and increases gastric acid secretion and GI motility to prepare the body for food intake. During meal consumption, the introduction of chyme (containing fatty acids, certain amino acids, and nutrients) from the stomach into the small intestine stimulates the I- and L-cells in the mucosa of the gastrointestinal tract to release various gut hormones such as cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), and peptide YY (PYY). These peptides then enter the bloodstream and travel to effector organs to perform a multitude of functions including signaling the release of various digestive enzymes and bile from the pancreas and gallbladder (role of CCK), stimulating the release of peptide hormones such as insulin or glucagon from the pancreas (role of GLP-1), or inhibiting gastric motility (role of PYY). Insulin and amylin are co-secreted from pancreatic beta cells in response to rising glucose levels and function to prevent post-prandial spikes in blood glucose levels. Between meals, adipocytes, or fat cells, release the protein hormones adiponectin and leptin that are responsible for regulating metabolic processes involved in energy homeostasis such as glucose regulation and fatty acid oxidation.

Interestingly, in addition to their unique roles in digestion and energy metabolism, these blood-borne peptides all possess the ability to act as hunger or satiety signals in the central nervous system. They can be simplistically categorized into two types: orexigenic—causing a sensation of hunger and promoting food intake (only known GI peptide to date is ghrelin), or anorexigenic—causing a sensation of satiety and inhibiting food intake (i.e. CCK, GLP-1, PYY, adiponectin, and leptin). However, due to the lipophilic nature of these peripheral signals, they are unable to cross the BBB; and therefore, alternative methods through which they communicate with the brain have been proposed, one being by actions in the sensory CVOs.

Over the past few decades there has been growing evidence to support the idea that sensory CVOs, initially the AP and more recently the SFO, are involved in the neural circuitry underlying hunger and satiation, specifically by acting as an integrative ‘gate keeper’ that senses various circulating hormones and transmits the information to areas of the hypothalamus and brainstem.

The presence of receptors in the AP and SFO for these various gastrointestinal hormones involved in energy homeostasis has been confirmed using various scientific approaches including *immunostaining*, *in situ hybridization*, as well as *pharmacological* approaches. Many of these peptide hormones cause increased c-fos expression (an indirect marker of neuronal activity) in the AP and SFO when injected intraperitoneally, and/or have been shown to cause changes in the excitability of AP and SFO neurons in dissociated or slice preparations. The AP expresses receptors for and can respond to: adiponectin, leptin, amylin, ANG, CCK, ghrelin, GLP-1,

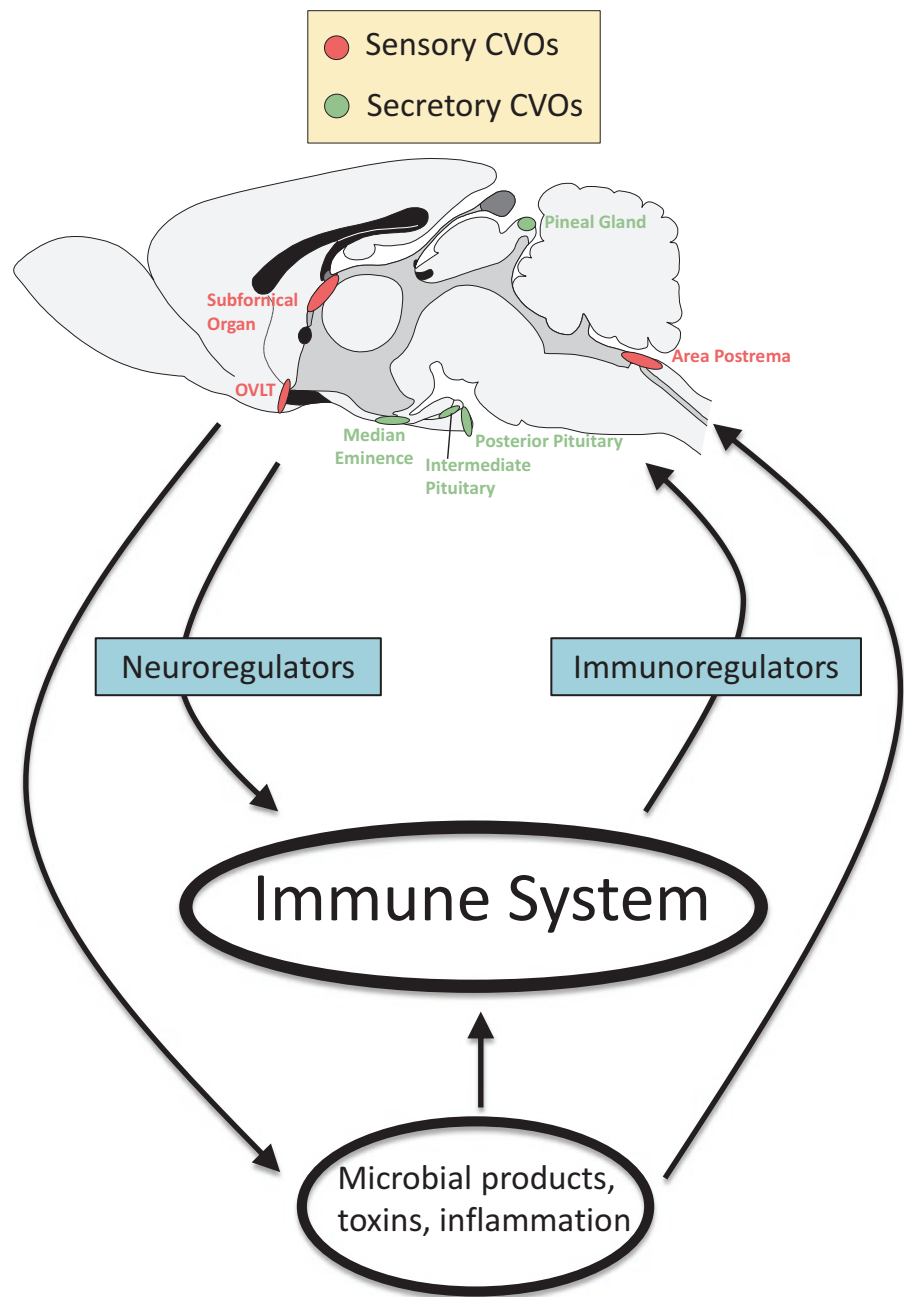
oxyntomodulin, vasopressin and PYY (Baraboi et al. 2010a); while the SFO can sense and respond to ANG, calcitonin, amylin, ghrelin, leptin, adiponectin, CCK, and PYY (see Fig. 3.3) as described in reviews (Cottrell et al. 2004; Hoyda et al. 2009; Smith et al. 2010). These data are important as they show that the receptors expressed by these sensory CVOs are in fact able to continuously monitor these circulating signals involved in energy homeostasis.

Behavioral studies have also demonstrated critical roles for the sensory CVOs in regulation of energy balance. Lesioning the AP results in significant hypophagia and loss of body weight (Hyde and Miselis 1983; Bird et al. 1983; Contreras et al. 1982), while Potes et al. demonstrated that lesioning noradrenergic AP neurons abolished subcutaneously injected amylin induced hypophagia (Potes et al. 2010). Jordi et al. assessed the effect of oral ingestion of 20 amino acids and found that food intake was most potently reduced by L-arginine (Arg), L-lysine (Lys), and L-glutamic acid (Glu), effects that were accompanied by increased neuronal activity in the AP and NTS. Interestingly, when the AP was surgically lesioned, the anorectic response and increased neuronal activity in the AP and NTS, in response to Arg and Glu, was abolished suggesting that the AP is not only a sensor of circulating GI peptide hormones released when food is ingested, but also a sensor of metabolic food products themselves (Jordi et al. 2013). Although the majority of work involving lesioning the SFO has focused on effects on fluid balance, a recent study has shown that, while lesion of just the SFO has no effect on body weight, combined lesion of SFO and AP has long lasting effects on body weight gain (Baraboi et al. 2010b). In addition, we have recently shown that electrical activation of SFO neurons in satiated rats induces feeding as well as drinking (Smith et al. 2010). Collectively, these data support the conclusion that the AP and SFO, through their connections to the brainstem and hypothalamus, play an important role in central nervous system control of energy homeostasis as components of an integrated network.

3.5.4 Immune Function and Temperature Regulation

The continuous crosstalk between the nervous and immune systems is critical to the integrated mechanisms through which the nervous system can regulate specific immune system functions through the production of neuroregulators (neurotransmitters, neuromodulators, and neuropeptides). Additionally, the peripheral immune system has the ability to regulate specific nervous system functions through the production of immunoregulators (immunomodulators and immunopeptides) (see Fig. 3.5). For example, classical neurotransmitters such as norepinephrine and serotonin are able

Fig. 3.5 Communication between the nervous and immune systems. The nervous system and immune system communicate and influence one another via neuro-immune and immuno-neural pathways utilizing neuroregulators and immunoregulators, respectively. Microbial products, toxins, and inflammation can trigger synthesis and release of immunoregulators from immune or CNS cells, signals that are integrated at the circumventricular organs



to induce immunosuppression (Walker and Codd 1985), while lymphocytes and leukocytes express receptors for and can synthesize biologically active neuroendocrine peptide hormones (i.e. growth hormone and adrenocorticotrophic hormone), which can not only influence nervous system function, but can also modulate the proliferation and differentiation of T-lymphocytes [for review see (Weigent and Blalock 1987)].

Immunomodulators, such as interleukin 1 (IL-1) and tumor necrosis factor (TNF), are important regulators of immune responses and inflammatory reactions. Bacterial endotoxins, such as lipopolysaccharide (LPS), can bind to

immune cells, such as monocytes and macrophages, and promote secretion of these pro-inflammatory cytokines. Interestingly, the biological actions of IL-1 and TNF are not restricted to the immune system. They have been found to have various actions in the nervous system, including pyrogenic, thermogenic, and somnogenic effects, influence on growth and differentiation, as well as suppression of food intake [for review see (Plata-Salaman 1991)]. This seems logical seeing as sickness is often associated with fever, tiredness, and decreased appetite. While the way in which these two systems communicate to coordinate integrated responses is not yet fully understood, the CVOs, with their

Table 3.1 Blood-borne immunomodulators that influence CVO neurons

	Data source	IL-1 β	TNF	LPS
SFO	<i>mRNA</i>		✓	✓
	<i>c-fos</i>	✓	✓	✓
	<i>Immunohistochemistry</i>	✓		
	<i>Intracellular Ca2+</i>			
	<i>Single Unit Recording</i>	✓		
AP	<i>mRNA</i>	✓	✓	✓
	<i>c-fos</i>	✓	✓	✓
	<i>Immunohistochemistry</i>	✓		
	<i>Intracellular Ca2+</i>	✓	✓	✓
	<i>Single Unit Recording</i>			
OVL	<i>mRNA</i>		✓	✓
	<i>c-fos</i>	✓	✓	✓
	<i>Immunohistochemistry</i>	✓		
	<i>Intracellular Ca2+</i>			✓
	<i>Single Unit Recording</i>		✓	

IL-1 β interleukin 1 beta, TNF tissue necrosis factor, LPS lipopolysaccharide

lack of a BBB, represent an access point for circulating cytokines and endotoxins to directly influence the CNS (see Table 3.1). Furthermore, because of their connections to critical autonomic control centers, the CVOs have been suggested as integrators for the multiple components of these coordinated responses.

Using in situ hybridization, receptors for IL-1 β (IL-1R) (Ericsson et al. 1995), TNF (p55) (Nadeau and Rivest 1999), and bacterial LPS (mCD14 and toll-like receptor 4 (TLR-4)) (Laflamme and Rivest 2001) have been shown to be expressed by the sensory CVOs. The SFO also expresses receptor mRNA for ciliary neurotrophic factor (Hindmarch et al. 2008), another mediator of the immune response.

In vitro and in vivo techniques that examine neuronal activity have been used to examine the effects of activation of these receptors. Peripheral administration of IL-1 β increases *c-fos* expression in the AP, OVL, SFO, PVN, NTS, and PBN (Brady et al. 1994; Day and Akil 1996), and has also shown to induce phosphorylation of extracellular signal-regulated protein kinase 1 and 2 (ERK1/2) in the CVOs, PVN, and SON (Nadjar et al. 2005). Peripheral administration of TNF- α has been shown to cause upregulation of mRNA for both TNF receptors (p55 and p75) in the CVOs, while also increasing *c-fos* expression in the CVOs, PVN, and NTS (Nadeau and Rivest 1999). Additionally, one hour after intravenous TNF- α administration, PVN expression of corticotropin-releasing factor increased, an effect that was associated with increases in the plasma corticosterone levels (Nadeau and Rivest 1999). Systemic administration of LPS has been shown to rapidly stimulate transcription of IL-6 mRNA and biosynthesis of IL-6 receptor (IL-6R) in the sensory CVOs (Vallieres and Rivest 1997). Pretreated OVL and AP microcultures with IL-10 antibodies before incubation with LPS show significantly enhanced LPS-induced increase

in TNF- α and IL-6 in the microculture supernatant (Harden et al. 2013), which suggests that the CVOs may have a key role to play in both the initiation and modulation of neuroinflammation. An in vivo example of CVO involvement during the development of neuroinflammation can be seen when Wuerfel et al. demonstrated increased signal intensity in the SFO and AP on gadofluorine M-enhanced MRI scans during autoimmune encephalomyelitis (Wuerfel et al. 2010).

Gerstberger et al. have shown that AP and OVL neurons respond to LPS, TNF- α , and IL-1 β with increases in intracellular calcium (Wuchert et al. 2008, 2009; Ott et al. 2010). Interestingly, the 2008 study also showed that LPS-induced calcium signaling can be suppressed by pre-incubating AP microcultures with LPS for 18 h, suggesting that AP cannot only sense circulating LPS but also has the capacity to develop endotoxin-tolerance. Patch clamp recordings from dissociated SFO neurons have also shown that physiological (subseptic) bath application of IL-1 β cause depolarization with increased spike frequency due to activation of a non-selective cationic current (Desson and Ferguson 2003). Additionally, OVL neurons in slice preparations have been shown to increase firing frequency in response TNF- α and interferon α -2 (Shibata and Blatteis 1991).

Finally, we turn our attention to CVO ablation and lesion studies that present evidence for CVO involvement in fever production and neuroendocrine activation during the immune response. The OVL was originally hypothesized to be involved in the febrile response due to its proximity and reciprocal neuronal connections to the median preoptic nucleus (MnPO) and the medial preoptic area (MPO), both critical hypothalamic sites for central thermoregulation and fever production (Nakamura and Morrison 2008; Lazarus et al. 2007). Blatteis et al. ablated the anteroventral third ventricular (AV3V) area, which included the

OVLt, in guinea pigs and sheep and found that these animals were no longer capable of developing a fever in response to systemic endogenous pyrogens (Blatteis et al. 1983, 1987). Conversely, Stitt (1985) found OVLt (not AV3V) lesion-induced fever enhancement in rabbits and rats; whereas, Takahashi et al. (1997) found that lesioning the OVLt or AP had no effect on fever while SFO lesion significantly reduce LPS-induced fevers. As might be expected, the cause for such discrepancies between results was then sought. A more recent study, which reappraised these previous experiments, demonstrated that lesioning the OVLt resulted in many “side effects,” which were not properly controlled for, including acute adipisia and gross emaciation, and chronic hypernatremia, hyperosmolality, a suppressed drinking response to hypertonic saline, and previously unrecognized long lasting hyperthermia (2 °C for >3 weeks) (Romanovsky et al. 2003). After recovery from acute, but not chronic, side effects, rats were still unable to elicit a febrile response to injected IL-1 β . The authors note, however, that rats were in a hyperthermic state at the time of injection, and that this may have been the underlying explanation for the lack of febrile response, not a disruption of a febrigenic-signaling pathway.

The previously described SFO ablation induced reduction of febrile response seen by Takahashi et al. in 1997 gained support in 1999 when Cartmell et al. who demonstrated that microinjection of the IL-1 receptor antagonist into the SFO attenuated LPS induced fever (Cartmell et al. 1999) also showed that antagonist injection into the OVLt had no effect on febrile response. This further supports the hypothesis that the attenuation of febrile response seen in previous studies may not have been attributed to a disruption in the febrigenic-signaling pathway, as was previously suggested.

Studies examining the neuroendocrine response to IL-1 β have suggested roles for the AP as the large elevations in adrenocorticotrophic hormone (ACTH) and corticosterone levels in the plasma in response to systemic IL-1 β and increases in fos expression in AP, NTS, and PVN are attenuated by AP lesion (Lee et al. 1998). This suggests that the AP and adjacent NTS play a critical role in sensing and transducing this circulating cytokine signal to the hypothalamic-pituitary-adrenal axis thereby indirectly influencing the release of ACTH and corticosterone into the bloodstream. Peripherally administered IL-1 β has also been shown to increase extracellular norepinephrine concentration in the PVN of the hypothalamus, an effect that is also attenuated in AP-lesioned rats (Ishizuka et al. 1997).

3.6 Review Questions

1. What are some defining characteristics of sensory CVOs?
2. What makes the sensory CVOs ideal integrative sites?
3. Can most cytokines and peptides cross the blood-brain barrier?

4. What are ways in which the peptides and cytokines can access the CNS?
5. Name two critical neuroendocrine nuclei in the hypothalamus onto which CVOs project.
6. (a) What are the differences between sensory and secretory CVOs?
(b) Why is it important that these areas lack a normal blood-brain barrier?
7. A female rat that is a subject in your study does not display the usual increase in blood pressure in response to ANG injection. Her estrous cycle has been disrupted and her febrile response to lipopolysaccharides (LPS) is much smaller than expected. Based on these observations, which CVO would you say has been damaged in the subject?

3.7 Answers

1. Lack a blood-brain barrier (fenestrated capillaries) High expression of peptide and hormone receptors. Extensive efferent projections, fewer afferent
2. The defining characteristics of: lack of a blood-brain barrier, high receptor expression and extensive efferent projections enable the sensory CVOs to interpret information from different systems, whether it be electrical or chemical, and convey this integrated information to autonomic control centers protected by the BBB.
3. No, many are too large or lipophobic
4. Diffusion across the BBB; Active transport across the BBB; Binding to receptors in the sensory CVOs, which aren't protected by the BBB
5. Paraventricular Nucleus (PVN); Supraoptic Nucleus (SON)
6. (a) Sensory CVOs detect signals circulating in the periphery; whereas, secretory CVOs secrete hormones directly into the circulation.
(b) In order to detect peripheral signals, sensory CVOs need direct access to the circulatory system. Similarly, secretory CVOs need direct access in order to secrete their respective hormones into circulation.
7. Subfornical Organ (SFO)

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Anterior Chamber and Retina (Structure, Function and Immunology)

4

William Rhoades, Leila Kump, and Eyal Margalit

Abstract

The goals of this chapter are to review the anatomy of the eye and its immune response, focusing on the anterior and posterior segments. Anterior segment diseases with known immune system involvement include keratitis, uveitis, dry-eye syndromes, uveal melanoma, and allograft rejection following corneal transplantation. Posterior segment diseases with immune system involvement include age-related macular degeneration, glaucoma, chorioretinal disorders and autoimmune retinopathies including cancer and melanoma associated retinopathies.

Keywords

Age related macular degeneration • Anterior chamber associated immune deviation • Autoimmunity • Complement • Immune deviation • Ocular immunology • Ocular inflammation

4.1 Anatomy and Physiology: The Eye

The eye is a sensory organ which uses its lens system to focus light from the surrounding environment onto the retina. The cornea, iris, and crystalline lens cooperate to form a focused image on the retina. The neurons of the retina capture the image and encode it for accurate transmission via the optic nerve to the visual centers in the brain. Figure 4.1 shows a horizontal cross-section of the human eye. Light

entering the eye is refracted by the cornea and the crystalline lens, and is aperture-limited by the iris. The lens is suspended by a ring of thin fibers (zonules) that are attached to a membrane encapsulating the lens. The refractive power of the lens can be adjusted by contraction or relaxation of a muscle ring called the ciliary muscle, which thickens the lens to allow accommodation.

The eye contains three compartments: the anterior chamber (AC), posterior chamber, and vitreous cavity. The AC is a space between the iris and the cornea, which is filled with aqueous humor. On average, it is 3 mm deep, with a volume of about 250 μ L. The posterior chamber is also filled with aqueous humor; its location is posterior to the iris and ciliary body and anterior to the lens and the vitreous face. The largest compartment of the eye is the vitreous cavity, which is filled with vitreous gel. Its average volume is 6 mL. This compartmental division is a practical one. It is used extensively in clinical practice of ophthalmology. Differential diagnosis of a large number of infectious and inflammatory disorders is based on the anatomic location of the process.

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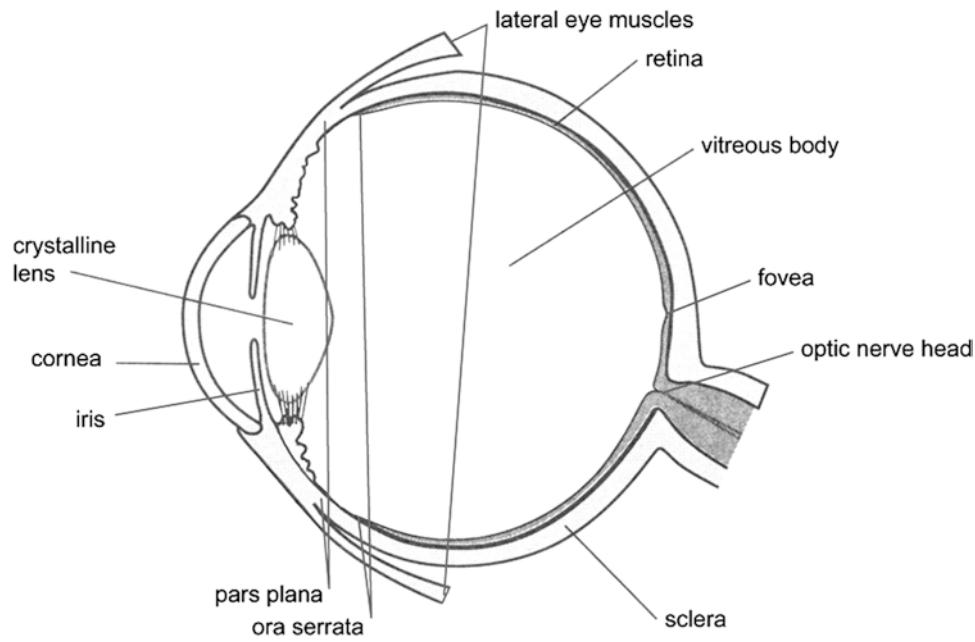


Fig. 4.1 Horizontal cross section through the human eye, showing the principal structures referred to in the text

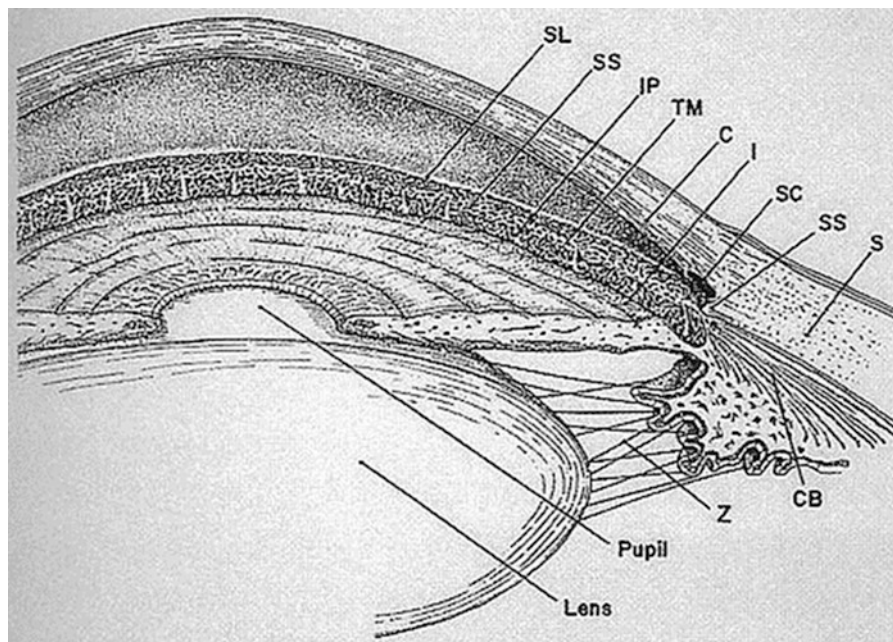


Fig. 4.2 Drawing of the structures of the angle of the AC and ciliary body. *SL* Schwalbe's line, *SS* scleral spur, *IP* iris process, *TM* trabecular meshwork, *C* cornea, *I* iris, *SC* Schlemm's canal, *S* sclera, *CB* ciliary body, *Z* zonular fibers

4.2 Anterior Chamber

4.2.1 Anatomy and Physiology

The cornea serves as the most anterior structure of the AC. Its exterior is covered by the precorneal tear film, which lubricates, nourishes and protects the corneal surface. The iris and

the pupil represent the posterior border of the AC. The AC angle is an important structure which is comprised of: Schwalbe's line, Schlemm's canal and trabecular meshwork, scleral spur, anterior border of the ciliary body and the iris (Fig. 4.2). Aqueous humor that fills the AC is produced by the ciliary epithelium located in the posterior chamber. The fluid flows through the pupil and is drained by the trabecular meshwork into Schlemm's canal and subsequently into the

episcleral vessels. This passage is named *the conventional pathway*. Aqueous humor is also drained by *the uveoscleral pathway* across the ciliary body into the supraciliary space.

4.2.2 Anterior Chamber Associated Immune Deviation (ACAID)

The phenomenon of immune privilege was initially described by Dutch ophthalmologist van Dooremal 1873. He noticed that tumor cells injected into the AC formed growing tumors unlike tumor cells injected into skin or other organs. Medawar discovered that transplants between genetically different individuals were usually destroyed. However, skin grafts placed in the AC and in the brain of rabbits survived much longer. Medawar called the AC and the brain “immune privileged sites.” Medawar explained that immune privilege was a special circumstance where the laws of transplantation immunology did not apply (Medawar 1948). Immune privilege was later defined as a prolonged, sometimes indefinite, survival of organ or tissue grafts at special body/organ sites (Barker and Billingham 1977). Additional examples of such immune privileged sites would be the cornea, lens, vitreous cavity and subretinal space; testis, ovary, adrenal cortex, pregnant uterus and certain tumors.

Anatomic structures were originally considered key elements of immune privilege in early studies. The existence of a blood-tissue barrier and the absence of afferent lymphatic drainage pathways led to the belief that antigens of the grafts placed in immune privileged sites remained physically sequestered from the immune environment. As such, the immune system never became aware of them. However, later studies demonstrated that the maintenance of immune privilege is actually a dynamic process combining immunoregulatory forces combined with anatomic structure to allow the survival of grafts in privileged sites (Niederhorn et al. 1980; Niederhorn 1990; Ksander and Streilein 1993; Tompsett et al. 1990; Streilein 1995). In other words, the blood-brain and blood-ocular barriers exist, but their creation and exis-

tence are actively maintained. The list of factors responsible for the ocular immune privilege is presented in Table 4.1.

It is now recognized that both active and passive factors contribute to sites with immune privilege. It has been known for over 100 years that the AC possessed qualities allowing a long-term survival of tissue and tumor grafts (Van Dooremal 1873). In the late 1970s Kaplan and Streilein discovered that antigenic cells placed into the AC were not only detected by the immune system, but also elicited a down-regulation of alloimmune responses (Kaplan et al. 1975; Kaplan and Streilein 1977).

It became clear that antigens are not physically sequestered from immune recognition in the AC, but that the consequence of their detection is a deviation from the expected immune response. Although AC injection of allogeneic lymphoid cells aroused the humoral arm of the immune response resulting in the production of antibodies against donor histocompatibility antigens, the cell-mediated component was impaired. Studies in the early 1980s led to the knowledge that a wide variety of antigens (alloantigens, tumor-specific antigens, soluble protein antigens, haptens, viral-encoded antigens) placed in the AC of mice produced a similar deviant pattern of systemic immune responses. Niederhorn, Streilein and Shadduck coined the term Anterior Chamber Associated Immune Deviation (ACAID) to designate this unusual response (Niederhorn et al. 1980). ACAID is an antigen-specific systemic immune response to eye-derived antigens, a response that represents a selective deficiency of T cell functions that mediate delayed hypersensitivity to the antigen Th1 cells and B cells that secrete complement-fixing antibodies. However, this systemic immune response retains primed, clonally expanding cytotoxic T cell precursors and B cells that secrete IgG1, a noncomplement-fixing antibody. Lymphoid tissues of mice with ACAID contain three populations of regulatory T cells: CD8⁺ T cells suppressing expression of delayed hypersensitivity; CD4⁺ T cells suppressing the induction of Th1 cell responses; and CD8⁺ T cells that inhibit B cells from switching to IgG isotypes that fix complement.

Table 4.1 Factors responsible for ocular immune privilege

Passive	Active: soluble factors	Active: cell-surface factors
Blood-tissue barrier	Transforming growth factor- β 2	CD95 ligand
Tissue fluids drain IV	Alpha-melanocyte stimulating hormone	CD55, CD59, CD46
Deficient lymphatics	Vasoactive intestinal peptide	
Reduced expression of major histocompatibility class I and II molecules	Calcitonin gene-related peptide	
Reduced antigen presenting cells with altered function	Somatostatin	
	Thrombospondin	
	Macrophage migration inhibitory factor	
	IL-1 receptor antagonist	
	Free cortisol	

In late 1980s experiments with samples of aqueous humor showed that it possessed some immunomodulatory properties, but these properties were not global, i.e. aqueous humor does not inhibit all immune reactions (Kaiser et al. 1989). Suppression of the following phenomena by aqueous fluid was found:

1. Activated macrophage production of nitric oxide and reactive oxygen intermediate (Taylor et al. 1998).
2. Neutrophil-mediated lysis of target cells (Miyamoto et al. 1996).
3. Lysis of target cells by natural killer (NK) cells (Apte and Niederkorn 1996).
4. Conventional antigen-presenting cell (APC) activation of Th1-type cells in-vitro (Takeuchi et al. 1999).
5. C1q from binding to antibody-coated erythrocytes.
6. C3 cleavage to C3b via the classical or the alternative complement pathways (Goslings et al. 1998).

Streilein and Kaplan have discovered that the spleen plays a crucial role in the immune deviation effects in rats (Streilein and Kaplan 1979). This was confirmed in later experiments in mice (Streilein and Niederkorn 1981; Wilbanks et al. 1991). Following an injection of the heterologous protein antigen ovalbumin (OVA) into the AC, an ACAID-inducing signal (AIS) is released to the blood stream. For example, when naïve mice received intravenous injection of blood from mice that had been injected with OVA into the AC 48 h prior, the naïve mice acquired OVA-specific ACAID. It was postulated that eye-derived antigen-bearing antigen presenting cells (APCs) escaped through the trabecular meshwork, and cells migrated via the bloodstream to the spleen. In the spleen, the regulatory cells that mediate ACAID eventually emerged. This hypothesis suggests that the eye contains nascent APCs that pick up the antigen and deliver it to the spleen via the blood. The spleen induces ACAID, without which, immune privilege in the AC cannot be sustained.

Further understanding of the ACAID phenomenon is attributed to Hara et al. who developed an in-vitro generated ACAID-inducing signal (Hara et al. 1992). These investigators incubated conventional APC (from peritoneal exudate) overnight in the presence of acid-treated aqueous humor and then pulsed with OVA. When injected into naïve mice these APCs induced ACAID. Since then, it became possible to perform experiments with ACAID-inducing APCs that could be grown in vitro. Takeuchi and Streilein conducted series of experiments that led them to conclude that ACAID-inducing APCs were formed by pulsing active-TGF β -2-pretreated-peritoneal-macrophages with OVA. The principal action of TGF-beta is to inhibit activation and proliferation of lymphocytes. It is a family of molecules named TGF β -1, TGF β -2 and TGF β -3. Cells of the immune system mostly produce TGF β -1. This family inhibits immunologic, inflammatory

responses and has immunosuppressive effect. The above-mentioned APCs were capable of activating T-cell receptors (TCR), transgenic OVA-specific CD4 cells, and CD8 T cells in vitro (Takeuchi et al. 1998, 1999).

A universal property of ACAID-inducing APCs created in vitro is the capacity to deviate naïve CD4+ and CD8+ T cells away from their typical differentiation pathway toward a pathway that might enable them to be regulators. Normal aqueous humor contains large amounts of thrombospondin (a glycoprotein involved in the clotting cascade). The gene controlling the formation of this protein is constantly active in ocular parenchymal cells. Thrombospondin and TGF β -2 may be the key players in creating immune deviation when antigens are introduced into immune-privileged sites.

Unique qualities of ACAID-inducing APCs have been analyzed by many researchers in order to understand their ability to alter the function of responding T cells (Takeuchi et al. 1998; Faunce et al. 2001; Masli et al. 2002). TGF β -2 treated peritoneal macrophages usually activate Th1 cells, and express normal levels of class I and II major histocompatibility proteins. However, TGF β -2 exposed APCs fail to upregulate CD40, and they secrete only small amounts of IL-12. IL-12 serves as a mediator and an inducer of innate immune responses to intracellular microbes. It also activates NK cells, promotes their cytolytic activity and development of Th1 cells. CD40 plays various roles in macrophage, dendritic cell and endothelial cell activation. Expression of IL-12 and CD40 is not upregulated even in the presence of responding T cells. Interestingly, the supernatants of TGF β -2-treated APCs contain active TGF β -1, which is usually produced by bone-marrow-derived cells.

In addition, treatment with active TGF β -2 has a profound effect on the genetic arrangement of APCs, as discovered by a study of macrophage hybridoma #59, which is a laboratory-created cell line for immunologic studies (Wetzig et al. 1982). It is apparent that the thrombospondin gene is an early target of TGF β -2 and is upregulated. Thrombospondin has a number of actions that promote the ACAID-inducing properties of TGF β -treated APCs listed here:

1. It binds to CD36 on APCs and tethers latent TGF β to the APC surface;
2. It binds to CD47 on APCs and on responding T cells, potentially forming a cellular bridge that stabilizes the APC/T cell interaction;
3. With CD47 on T cells, thrombospondin alters signaling through the TCR in a manner that deviates the cell's functional program; and
4. It promotes conversion of latent TGF β to its active form in the environment between APC and T cells.

As mentioned above, the spleens of mice that receive OVA into the AC acquire three populations of regulatory T

cells. These cells are produced within 7 days (Wetzig et al. 1982; Niederkorn and Streilein 1983; Waldrep and Kaplan 1983; Wilbanks and Streilein 1990). One population is CD4+. When it is adoptively transferred into naïve recipients, it suppresses the induction of OVA-specific delayed hypersensitivity. A second population of regulatory cells is CD8+, which inhibits the expression of delayed hypersensitivity. So, generation of ACAID correlates with regulatory T cells that suppress the induction and expression of delayed hypersensitivity. A third population of regulatory CD8+ cells arises following AC injection of OVA. This population prevents OVA-specific B cells from class-switching their immunoglobulin isotype to the isotypes that fix complement: IgG2a, IgG2b, and IgG3.

The second population of regulatory cells, efferent CD8+ T cells that suppress delayed hypersensitivity expression, has gained a lot of attention because of its uniqueness to ACAID. These cells are relatively easy to detect and evaluate. They can be evaluated systemically by transferring large numbers of spleen cells intravenously into mice previously sensitized to OVA and challenged for delayed hypersensitivity a few hours later. The regulator cells can be also evaluated locally in a “local adoptive transfer reaction” in which putative regulator cells are mixed with OVA-specific T cells and antigen, and the mixture is injected intradermally into the ear pinnae of naïve mice. Thus, researchers have been able to elucidate a mechanism of ACAID by demonstrating the production of T regulatory cells that inhibit the local expression of delayed hypersensitivity.

In order to induce immune deviation only two cells need to interact: the “tolerogenic” APC and the T cell. These two cells interact under the influence of innate immune cells within the lymphoid organs. It has been found that ACAID does not occur in the absence of gamma/delta T cells (Skelsey et al. 2001; Xu and Kapp 2001), natural killer T (NKT) cells (Sonoda et al. 1999), or B cells (D’Orazio and Niederkorn 1998b). The innate cells that have been most exclusively studied are the APC and NKT cells. The NKT cell is important in peripheral tolerance induction in association with the AC (Sonoda et al. 1999). The antigen-transporting cells may direct the multicellular organization of the innate cells (specifically, NKT cell) with the appropriate adaptive immune cell precursors. The soluble factors secreted by the antigen-transporting cell recruit specific cell types and factors to create an immunosuppressive environment favorable to the induction of tolerance. The antigen that is injected into the AC is carried from the eye by the indigenous APCs. These transporting APCs are unique, possibly because they are bathed in eye-specific immunosuppressive factors that induce their special phenotype. It has been shown that the eye-derived macrophages selectively increased the chemokine MIP-2 (Faunce et al. 2001). While MIP-2 was previously thought to be a neutrophil

chemoattractant, it was found that MIP-2 was a strong chemoattractant for NKT cells as well. NKT cells are absolutely required for the development of the efferent T regulator cell in ACAID. When NKT cells were removed by use of antibodies *in vivo*, or when NKT deficient mice were used, the efferent T regulator cells were not generated (D’Orazio and Niederkorn 1998a).

4.2.3 ACAID and Other Forms of Immune Regulation and Tolerance

Immunologic tolerance is defined as unresponsiveness to an antigen and it is induced by prior exposure to that antigen. When specific lymphocytes encounter antigens, three possible outcomes may follow: the lymphocytes are activated, leading to immune responses; the lymphocytes are inactivated or eliminated, leading to tolerance; or the antigen is ignored. Different forms of the same antigen may induce an immune response or tolerance or may elicit no response. Tolerance comes in many forms. Central tolerance ensures that the repertoire of mature lymphocytes cannot recognize ubiquitous self antigens, which are antigens most likely to be present in the degenerative lymphoid organs. This mechanism is mainly responsible for the elimination of self-reactive lymphocytes from the mature repertoire, and thus for self/nonself discrimination and therefore suppression of autoimmune disease. Peripheral tolerance, also called “active tolerance,” is induced by recognition of antigens without adequate levels of the costimulators that are required for lymphocyte activation. T cell activation requires not only the recognition of antigen, but also the recognition of costimulators. These are also called “signal 2.” If costimulators are not present while the antigen is being recognized, T cell anergy occurs. This also occurs in response to persistent and repeated stimulation by self-antigens in the peripheral tissues.

ACAID is a special form of peripheral tolerance. About 98% of the antigen that is inoculated into the eye is carried into the bloodstream within 6 h of injection (Wilbanks and Streilein 1989). It was initially suggested that ACAID is just a manifestation of intravenously induced tolerance. However, ACAID was demonstrated to be distinctly different from intravenously induced tolerance. Intravenous tolerance is mediated by a CD8+ afferent T suppressor/regulator cell, and in intravenous tolerance no regulatory efferent T cell is generated (Heuer et al. 1982). In ACAID, the afferent regulatory T cell is CD4+ and the efferent regulator is CD8+. Further, ACAID does not occur in mice that are deficient in NKT cells. Intravenously induced tolerance to the same antigens can be induced readily in NKT cell knockout (KO) mice (Sonoda et al. 1999).

Both ACAID and oral tolerance produce efferent CD8+ T regulator cells that are dependent on intact NKT cells. While

low dose oral tolerance and ACAID correlate with the presence of efferent CD8+ regulatory cells, the cells are not functionally identical. The efferent T cells of oral tolerance respond to antigen by secreting IL-10 and TGF- β , whereas the efferent T suppressor cells of ACAID secrete TGF- β only (Weiner 1997). However, NKT cells are needed for both forms of CD8+ T regulatory cell-mediated tolerance.

T helper cells were found to comprise two functionally distinct types (type 1 and 2) (Mosmann and Coffman 1989; Mosmann 1992). Th1 cells responded to antigen stimulation by secreting IL-1 and IFN γ . Th1 cells mediate delayed hypersensitivity reactions, and promote the switch of B cells to Ig isotypes that fix complement (IgG2a/b, IgG3 in mice). Th2 cells respond to antigen stimulation by secreting IL-4, IL-5, IL-6, IL-10, and IL-13, but they fail to secrete IFN γ . Th2 cells promote humoral responses rich in noncomplement-fixing IgG1, IgA, and IgE antibodies. Also, Th1 and Th2 cells are able to cross-regulate each other: Th1 cells suppressing Th2 cells and vice versa. Cross-regulation of Th1 and Th2 cells has been called “immune deviation.” It was speculated that ACAID is a Th2 response evoked by an unusual route of antigen delivery (the eye). It has been reported that mice genetically deficient in IL-10 production fail to acquire ACAID (D’Orazio and Niederkorn 1998a). Some experiments dispute the idea that ACAID is a Th-2 mediated response (Streilein et al. 2001). It is currently thought that ACAID represents a unique form of tissue-dependent regulation of systemic immunity that resembles, but is mechanistically different from tolerance induced by some other routes and procedures. ACAID is a form of peripheral tolerance, unlike central tolerance, where clonal deletion and/or anergy occur within the thymus. Once induced, ACAID persists for a very long period of time. ACAID is an actively acquired and actively maintained manifestation of ocular immune privilege.

4.2.4 ACAID, Ocular Immune Diseases and Implications for Therapy

ACAID may have either beneficial or deleterious effects on ocular inflammatory disease. Ocular inflammation that is elicited by T cell-mediated delayed hypersensitivity and by complement fixing antibodies. ACAID tries to protect the eye from inflammation related injury by selectively suppressing innate (production of phagocytic cells and NK cells, blood proteins and cytokines) and adaptive (lymphocyte and antibody production) immune responses of the intense proinflammatory type (when large amounts of proinflammatory cytokines are present and are ready to excite inflammation). For certain infectious pathogens the entire array of immune defense mechanisms is mobilized in order to eliminate the exciting agent. Because of immune privi-

lege and ACAID, resistance to infectious agents cannot be mediated by delayed hypersensitivity T cells and by complement-fixing antibodies because these components are not active in such an environment. Thus, immune privilege and ACAID could have both positive and negative effects in the eye. ACAID might play a role in protecting the eye against autoimmune attack directed at strong ocular autoantigens such as arrestin, interphotoreceptor retinol binding protein, and rhodopsin.

Some speculate that ACAID-based immunotherapy may be beneficial in immune-mediated diseases of the eye and a variety of other organs. This is due to the fact that the immune deviation of ACAID produces T regulatory cells that are effective in inhibiting both Th1 and Th2 responses (both primary and secondary responses). Cd1d-reactive NKT cell-dependent tolerance or ACAID induced by inoculation of antigen into the eye may contribute to self-tolerance and prevention of autoimmune responses in organs and tissues in general.

There are reports of correlations of defective or deficient NKT cells in a number of autoimmune diseases in mice and humans (Sumida et al. 1995; Baxter et al. 1997; Wilson et al. 1998; Illes et al. 2000; Mieza et al. 1996; Zeng et al. 2000; Shi et al. 2001; Nagane et al. 2001). In humans, diabetes, systemic sclerosis, myasthenia gravis, and multiple sclerosis and in mice, a lupus model, are associated with NKT cell deficiencies. It was demonstrated that the adoptive transfer of NKT cells prevented diabetes in NOD mice. However, it is not clear whether the effectiveness of this treatment was actually due to the establishment of immune deviation of T cell-mediated active tolerance.

It is known that ACAID contributes to ocular tumor survival and long-term survival of orthoptic corneal allografts. Some experiments showed that removal of NKT cells prevented the continued acceptance of ocular tumors and caused the elimination of the immune privilege of the eye. Without functioning NKT cells, mice were not able to accept orthoptic corneal allografts for prolonged periods of time (Sonoda et al. 2002). This data demonstrates the essential role of NKT cells in the generation of ACAID. New information about ACAID may lead to application of ACAID mechanisms in prevention and treatment of immune-mediated inflammatory diseases in humans.

4.2.5 Corneal Transplantation and Ocular Immune Responses

Corneal transplantation is the most common form of solid organ transplantation today. While very successful in patients without corneal neovascularization, it can be less effective in patients with highly vascularized corneal beds (Maguire et al. 1994). T cell mediated graft rejection can be promoted

by chemotactic cytokines and receptors to antigen presenting cells in the cornea in highly vascularized eyes, indicating that when physical barriers are altered and neutrophils are recruited to the allogenic graft, ACAID processes may not be able to prevent rejection. Recent experiments have also shown that neutralizing certain chemokines can lead to prolonged graft survival (Amescua et al. 2008). These experiments show that transplant rejection in the AC can also be mediated by chemotactic factors that have a role in ocular immune responses to allogeneic transplantation. Further research may provide more insight into ocular transplant outcomes.

4.3 Retina

4.3.1 Anatomy

A more complete coverage of this topic appears in Sect. 4.4 of this book. In short, the retina is a thin transparent layered structure lining the posterior eye wall. The main cell types include photoreceptors (rods and cones), which capture the light; bipolar and ganglion cells, which pass the visual signal on towards the optic nerve, and horizontal and amacrine cells, which provide lateral interactions among cells in neighboring locations. Figure 4.3 demonstrates a diagram of cross-sectioned retinal layers (A), a preparation of normal donor retina (B), and tissue from a donor who suffered from a retinal degeneration, causing the disappearance of the photoreceptor layer (C).

The layers of normal cross-sectioned retina (from outer to inner retina in Fig. 4.3a) are:

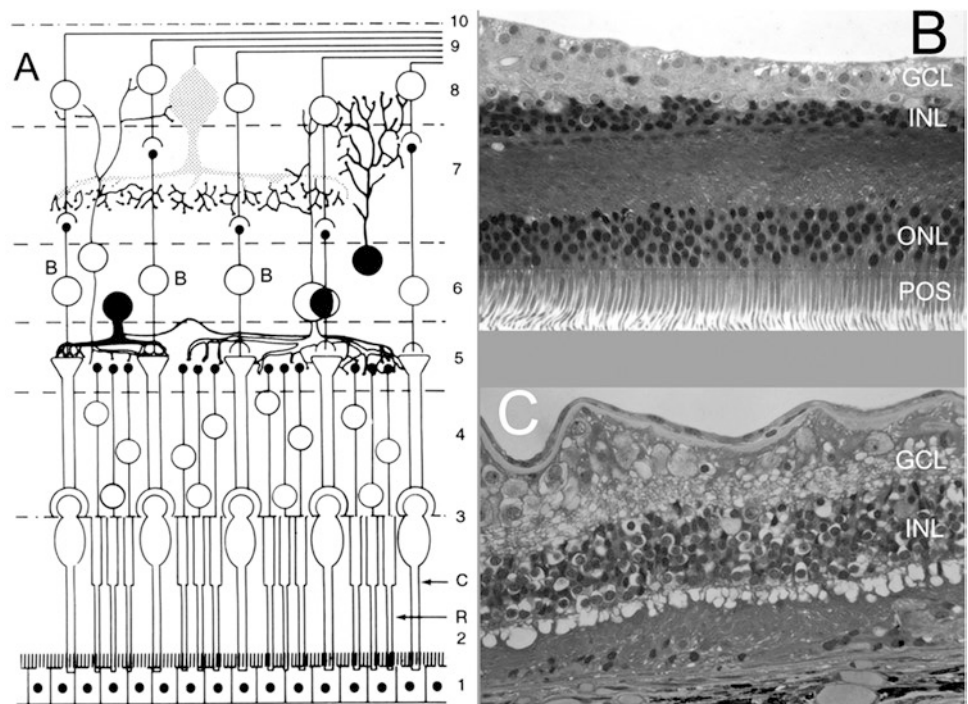
1. Retinal pigment epithelium (RPE) and its basal lamina
2. Rod and cone inner and outer segments
3. External limiting membrane
4. Outer nuclear layer
5. Outer plexiform layer
6. Inner nuclear layer
7. Inner plexiform layer
8. Ganglion cell layer
9. Nerve fiber layer
10. Internal limiting membrane

A single layer of RPE cells fulfills the role of sustaining the metabolism of the photoreceptors: The metabolic level of the photoreceptor outer segments is among the highest in the human body. RPE cells supply nutrients and oxygen, regenerate phototransduction products, and digest debris shed by the photoreceptors.

Photoreceptors, the cells capturing the incoming light, come in two classes: rods, which allow vision at low light levels, and cones, in short, medium, and long wavelength types to allow color perception. In both classes of cells the actual light capture and conversion takes place in the outer segment—visible as a band with long/thin elements (rods) in Fig. 4.3b. The cell's inner segment provides the transduction to secondary neurons and regulates cell function.

The outer plexiform layer (OPL) contains the contacts between photoreceptors, horizontal and bipolar cells, allowing

Fig. 4.3 (a) Schematic representation of the cell layers in the retina. Light entering the retina passes through the nerve fiber (9), ganglion cell (8), inner nuclear (6)—containing bipolar cells (B), and outer nuclear (4) layers, before being absorbed in the outer segments of the rods (R) and cones (C). (b) Cross section through the retina near the fovea, showing healthy photoreceptor outer segments (POS), multiple layers of photoreceptor cell nuclei in the outer nuclear layer (ONL; labeled black), bipolar cell nuclei in the inner nuclear layer (INL) and ganglion cell bodies in the ganglion cell layer (GCL). (c) Retina of a patient with retinal degeneration, and poor vision, showing lack of photoreceptor outer segments and cell bodies in comparison with B



the first stage in retinal image processing to take place. This processing provides coupling between neighboring rods and/or cones, allowing for the smooth transition between rod and cone function during light/dark adaptation and for dealing with differences, and rapid changes, in illumination.

The inner nuclear layer (INL) contains the cone and rod bipolar cells. Cone bipolar cells form the first stage of image processing in the visual system, combining output from different cone types for color processing. In the periphery, they gather information from multiple cones, and save image transmission bandwidth by reducing resolution. All rod bipolar cells receive input from multiple rods, allowing reliable signal processing at very low light levels. Also located in the INL are the cell bodies of horizontal and amacrine cells.

In the inner plexiform layer (IPL), extensive interactions take place through synaptic contacts: between bipolar cell axons and retinal ganglion cell dendrites; between rod and cone on-bipolar cells through rod amacrine cells (thus merging the rod output signals into the cone pathway); between amacrine, ganglion, and bipolar cells (providing inhibitory feedback to strengthen ganglion cell properties); and between bipolar cells and interneurons (performing a feedback function between neighboring bipolar cells).

The ganglion cell layer (GCL) contains the cell bodies of retinal ganglion cells, with their axons running across the retinal surface (nerve fiber layer) towards the optic nerve head, and on through the optic nerve to the lateral geniculate nucleus in the mid-brain. The nerve fiber layer and a thin membrane (the inner limiting membrane) form the most superficial retinal structures.

In addition to the neuronal cell types responsible for visual signal transmission the retina has several types of supporting cells, similar in function to the RPE cells supporting the photoreceptor outer segments. The most important of these are the Mueller cells, whose principal role appears to be to buffer and balance electrolyte concentrations in the extracellular space, in response to the activity of the retinal neurons, especially photoreceptors.

Figure 4.3c shows a cross-section of the retina, but this tissue came from a donor who had lost useful vision due to a retinal degeneration. There is a complete loss of photoreceptor outer segments, the almost complete absence of cells in the ONL. However, the inner retinal cells are preserved in substantial numbers.

4.3.2 Anatomy and Physiology: Retino-cortical Pathway

The eye and the central nervous system are connected through the fibers of the optic nerve. These fibers, with a diameter of about 1 μm , are actually the axons of retinal gan-

glion cells. Inside the eye, the fibers run along the retinal surface towards the optic nerve head in a characteristic pattern, such that fibers of the upper and lower retinal halves remain separated, and fibers close to the horizontal meridian, but far from the nerve head, arc away from this line to allow room for fibers originating closer to the nerve head. This orderly arrangement causes the fibers from the foveal area (which form 15–20 % of all nerve fibers) to be located in the temporal quadrant of the optic nerve, at least for the initial portion of its trajectory.

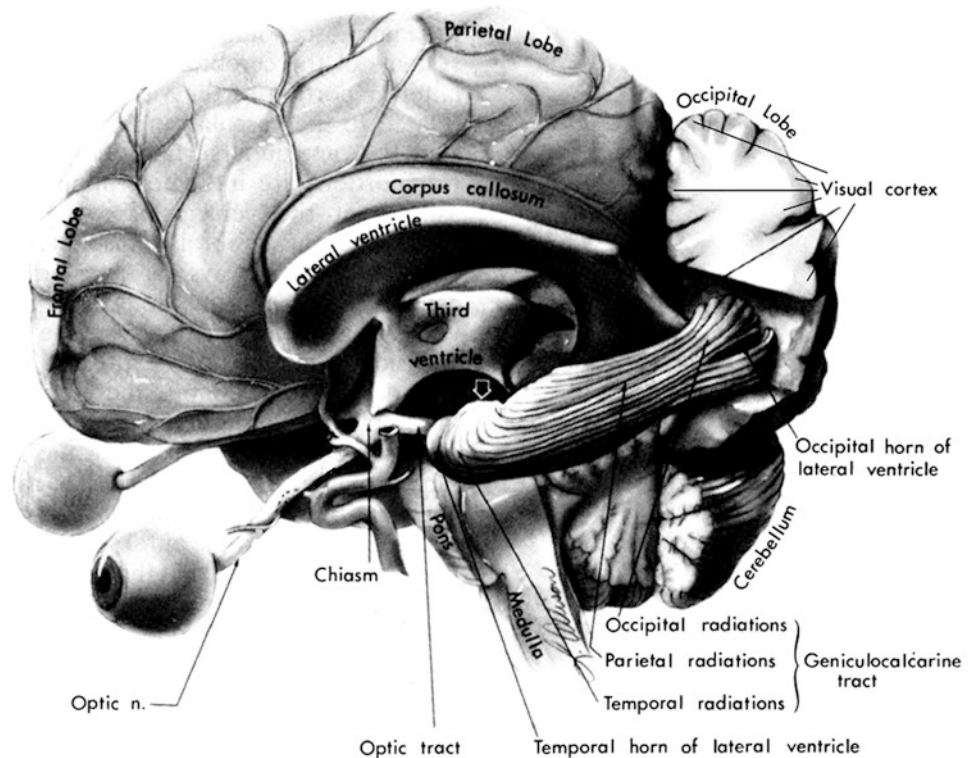
After the axons enter the optic nerve, each fiber is encapsulated with a myelin sheath, formed by oligodendrocytes; this sheath decreases the membrane conductance of the axons, increasing the conduction velocity and the length over which impulses can be conducted without severe attenuation. Only at the so-called Ranvier nodes is the myelin sheath interrupted, allowing the impulses to be reinforced by virtue of the gating properties of the local membrane.

A cross-section through the human visual pathways can be seen in Fig. 4.4. One may note that the predominant pathway leads from the eye to the lateral geniculate nucleus (LGN) of the thalamus, and from there to the occipital cortex, while less important numbers of fibers branch off to a tectal area, the superior colliculus (SC), and to a number of pre-tectal nuclei. We will briefly discuss these subcortical pathways below.

The LGN and cortical areas exist in duplicate in the two halves of the brain. Each deals with one half of the visual world: the optic nerves from the two eyes meet at the optic chiasm, where fibers from the two nasal retinas cross over to combine with those from the temporal retina of the fellow eye; consequently each LGN and cortical hemisphere receive visual information from two corresponding retinal halves on their own side, and thus from the contralateral half of the visual field.

The LGN has a layered structure, with layers devoted to nerve fibers coming from each eye, and subsequent pairs of layers receiving axons of different ganglion cell types. Significant interaction between layers does not occur; therefore, binocular interactions such as those required for stereopsis do not take place until the level of the visual cortex. The gateway function of the LGN, which in other mammals such as the cat appears to play a crucial role in adaptation and attention, and through which signals from the two eyes can mutually inhibit each other, is thought to be less prominent in primates, including humans. Yet anatomical feedback connections from a number of subcortical nuclei onto the LGN are as extensive in monkey as in cat, and gating functions related to circadian rhythms and other systemic conditions are therefore plausible in primates as well. Forward pathways from the LGN lead to the primary visual cortex (V1, also called striate cortex; these fibers form the optic

Fig. 4.4 Structure and location of the human primary visual pathways, in relation to other major brain structures. The left cerebral hemisphere, with the exception of the occipital cortex, has been removed; the left LGN is hidden by the optic radiations (arrow)



radiation), but also to higher visual cortical areas and to subcortical areas such as the superior colliculus (SC). The role of the extrastriate cortical pathways is still a topic of study; patients with lesions to the visual cortex suffer vision loss. The roles of the tectal pathways, including mutual connections between cortical areas, the SC and the pulvinar, are also not completely understood.

4.3.3 Anatomy and Physiology: Visual Cortex

The visual cortex occupies the occipital and parts of the parietal and temporal lobes of the cerebral cortex. Like the entire cortex, it forms a highly folded structure, with a thickness of approximately 1.5 mm. It is surrounded by the cerebrospinal fluid, several layers of meninges—pia mater, arachnoid, dura, and the skull.

Like the retina, the visual cortex is a layered structure, in which different cell groups perform different tasks. Along its two-dimensional surface, one finds an orderly mapped representation of the outside world. Unlike the retina, the cortex consists of multiple areas, hierarchically organized, each of which performs a partial processing task in the analysis of the scene around us. Over 30 visual cortical areas per hemisphere are recognized in monkey, and a smaller number of more expanded areas are thought to exist in humans.

The first cortical representation, in the striate cortex, is shown schematically in Fig. 4.5. It presents a straightforward map of the visual world, but contains four major transformations.

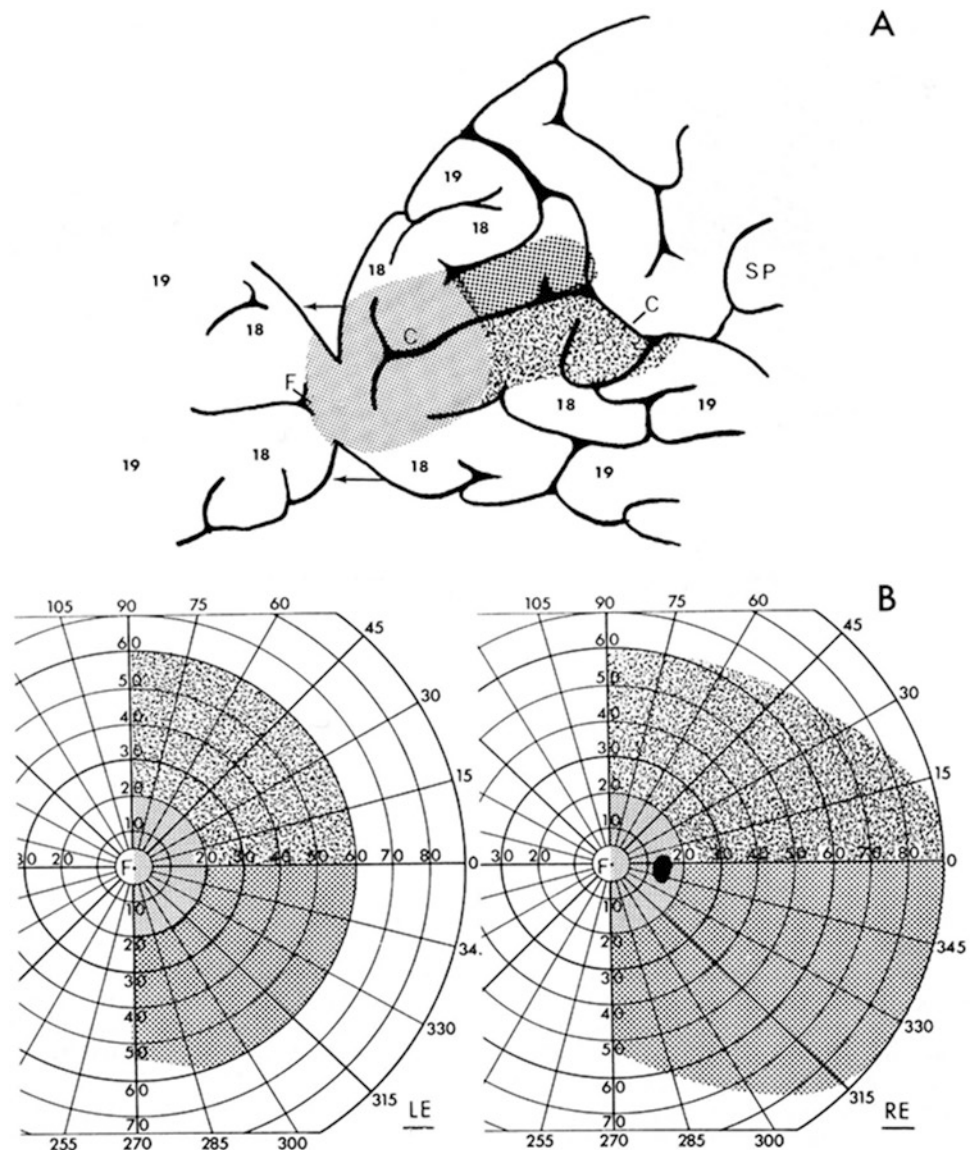
The projection from the LGN (and thus retinal ganglion cells) onto V1 input cells has approximately constant density, which means that the central visual field is highly over-represented in the visual cortex: Roughly 20% of V1 represents the retinal fovea, and thus the central 1–2° of the visual field, with rapid drop-off of the density towards the periphery.

As explained previously for ganglion cell and LGN characteristics, even the signals arriving in the cortex do not carry simple point-to-point representations of the visual field. From the complex processing network in V1 hypercolumns emerge feed-forward signals to higher cortical areas. In turn, feedback signals from these higher areas further influence local signal processing. Moreover, subcortical signals modulate the activity level in response to state changes such as sleep and arousal.

4.3.4 Anatomy and Physiology: Subcortical Pathways

While the visual pathway to the LGN and striate cortex receives the great majority of retinal ganglion cell axons, subcortical pathways exist as well, formed by optic nerve fibers projecting to pretectal nuclei and to the pulvinar. In primates, the projections to the pregeniculate nucleus and pulvinar are thought to be of minor importance, and may be thought of as anatomical evolutionary remnants. For example, in lower mammals, ablation of striate cortex at birth allows these projections to greatly increase in density, leading to the development of crude functional vision, but simi-

Fig. 4.5 V1 projection of the visual field. The medial wall and part of the occipital surface of the left cerebral hemisphere (A) and the corresponding visual fields for the two eyes (B) are shown. Note that the projection of the fovea (*F*) and a narrow surrounding hemicircle of the visual field project onto the occipital cortex, with the projection of the vertical meridian adjacent to area V2, whereas more peripheral areas—including most of the macula—projects to the medial wall of the cortex, with much of the projection buried in the calcarine fissure (*C*). Also note that the left hemisphere receives information from the right visual hemifield, that the superior visual field projects to the inferior part of V1—i.e., gross localization is preserved from retina to V1, and that corresponding retinal locations in the two eyes project to the same cortical location. No such locations exist for the far nasal segment of the right retina (60° – 90°), as the bridge of the nose blocks the corresponding area in the left eye



lar experiments in newborn monkeys show neither the proliferation of projections nor appreciable acquisition of visual function (Cowey et al. 1994, 2001).

However, other projections in primates, in particular those to the pretectal nucleus of the optic tract (NOT) and the terminal nuclei (TN) of the accessory optic system, have been demonstrated to play an important role in the rapid control of eye position through vestibulo-ocular reflex, saccades, and sustained fixation.

Detailed studies of anatomy and physiology of the primate eye movement system over the last several decades in awake, trained animal models have shown that the NOT receives information on “retinal slip,” i.e., generalized displacement of the retinal image (Mustari and Fuchs 1989). This retinal slip signal is encoded as a velocity signal, and serves as input to the neural integrator in the nucleus prepositus hypoglossi

(Mustari and Fuchs 1989). Pathways between the NOT and primary visual cortex (as well as multiple similar projections between cortical and subcortical structures) are also known to exist, and have been shown to compensate in part for lesions to the NOT or its retinal input (Mustari and Fuchs 1989; Hoffmann 1996).

4.4 Retinal Immunology

Distinctive immunologic characteristics of the eye were well documented and led to the concept of immunologic privilege. The success of corneal transplantation established the eye as a site for tissue transplantation (von Hippel 1888). Later, the harvesting and transplanting photoreceptors or retinal pigment epithelial (RPE) cells into subretinal space

further demonstrated this characteristic (Silverman et al. 1992; Silverman and Hughes 1989; Kaplan et al. 1997). This is influenced by the existence of immunologic privilege in the subretinal space and immunogenicity of the transplanted tissue.

As mentioned above, immune privilege in the eye is thought to be the result of a combination of anatomic features with a special molecular environment. As inflammation can cause tissue damage, tissues with limited regenerative capacity such as the neurons in the brain and retina use immune privilege for protection. An absence of lymphatic drainage, blood-ocular barrier, the presentation of antigen to the host via the circulation (camero-splenic axis), and the reduced expression of MHC class I and II molecules on resident cells in the AC are the anatomic features that contribute to immune privilege (Streilein et al. 1997). In a similar manner, the subretinal space is protected by a blood-retina barrier, via tight junctions between RPE cells, as well as the vascular endothelium of the retinal circulation. The subretinal space has no lymphatic drainage and has reduced expression of MHC class I and II molecules on parenchymal cells of the neurosensory retina (Wang et al. 1987).

Some of the molecular mechanisms necessary to achieve and maintain immune privilege in the AC also exist in subretinal space.

1. Complement inhibition: There have been several complementary regulatory proteins identified immunohistologically in the eye. Their role is to protect intraocular tissue from complement-mediated destruction. There is a selective expression of CD59 (inhibiting formation of complement) in the neurosensory retina, but not membrane cofactor protein (MCP or CD 46) or decay-accelerating factor (DAF or CD55), which are expressed within the AC, and both of which participate in the regulation of complement activation (Bora et al. 1993).
2. Fas ligand-mediated apoptosis: Fas-ligand is a membrane protein that is a member of the TNF family of proteins expressed on activated T cells. Fas ligand binds to Fas, thus stimulating a signaling pathway leading to apoptotic cell death of the Fas-expressing cell. Fas ligand is expressed in various ocular tissues, including the RPE and neurosensory retina. Experiments in vivo and in vitro suggest that constitutive expression of Fas ligand in a tissue leads to a deletion of Fas+ T cells that enter the tissue (Griffith et al. 1997; Jorgenson et al. 1998).
3. Immunosuppressive molecules: Aqueous humor contains several immunomodulatory substances, including TGF- β_2 , free cortisol, IL-1 receptor antagonist, substance P and vasoactive intestinal peptide. It has been shown that RPE secretes TGF- β_2 as well. This immunosuppressant is found in the vitreous gel of the eye.

Thus, the existence of a limited immune privilege in the subretinal space is the result of antigen-specific inhibition of cellular and humoral responses. After the introduction of an antigen into the subretinal space, a suppression of delayed-type hypersensitivity to soluble-protein antigens and a delayed rejection of allogeneic RPE cells are seen. These phenomena are the result of the inhibition of cell-mediated immunity. In the AC, delayed-type hypersensitivity is inhibited while the cytotoxic antibody response is exaggerated. In the subretinal space, both antibody production and delayed-type hypersensitivity are inhibited. This is an important difference between the immunologic privilege process within the AC and the subretinal space.

Most of the experimental studies involving placement of alloantigens in the subretinal space have been performed in rodents, and much about the nature of the immune privilege in the subretinal space remains unknown. Also, the immunogenetic disparity between donor and host is not known, so naturally, a question arises, whether immunosuppression is necessary at the time of allogeneic transplant. The survival of the allogeneic transplant is dependent on the presence of immune privilege at that site, as well as the immunogenicity of the transplanted tissue. There are studies demonstrating that RPE cells express MHC class I but not class II molecules. They can express class II molecules when stimulated with interferon- γ (Liversidge et al. 1988, 1998). The RPE also express ICAM-1, a molecule necessary for T-cell activation (Liversidge et al. 1990). The expression of MHC class II and ICAM-1 on the apical plasma membrane of cultured RPE suggests that these molecules may play an important role in the presentation of antigen to the host (Percupo et al. 1990; Osusky et al. 1997).

4.4.1 Retinal Antigens and Autoimmunity

In 1968 Wacker and Lipton developed an excellent animal model of experimental autoimmune uveitis (EAU) (Wacker and Lipton 1968). The EAU model has been used for several decades in eliciting immune mechanisms, the identification of pathogenic epitopes of autoantigens in the eye in animals, and the evaluation of therapeutic strategies.

Retinal antigens, such as S-antigen (arrestin), interphotoreceptor retinoid-binding protein (IRBP), rhodopsin, recoverin, and phosducin, appear to hold uveitogenic properties. Immunization with these antigens or their fragments can induce ocular inflammation in susceptible strains of laboratory animals. New eye autoantigens have been discovered, such as uveal autoantigen with coiled domains and ankyrin repeats (UACA) in patients with Vogt-Koyanagi-Harada disease (Yamada et al. 2001).

EAU models using S-antigen and IRBP (peptides derived from these proteins) contributed mostly to our knowledge of

retinal autoimmunity. S-antigen, a 48-kDa protein (arrestin), is one of the antigens used to induce EAU (Singh et al. 1988). It is the first retinal autoantigen that has been implicated in the pathogenesis of uveitis (Hirose et al. 1989; Merryman et al. 1991). The main action of arrestin is blocking the interaction of rhodopsin with the G-protein transducin in the phototransduction cascade. Immunization of susceptible animals with S-antigen induces a predominantly CD4+ T-cell-mediated inflammatory response in the retina, uveal tract, and the pineal gland. S-antigen has been implicated in the pathogenesis of uveitis through molecular mimicry. It has been demonstrated that several exogenous antigens, such as baker's yeast, *Escherichia coli*, hepatitis B virus, streptococcal M5 protein, Moloney murine sarcoma virus, and baboon endogenous virus, and several endogenous antigens, such as human leukocyte antigen B-derived peptide, tropomyosin antigens share sequence homology with uveitogenic peptide M of S-antigen (Shinohara et al. 1990). Antistreptococcal monoclonal antibodies were found to recognize several uveitogenic peptides of S-antigen, thus suggesting that immunological mimicry between self and exogenous antigens from an infectious agent may be a potential mechanism in the pathogenesis of uveitis in humans (Lerner et al. 1995).

IRBP is a major protein (1264 amino acid residues) of the interphotoreceptor matrix. It functions as a transporter of retinoids between the retina and RPE. It is found in both the eye and the pineal gland. A spectrum of disease ranging from hyperacute to chronic relapsing disease could be induced with variable doses of this antigen. The inflammation is located at the photoreceptor layer, producing histopathology similar to that seen in uveitis, retinal vasculitis, granuloma, focal serous detachments, loss of photoreceptors, and formation of sub-RPE infiltrates resembling to Dalen-Fuchs nodules (Caspi et al. 1988). The relatively long duration of disease activity in the murine IRBP EAU model makes it a good model for evaluation of therapeutic strategies in established disease (Chan et al. 1990).

Retinal autoimmunity is a complex mechanism that has previously been thought to be always pathological. However, retinal autoimmunity appears to have certain neuroprotective qualities. One such study showed that vaccination with peptides derived from IRBP resulted in protection of retinal ganglion cells from glutamate-induced death or death as a consequence of optic nerve injury (Mizrahi et al. 2002). It is evident that the immune system not only protects the body against invading pathogens but also protects it from toxic substances released by the body's own tissues during stress and trauma. So, the autoreactive cells that induce neuroprotection and those that induce autoimmune disease may share the same qualities, indicating their potential to be protective and destructive at the same time (Kipnis et al. 2002). Lymphocytes reactive to retinal antigens have been found in

healthy individuals (Mizrahi et al. 2002; Nussenblatt et al. 1980). The presence of circulating autoreactive cells in healthy humans suggests that immunoregulatory mechanisms are probably in place to prevent retinal autoimmunity. It appears that the ability to protect the eye from inflammation and injury does not purely depend on mechanisms of immune privilege but instead on a precise regulation of autoimmunity.

4.4.2 Autoimmune Retinopathy (AIR)

AIR is an umbrella term for several disorders with common clinical findings. AIR patients experience acute and progressive vision loss without other known causes in the setting of circulating anti-retinal antibodies in the patient serum. These antibodies can cross the blood-retina barrier and can be found in intraocular fluids as well (Adamus 2008). AIR patients have damage throughout the retinal layers, including Muller cells, bipolar cells, and photoreceptor cells. There is vision loss, electroretinographic changes, and often family history of autoimmune disease. In a recent case series, Heckenlively and colleagues have shown that many patients respond to immunosuppressive regimens (Ferreira et al. 2009). AIR disorders include cancer associated retinopathy (CAR), melanoma associated retinopathy (MAR), and non-paraneoplastic autoimmune retinopathy (npAIR). CAR and MAR are rare paraneoplastic diseases thought to be caused by homology between tumor and retinal proteins with resultant autoimmunity. npAIR share a similar phenotype with CAR and MAR, but is not related to underlying malignancy. There are many retinal antigens associated with AIR. Recoverin, α -enolase, and Carbonic anhydrase II (CA II) are associated with CAR and npAIR. Tubby-like protein 1 (TULP-1) and Heat shock cognate protein 70 (HSC70) have been associated with CAR. Arrestin (S-antigen) and Transducin have been associated with MAR. Collagen II, Cardiolipin, and Phosphatidylinositol have been associated with npAIR. The mechanisms involved in AIR are currently under further study.

4.4.3 Age Related Macular Degeneration (ARMD) and the Ocular Immune Response

The retinal pigment epithelium (RPE) cells have many tasks, including phagocytosis of damaged photoreceptors and metabolic waste material, and support of the photoreceptor cells. They help form the blood-retina barrier to keep the systemic immune system from retinal antigens, and inhibit immune cells to prevent inflammation. Recent studies have indicated that at least in a subset of patients, age related macular degeneration involves autoimmune processes. Complement proteins

have been found in drusen, autoantibodies to retinal proteins have been discovered in ARMD (de Jong 2006), and certain polymorphisms in genes encoding complement proteins (e.g. Complement factor H (CFH) and others) have been associated with the presence of disease (Haines et al. 2005).

Recent research has also shown a link between oxidative stress and the formation of lipid peroxidation products such as malondialdehyde (MDA). Weismann and colleagues have shown that the pro-inflammatory MDA is bound by CFH, and that mutations in CFH lead to impaired binding by the immune system, allowing oxidative stress to accumulate and potentially cause damage and inflammation (Weismann et al. 2011). There is also evidence that oxidative stress can alter lipid peroxidation products into retinal antigens capable of setting off an autoimmune reaction. Carboxyethylpyrrole (CEP) is a product formed from docosahexanoic acid (DHA) under oxidative conditions. In the highly oxidative milieu of the retina, proteins become adherent to CEP forming antigenic moieties which have been shown capable of inducing anti-CEP autoantibodies in a systemic adaptive immune response (Hollyfield et al. 2008). Although the exact mechanisms of ARMD pathology are as yet not completely clear, there is evidence that proinflammatory macrophages (M1 macrophages) may be involved with retinal damage, and it is hypothesized that complement deposition in the setting of oxidative damage may play a role in guiding these destructive cells toward the RPE.

Current research in animal models is focused on elucidating the role of T cells and the adaptive immune response in ARMD. It is hoped that further understanding of the humoral and adaptive immune responses in relationship to ARMD may lead to novel prevention and treatment strategies. In humans, there have been several clinical trials to assess complement inhibitors as a treatment for the dry form of ARMD. Clinical trials are still ongoing to assess if they can be effective in treating or preventing ARMD (Perez et al. 2008).

4.5 Summary

The eye has been recognized as an immune-privileged site for more than 100 years. Medawar demonstrated this by showing a prolonged, often indefinite, survival of organs or tissue grafts in the anterior chamber of the eye (Medawar 1948). Immune privilege is a dynamic process in which immunoregulatory mechanisms combined with anatomical factors maintain the vitality of grafts in privileged sites. ACAID is the best-studied immune-privilege phenomenon in the eye. Although it is an anterior-chamber phenomenon, there is enough evidence to show that there are some of the same mechanisms at work in the vitreous and sub-retinal space (Jiang et al. 1993). Therefore understanding

ACAID could possibly further our knowledge of retinal autoimmunity.

Historically retinal autoimmunity has been considered pathogenic. However, later studies have demonstrated the presence of retinal autoantibodies in normal controls (Yamamoto et al. 1993). Animal optic nerve injury studies suggested possible beneficial roles of retinal autoimmunity in controlling collateral damage to the retinal ganglion cells (Kipnis et al. 2002). Thus, retinal autoimmunity can be viewed as both protective and destructive phenomenon.

Current research is focused on the role of immunity in ARMD, AIR, and corneal transplantation. Subretinal embryonic stem cell research is another field made possible by the eye's unique immune status. Recent research has also shown progress in the field of stem cell transplantation in patients with ARMD and Stargardt's macular dystrophy (Schwartz et al. 2015). Ocular immunology will likely play a major role in the next set of breakthroughs for some of the most vexing eye diseases.

4.6 Review Questions

1. How many compartments the eye contains?

- (a) Three: cornea, anterior chamber and posterior chamber.
- (b) Two: anterior and posterior chamber.
- (c) Four: cornea, anterior chamber, posterior chamber and vitreous cavity.
- (d) *Three: anterior chamber, posterior chamber and vitreous cavity.*

2. What is the volume of vitreous cavity on average?

- (a) 2 mL
- (b) 4 mL
- (c) *6 mL*
- (d) 8 mL
- (e) 10 mL

3. Anterior chamber angle includes all of the following structures except:

- (a) Schwalbe's line
- (b) Sclera
- (c) Ciliary body
- (d) *Lens*
- (e) Iris
- (f) Schlemm's canal
- (g) Trabecular meshwork

4. Aqueous is produced by:

- (a) *Ciliary epithelium*
- (b) Corneal endothelium
- (c) Schlemm's canal
- (d) Trabecular meshwork
- (e) Iris roots

5. **All of the following are immune privileged sites except:**
 - (a) Cornea
 - (b) *Conjunctiva*
 - (c) Lens
 - (d) Vitreous cavity and subretinal space
 - (e) Testis and ovary
6. **Which statement is not true regarding immune privilege?**
 - (a) The phenomenon of immune privilege was initially described in the nineteenth century.
 - (b) Immune privilege is defined as a prolonged, sometimes indefinite, survival of organ or tissue grafts at special body/organ sites.
 - (c) Immune privilege is a dynamic process maintained by anatomic structures and immunoregulatory forces.
 - (d) *Grafts placed in immune privileged sites remain sequestered from the immune environment.*
 - (e) Both active and passive factors of immune privileged sites and tissues contribute to the privileged status.
7. **All the following is true about anterior chamber immune associated deviation (ACAID) except:**
 - (a) ACAID is an antigen-specific systemic immune response to eye-derived antigens
 - (b) ACAID is an actively acquired and actively maintained manifestation of ocular
 - (c) *In ACAID aqueous humor possesses immunomodulatory properties and inhibits all immune reactions*
 - (d) ACAID is a form of peripheral tolerance, unlike central tolerance, where clonal deletion and/or anergy occur within the thymus
 - (e) Once induced, ACAID persists for a very long period of time.
8. **Visual cortex occupies the following structures:**
 - (a) *Occipital and parts of the parietal and temporal lobes of the cerebral cortex*
 - (b) Parietal and parts of occipital and temporal lobes of the cerebral cortex
 - (c) Lateral geniculate body, occipital and frontal lobes of the cerebral cortex
 - (d) Frontal and parts of occipital and temporal lobes of the cerebral cortex
 - (e) Occipital and parts of frontal and parietal lobes of the cerebral cortex
9. **Which substance found in the eye is not uveitogenic?**
 - (a) S-antigen (arrestin)
 - (b) *TGF- β*
 - (c) Interphotoreceptor retinoid-binding protein (IRBP)
 - (d) Recoverin
 - (e) Phosducin

10. **Which antigen is not associated with AIR?**

- (a) Recoverin
- (b) TULP-1
- (c) HSC70
- (d) α -enolase
- (e) *Vasoactive intestinal peptide*

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Abstract

The retina is an outpost of the central nervous system (CNS) with neuronal structures and proteins specialized for the transduction of light signals into a neural code that the brain can interpret. Mutations of proteins involved in phototransduction or synaptic transmission through the retina produce visual deficits ranging from subtle color vision defects to complete blindness. Although normally isolated from blood-mediated immune responses by the blood-retinal barrier, inflammatory responses, including gliosis and those mediated by the complement system, are important contributors to retinal degeneration, particularly age-related macular degeneration (ARMD). Studying immune responses in the retina and their pharmacological manipulation therefore presents a promising avenue for the treatment and prevention of retinal degeneration.

Keywords

Amacrine cell • Bipolar cell • Choroid • Cone • Fovea • Horizontal cell • Magnocellular ganglion cells • Melanopsin • mGluR6 • Müller cells • Opsin • Outer segment • Parvocellular ganglion cells • Photoreceptor • Retina • Retinal ganglion cell • Retinal pigment epithelium • Ribbon • Rod • Transducin

5.1 Introduction

The retina, like other parts of the Central Nervous System (CNS), derives embryologically from the neural tube. Retinal neurons and glia therefore have many properties in common with other CNS tissue, but they also exhibit specialized response properties and proteins that have evolved to serve the retina's function in transducing light energy into nerve signals and analyzing the visual image. For example, to encode small changes in light intensity, many retinal neurons respond to light with graded changes in membrane potential and do not exhibit sodium-dependent action potentials. In

addition to possessing the specialized proteins needed for phototransduction (e.g., rhodopsin and transducin), the retina contains a number of proteins specialized for the transmission and processing of visual information such as the metabotropic glutamate receptor, mGluR6, in ON type bipolar cells; type C γ -aminobutyric acid (GABA_C) receptors (Lukasiewicz et al. 2004); and CaV1.4 calcium channels in photoreceptors. This chapter summarizes some of the special features of retina that help it to transduce and process light.

5.2 Anatomy

The retina is a thin sheet of neural tissue (150–400 μ m thick) at the back of the eye upon which the visual image is focused by the cornea and lens (Fig. 5.1a). Behind the neurosensory retina is a layer of pigmented epithelial cells known as the retinal pigment epithelium (RPE, Fig. 5.1) and beyond the RPE is a dense bed of capillaries within the choroid.

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Surrounding the choroid is the sclera, made up of densely woven collagen fibers that help to encase and protect the globe.

The retina has a highly organized laminar structure that is similar in all vertebrate species (Fig. 5.1b; Dowling 2012; Rodieck 1998). It is oriented so that photoreceptors lie at the back, adjacent to the RPE. Thus, to reach the photoreceptors light must generally pass through overlying layers of the retina. The photoreceptor (PR) layer consists of the outer and inner segments of rod and cone photoreceptors. Separating the inner segments and photoreceptor cell bodies is the outer limiting membrane (OLM) formed from apical processes of glial Müller cells. The outer nuclear layer (ONL) contains the cell bodies of photoreceptors. Photoreceptors make synaptic contact with horizontal and bipolar cells in the outer plexiform layer (OPL). The inner nuclear layer (INL) contains cell bodies of horizontal, bipolar, and amacrine cells. Synaptic contacts among bipolar, amacrine and retinal ganglion cells are made in the inner plexiform layer (IPL). Anterior to the IPL, closer to

the front surface of the retina, is the ganglion cell layer (GCL), which contains cell bodies of retinal ganglion cells. Axons from retinal ganglion cells create a nerve fiber layer (NFL) at the inner surface of the retina. The axons join together as they exit the eye to form the optic nerve, which projects to higher visual centers. The absence of retina and photoreceptors at the optic nerve head creates a small blind spot (Fig. 5.1a). The vitreal surface of the retina is bounded by an inner limiting membrane (ILM) formed by Müller cell endfeet.

The retina contains >60 types of retinal neurons grouped into five major classes: photoreceptors (3–4 types), horizontal cells (generally two types), bipolar cells (12–13 types), amacrine cells (~30 types), and ganglion cells (~20 types) (Masland 2012; Euler et al. 2014). Photoreceptors release L-glutamate to stimulate bipolar cells, which in turn release L-glutamate onto ganglion cells forming a throughput pathway for the transmission of light signals through the retina (Fig. 5.2). Horizontal and amacrine cells are predominantly

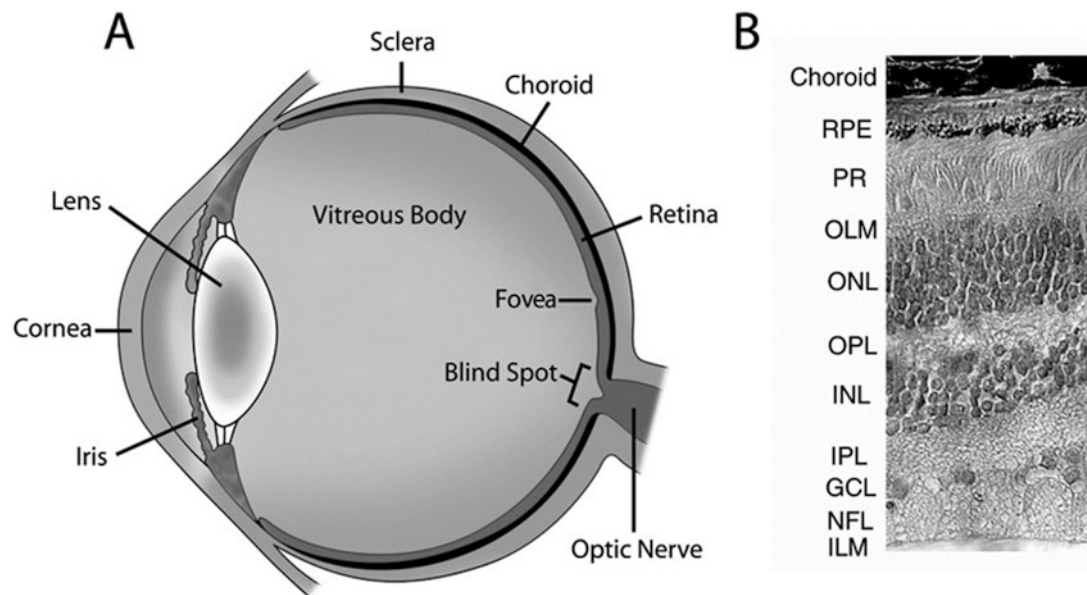
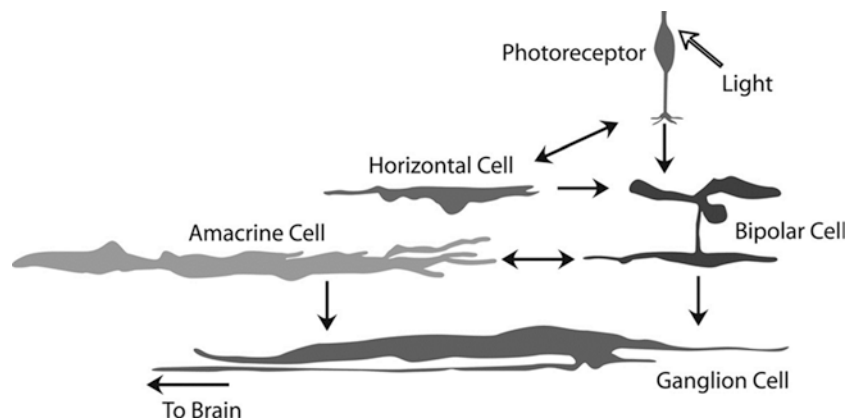


Fig. 5.1 (a) Schematic diagram of a primate eye. (b) Cross section of primate retina showing the different layers

Fig. 5.2 The five major retinal cell types (photoreceptors, bipolar cells, horizontal cells, amacrine cells, and ganglion cells) and their synaptic connections



inhibitory interneurons with processes extending laterally in the outer and inner plexiform layers, respectively. As light signals are transmitted from photoreceptors to bipolar cells to ganglion cells, they are modified by inhibitory synaptic feedback from horizontal cells onto photoreceptors and bipolar cells in the outer retina and from amacrine cells onto bipolar and ganglion cells in the inner retina. Photoreceptors and second order retinal neurons (bipolar and horizontal cells) respond to light with graded changes in their membrane potential (Dowling 2012). Action potentials are typically observed only in third order neurons of the retina (amacrine and ganglion cells). The use of graded responses by cells early in the visual pathway is likely related to the ability of graded responses to transmit more information than a spike code (Laughlin 2001). By contrast, the use of action potentials by ganglion cells is necessary to propagate information over the greater distances needed to reach higher visual centers.

5.3 Cell Types

5.3.1 Rod and Cone Photoreceptor Cells

5.3.1.1 Outer Segments and Phototransduction

There are two major types of photoreceptors cells: rods and cones (Ebrey and Koutalos 2001). The structure and phototransduction proteins of rods are specialized to allow them to respond to dim lights. Cones are less sensitive to light, but provide high acuity and color vision. Rods and

cones are named for their rod- and cone-shaped outer segments, respectively. The outer segments of both cell types contain the proteins necessary for phototransduction, packaged into disc-shaped organelles. Cone discs are formed by invaginations of the outer segment plasma membrane whereas rod discs are completely sequestered within the outer segment.

The principal job of photoreceptors is to transduce light into an electrical signal. Phototransduction involves a cascade of enzymes in the outer segment (Arshavsky et al. 2002) (Fig. 5.3). It is initiated by absorption of a photon by rhodopsin (or cone-specific opsins). Rhodopsin is a G-protein coupled receptor with homology to other G-protein coupled receptors (GPCRs) (e.g., beta-adrenergic, muscarinic, etc.). However, unlike GPCRs that are activated by the binding of a neurotransmitter ligand, the activating ligand of opsin is a light-sensitive chromophore molecule, vitamin A aldehyde (or retinal), bound within a pocket of the opsin protein. Absorption of a photon by this chromophore initiates a conformational transition of 11-cis-retinal, which is bent around the 11-cis carbon position, into all-trans retinal, which has a straight chain configuration (Fig. 5.3). The conformational change in retinal produces conformational changes in the seven trans-membrane domains of rhodopsin, causing it to assume an active configuration known as metarhodopsin. The activated GPCR, metarhodopsin, stimulates exchange of guanosine triphosphate (GTP) for guanosine diphosphate (GDP) on the associated G-protein, transducin (G_T). Activated alpha subunit of transducin (α_T) stimulates the activity of a cGMP-specific phosphodiesterase, which

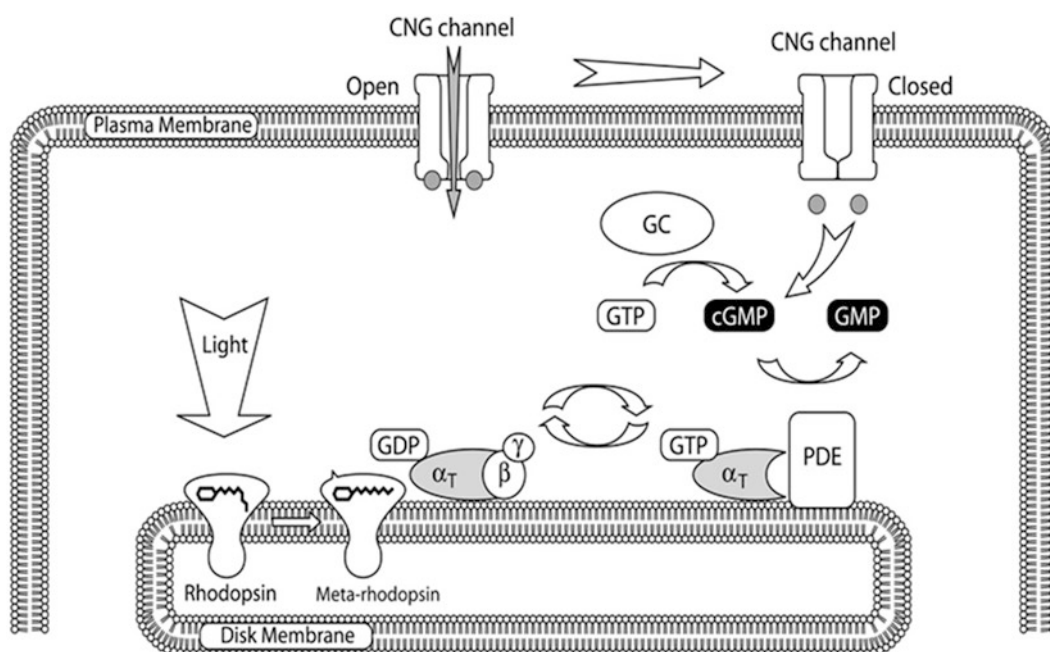


Fig. 5.3 The enzymatic cascade responsible for phototransduction

hydrolyzes cGMP. The plasma membrane of outer segments contains non-selective cation channels permeable to Na^+ , K^+ , and Ca^{2+} that are opened by the binding of cGMP to their intracellular face. Light-induced reduction in cytoplasmic [cGMP] causes some cGMP-gated cation (CNG) channels to close. When open in darkness, Na^+ and Ca^{2+} influx through CNG channels causes photoreceptors to depolarize. Conversely, light-induced closure of these channels causes photoreceptors to hyperpolarize. Because brighter lights cause a larger number of CNG channels to close, the amplitude of membrane hyperpolarization is graded with intensity.

The enzymatic transduction cascade initiated by photon absorption is terminated by two key mechanisms: (1) Light-activated meta-rhodopsin is inactivated in a process that begins with its phosphorylation by rhodopsin kinase. Following phosphorylation of rhodopsin at three sites by rhodopsin kinase, the protein arrestin binds to rhodopsin and arrests further activity. The actions of rhodopsin kinase are analogous to the regulation of beta-adrenergic receptors by beta-adrenergic receptor kinase. (2) Light activated transducin is also shut down by its intrinsic GTPase activity, which converts GTP into GDP and thereby de-activates the G protein. The intrinsic GTPase activity of transducin is accelerated by binding of the accessory proteins, RGS/G β 5, to the transducin alpha subunit.

Tremendous amplification of the light signal by phototransduction in rods allows them to detect absorption of a single photon (Field et al. 2005). To accomplish this feat, each activated metarhodopsin molecule catalyzes hundreds to thousands of transducin molecules and although each transducin activates only a single phosphodiesterase (PDE) molecule, every PDE molecule hydrolyzes thousands of cGMP molecules. The net result is that activation of a single rhodopsin molecule by a single photon of light causes the degradation of 10^5 – 10^6 cGMP molecules causing many cGMP-gated cation channels to close and producing a small but detectable change in membrane potential.

Mutations in phototransduction proteins are a major cause of retinitis pigmentosa and other photoreceptor degenerations (Kennan et al. 2005). Mutations in rhodopsin, phosphodiesterase and cGMP-gated cation channels can all produce retinitis pigmentosa. Mutations in rod-specific transducin produce night blindness whereas mutations in cone-specific transducin produce achromatopsia (rod monochromacy). Similarly, by preventing cones from responding to light, mutations in cone-specific cGMP-gated cation channels also lead to achromatopsia. For an updated list of genes involved in retinal diseases, consult the “Retinal Information Network” (<http://www.sph.uth.tmc.edu/Retnet/>).

5.3.1.2 Light Adaptation

Photoreceptors can respond to only a limited range of intensities before their responses saturate. To maintain responsiveness over the large range of intensities encountered in the world, the phototransduction apparatus adapts its sensitivity to increased light levels to maintain a constant relative response to increments in illumination ($\Delta I/I = \text{constant response}$) (Fain et al. 2001; Burkhardt 2001). This process of light adaptation is largely due to calcium-sensitive adjustments of the phototransduction enzyme cascade. As mentioned above, cGMP-gated cation channels are permeable to Ca^{2+} allowing Ca^{2+} to enter the outer segments when these channels are open in darkness. This steady Ca^{2+} influx is countered by the extrusion of Ca^{2+} from outer segment by $\text{Na}^+/\text{Ca}^{2+}$ exchangers. The closing of cGMP-gated channels in response to light diminishes the influx of Ca^{2+} but its efflux via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger continues. The concentration of Ca^{2+} in the outer segment therefore diminishes in light. This decrease in $[\text{Ca}^{2+}]$ enhances the activity of guanylyl cyclase stimulating the production of cGMP. The resulting increase in cGMP opens cGMP-gated channels leading to depolarization of the photoreceptor membrane potential. In cones, decreased $[\text{Ca}^{2+}]$ also increases the affinity of cGMP for cGMP-gated cation channels to further promote their opening. The membrane depolarization that results from re-opening of cGMP-gated cation channels restores the operating range of the photoreceptor cell, allowing it to hyperpolarize in response to another flash of light.

While calcium-dependent adaptation is sufficient to maintain the sensitivity of cones over a large range of intensities, at extremely high intensities there is also a contribution from photochemical adaptation in which a significant fraction of unbleached chromophore (11-cis-retinal) is bleached to all-trans-retinal (Burkhardt 1994). At most light levels, there is a sufficient reservoir of 11-cis-retinal in the outer segments so that the bleaching of chromophore molecules does not appreciably limit the sensitivity of the opsin molecule, as long as levels of 11-cis-retinal are soon restored. (As presented later, restoration of chromophore levels after bleaching involves participation of the RPE and Müller cells.) However, at extremely high light levels, the rate and amount of bleaching is sufficient to limit the availability of chromophore and thereby limit the sensitivity of opsin to light.

The combined effects of calcium-dependent and photochemical adaptation allow cones to maintain constant incremental sensitivity to light over 10^7 -fold changes in intensity (Burkhardt 1994). Rods, sensitive to dimmer lights, maintain relatively constant sensitivity over a 1000-fold change in intensity. Rod and cone systems together therefore allow the visual system to perform the impressive feat of maintaining relatively constant incremental sensitivity to light over a ten billion-fold range of intensities. For comparison, pupillary constriction or dilation contributes a 16-fold change in

sensitivity, although it has the advantage of being relatively rapid, occurring within a couple hundred ms compared to minutes for full light adaptation.

5.3.1.3 Photoreceptor Inner Segment, Soma, and Synaptic Terminal

The inner segments of photoreceptors are packed with mitochondria that fuel their tremendous metabolic demands. Sodium ions continuously entering the outer segments through cGMP-gated cation channels in darkness are extruded by Na/K-ATPases in the inner segments. The continuous consumption of adenosine triphosphate (ATP) by these pumps makes photoreceptor cells among the most metabolically active in the body.

Below the inner segment is the soma and nucleus, which connects at its base to the axon and synaptic terminal. Photoreceptors release glutamate at ribbon synapses (Heidelberger et al. 2005). Synaptic ribbons are specialized for sustained release of neurotransmitter and are also found in the terminals of retinal bipolar cells, as well as vestibular and cochlear hair cells. Synaptic ribbons receive their name because of their planar structure in photoreceptor terminals, but bipolar and hair cell ribbons are more spherical in shape.

The ribbon is composed mainly of the structural protein, Ribeye, but also includes other proteins such as a kinesin motor protein KIF3A, Rab3-interacting protein RIM, and Munc13. Ribbons are attached to the synaptic active zone by bassoon, and its structural relative piccolo, through interactions with “cytomatrix at the active zone” or CAST proteins. Although the ribbon appears to anchor a readily releasable pool of vesicles, molecular motors do not appear to be involved in vesicle movements near the active zone. RIM protein mutations have been implicated in an autosomal dominant rod-cone dystrophy (Johnson et al. 2003).

Glutamate release from synaptic terminals of photoreceptor and bipolar cells is regulated by calcium influx through L-type calcium channels (Heidelberger et al. 2005). The use of L-type channels at ribbon synapses contrasts with the reliance on N, P and Q type channels for neurotransmission at conventional synapses of spiking neurons. A retina-specific L-type channel, CaV1.4, is localized to rod terminals. Mutations in this channel produce congenital stationary night blindness (Bech-Hansen et al. 1998).

5.3.1.4 The Fovea

In humans, the center of the visual image is focused on the fovea in the macula lutea, the region of highest acuity in the retina (Kolb et al. 2005). Although primates are the only mammals with a fovea, many birds and lizards also possess a fovea. Unlike more peripheral retina where light must pass through overlying retinal layers to reach the photoreceptors, overlying neurons are displaced at the fovea to diminish light

scattering. At the center of the resulting foveal pit, the only structures between the outer segments and vitreal surface are cone axons. Cone axons contain xanthophyll pigments that give the macula lutea its characteristic yellow color. The fovea contains only cones and, at its very center, is even free of blue-sensitive cones. Visual acuity, the ability to resolve fine spatial details, is limited by the spacing between cones in the fovea and the density of cones parallels visual acuity in the retina. Acuity can be as high as 20/10 at the foveal center, but falls off rapidly towards the retinal periphery. Although we are not typically conscious of these movements, the eye is constantly in motion, making small microsaccades to allow the high acuity fovea to scan various points and thus construct a high-resolution image at higher visual centers. Loss of the macular region (e.g., in macular degeneration) thus leads not only to a loss of central vision but also to a general loss of high acuity vision.

5.3.1.5 Cones and Cone Opsins

The retinas of Old World primates have three cone subtypes with different spectral sensitivities: short wavelength (S or blue-sensitive), middle wavelength (M or green-sensitive), and long-wavelength (L or red-sensitive) cones. Trichromacy evolved in Old World primates 40 million years ago with duplication of a single M/L ancestral pigment gene followed by divergence into separate M and L pigments. Differences in spectral sensitivity among different cone types arise from the presence of different cone opsins. Cone opsins are 40 % homologous to rhodopsin, S cone opsins are 40 % homologous with M and L cone opsins, and M and L cone opsins are 97 % homologous with one another. The differences in spectral absorbance among different opsins result from differences in a small number of amino acid residues that alter the position of hydroxyl groups close to 11-cis-retinal. The 30 nm difference in spectral absorbance between primate M and L cones is determined primarily by only three amino acids: alanine vs. serine at position 180 (~4 nm), phenylalanine vs. tyrosine at position 277 (~10 nm) and alanine vs. threonine at position 285 (~16 nm) (Deeb 2005).

Different photopigments are encoded by genes located on different chromosomes (Deeb 2005). The gene for rhodopsin is found on chromosome 3, the S cone pigment gene on chromosome 7, and M and L cone pigment genes on the X chromosome. M and L cone pigments are found in a tandem array on the X chromosome. Recombination between these genes on adjacent X chromosomes is the most frequent cause of color vision anomalies. For this reason, most color vision defects involve red–green color vision and are X-linked recessive. Males with anomalous M or L cone pigments are most common (anomalous trichromats), with a frequency of ~6 % among European males, while 2 % are entirely lacking in one pigment or the other (dichromats).

5.3.2 Horizontal Cells

Horizontal cells are laterally arborizing interneurons in the outer retina that receive excitatory synaptic inputs from photoreceptors and make inhibitory synapses onto cones and bipolar cells. The neurotransmitter released from horizontal cells is predominantly GABA. A light-evoked reduction of glutamate release from photoreceptors causes horizontal cells to hyperpolarize by reducing the activation of AMPA-type glutamate receptors. With the exception of certain fish retina, there appear to be no N-methyl-D-aspartate (NMDA) receptors in horizontal cells of adult retina (Thoreson and Witkovsky 1999). Rats and mice have only one type of horizontal cell, but most other mammals have two types (Masland 2012). Because both types hyperpolarize to light of all visible wavelengths, they are sometimes referred to as luminosity-type horizontal cells. Many non-mammalian vertebrates have additional chromaticity or color-opponent horizontal cell types that depolarize to certain wavelengths and hyperpolarize to other wavelengths.

Horizontal cells have large receptive fields due to extensive gap junction coupling among cells. Large receptive fields allow horizontal cells to measure illumination from a wide area. Inhibitory feedback from horizontal cells to cones and bipolar cells subtracts the mean luminance level measured over a wide area from signals transmitted to the inner retina about local luminance changes (Thoreson and Mangel 2012). As discussed later, this negative feedback contributes to formation of the center-surround arrangement of visual receptive fields, important for the detection of edges.

5.3.3 Bipolar Cells

Bipolar cells transmit signals from photoreceptors to ganglion cells. They receive glutamatergic input from photoreceptors and inhibitory inputs from amacrine cell contacts at their terminals in the inner retina. Some may also receive inhibitory influences from horizontal cell contacts at their dendrites in the outer retina. Bipolar cells release L-glutamate at ribbon synapses that contact amacrine and ganglion cells in the inner plexiform layer. There are 12–13 types of cone bipolar cell and a single type of rod bipolar cell in mammalian retina (Masland 2012; Euler et al. 2014). The different cone bipolar cells can be grouped into two major physiological subtypes: cone ON bipolar cells that depolarize to light and cone OFF bipolar cells that hyperpolarize to light. Rod bipolar cells in the mammalian retina are a single ON, depolarizing type. In lower vertebrates, ON and OFF bipolar cells receive a greater mixture of rod and cone inputs.

ON and OFF responses of bipolar cells result from the presence of different glutamate receptors in the two cell types. OFF bipolar cells possess KA and AMPA-type iono-

tropic glutamate receptors, but not NMDA receptors (Thoreson and Witkovsky 1999). Thus, like horizontal cells, the synapse from cones to OFF bipolar cells is sign-conserving, i.e., light-evoked hyperpolarization of the cone reduces the depolarizing influence of AMPA/KA receptors thereby causing the OFF bipolar cell to hyperpolarize.

ON bipolar cells do not possess ionotropic glutamate receptors but are instead activated by a metabotropic glutamate receptor, mGluR6 (Slaughter and Awatramani 2002). mGluR6 is a G-protein coupled receptor that acts via the G protein, G_o , to close TRPM1 non-selective cation channels (Koike et al. 2010; Morgans et al. 2010). Thus, light-induced cessation of glutamate release from photoreceptors causes these cation channels in ON bipolar cells to open and thereby depolarizes the cell. By contrast with OFF bipolar and horizontal cells, the synapse from photoreceptors to ON bipolar cells is therefore sign-inverting. Mutations in mGluR6, TRPM1, and other proteins associated with the mGluR6 receptor complex such as nyctalopin disrupt rod bipolar cell function and thus cause a complete form of congenital stationary night blindness. In addition to ON bipolar cells, TRPM1 (“melastatin”) channels are expressed in melanocytes and their expression declines in metastatic melanoma. Circulating autoantibodies to TRPM1 can cause night blindness in melanoma-associated retinopathy by inhibiting rod bipolar cell function (Xiong et al. 2013).

ON and OFF bipolar cells excite ON- and OFF-type ganglion and amacrine cells, respectively. ON and OFF pathways remain segregated into the lateral geniculate nucleus of the thalamus suggesting this segregation has important functional significance. Saturating mGluR6 with the selective agonist, L-2-amino-4-phosphonobutyric acid (L-AP4), produces an acute deficit in the perception of positive contrast (i.e., bright spots on a dark background) (Schiller et al. 1986). It has therefore been suggested that ON bipolar cells preferentially encode information about positive contrast and OFF bipolar cells preferentially encode information about negative contrast. However, ON and OFF bipolar cells can respond equally well to positive and negative contrast steps (Burkhardt 2001). Furthermore, loss of cone ON bipolar cell function by mutations in mGluR6, TRPM1, or nyctalopin does not produce obvious deficits in contrast perception at photopic light levels (Dryja et al. 2005). Thus, the role played in contrast perception by the segregation of different ON and OFF pathways remains unclear.

5.3.4 Amacrine Cells

Amacrine cells are laterally interconnecting interneurons and most contain the inhibitory neurotransmitters GABA, glycine or both. Amacrine cells are excited by glutamate released from bipolar cells. This glutamate acts principally

on KA and AMPA receptors although many amacrine cells also possess NMDA receptors (Thoreson and Witkovsky 1999). By anatomical and neurochemical criteria, amacrine cells can be classified into ~30 different types (Masland 2012; Wässle 2004). Almost every type of neurotransmitter is present in at least one type of amacrine cell. Physiological responses of amacrine cells include transient and sustained depolarization at light onset (ON cells), light offset (OFF cells), or both (ON/OFF cells). By contrast with the graded light responses of bipolar, horizontal and photoreceptor cells, many amacrine cells exhibit sodium-dependent action potentials making their responses much more transient.

Many amacrine cells have relatively dedicated functions. Some examples include:

1. Large, radially symmetric starburst amacrine cells help create directional selectivity in directionally selective ganglion cells (Taylor and Vaney 2003).
2. Dopaminergic amacrine cells with widespread dendritic arborizations increase release of dopamine in response to increases in global illumination. Dopamine diffuses throughout the retina to influence cells as far away as the RPE. The increased release of dopamine by light modifies cell function to optimize retinal responses in daylight (Witkovsky 2004).
3. All amacrine cells transfer rod signals from rod bipolar cells to ganglion cells (Bloomfield and Dacheux 2001).

5.3.5 Ganglion Cells

Retinal ganglion cells are the output cells of the retina. Their axons course along the vitreal surface of the retina and bundle together to exit the eye as the optic nerve. Ganglion cells are excited by glutamate released from bipolar cells acting on both NMDA and non-NMDA (KA- and AMPA-type) glutamate receptors (Thoreson and Witkovsky 1999).

There are about 20 types of ganglion cells in mammalian retina (Masland 2012; Wässle 2004). The two most common types in primate retina are M (magnocellular) cells and P (parvocellular) cells. The various types of ganglion cells generally remain segregated in their projections to the lateral geniculate nucleus (LGN): M ganglion cells project to M cell layers of the LGN, P cells to the P cell layers, and bistratified cells project predominantly to the koniocellular (interlaminar) regions. Primate M and P cells are analogous to Y and X cell types in cat retina.

M cells have large cell bodies and large dendritic arborizations resulting in large receptive fields (Rodieck 1998). M cells are classified anatomically as parasol ganglion cells. The large receptive fields of M cells limit their contribution to fine feature analysis. Instead, their output is primarily related to motion and other changes in illumination.

In contrast to M cells, P cells have small cell bodies with small dendritic arborizations resulting in small receptive fields. P cells are also wavelength-selective. P cell output thus contributes to fine feature analysis and color vision. Small P cells are classified anatomically as midget ganglion cells.

S cones do not provide direct inputs into M and P-type ganglion cells, which receive inputs only from M and L cones. S cones instead provide inputs into two different types of ganglion cells: blue OFF cells and small bistratified blue ON cells.

There is also a population of ganglion cells that are intrinsically light sensitive. These cells do not require photoreceptor inputs in order to respond to light (Berson et al. 2002; Fu et al. 2005; Lucas 2013), although they do receive rod- and cone-driven synaptic inputs. The intrinsic light-sensitivity of these cells is conferred by the presence of a photopigment, melanopsin. Intrinsic light responses of these cells are slow and show minimal adaptation. Although the mechanism of phototransduction employed by melanopsin remains under investigation, it appears to involve transient receptor potential (TRP) channels similar to those employed in invertebrate phototransduction. Melanopsin-containing ganglion cells are large but few in numbers. They project to areas responsible for controlling non-visual responses to light such as the suprachiasmatic nucleus for circadian rhythm entrainment and the olivary pretectal nucleus for controlling pupil constriction. Recent work indicates that melanopsin photosensitivity also contributes to vision (Schmidt et al. 2014; Estevez et al. 2012; Ecker et al. 2010).

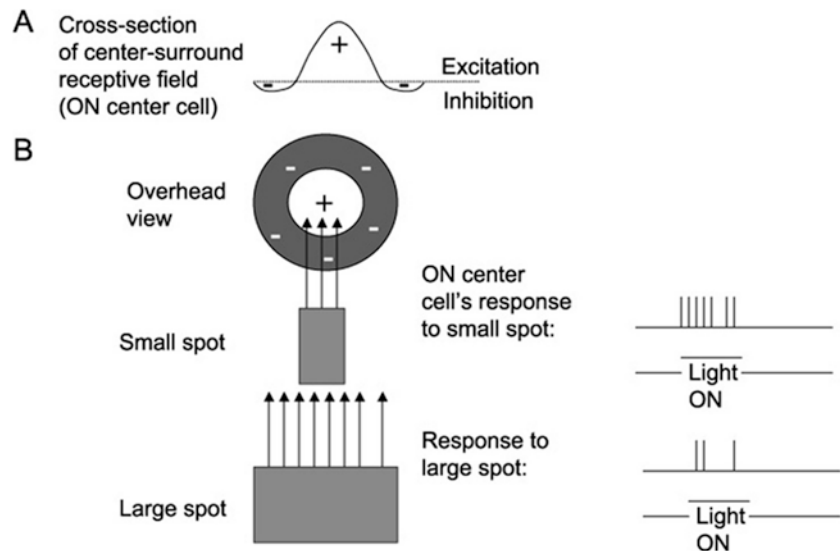
5.4 Circuitry

In addition to transducing the incoming light, the retina plays an important role in the initial process of analyzing visual information. In the following section, we consider the retinal circuitry employed for analysis of edges, color, directional selectivity, and scotopic vision.

5.4.1 Edge Detection and Center-Surround Receptive Fields

The detection of contrast edges is enhanced by the center-surround arrangement of receptive fields in cones, bipolar cells, and ganglion cells (Kuffler 1953; Baylor et al. 1971). How does this center-surround organization improve edge detection? Consider an ON type ganglion cell (Fig. 5.4) in which the circular center of the receptive field is excited by light. Its center-surround arrangement is imparted by the presence of an annular inhibitory region flanking this central excitatory region. A small spot of light illuminating only the excitatory center strongly excites this cell but an annulus of

Fig. 5.4 Center-surround receptive fields in the retina enhance responses to small spots, edges, and bars of light. (a) Cross section of the center/surround receptive field of an ON center cell. Light falling on the center of the receptive field excites the cell whereas light falling in the periphery produces inhibition. (b) Overhead view of the center/surround arrangement of a receptive field of an ON center ganglion cell illustrating differences in the trains of action potentials evoked by a small spot or full field illumination



light falling only on the inhibitory surround strongly inhibits it. Full field illumination, which stimulates both the excitatory center and the inhibitory surround, thus produces smaller changes in ganglion cell output. A bar or edge of light illuminating the entire excitatory center along with small portions of the inhibitory surround evokes a stronger response than full field illumination, but not as strong as a spot of light illuminating just the excitatory center. The net result of this center-surround receptive field arrangement is that cells respond more strongly to spots, bars and edges than to full field illumination. These same kinds of considerations can be extended to OFF type cells that are excited by light decrements in the center and inhibited by light decrements in the surround. Another way to think about center-surround inhibition is that inhibitory feedback from the broad receptive fields of horizontal and amacrine cells subtracts the mean luminance level from signals transmitted to ganglion cells about local luminance changes. In machine vision, the mathematical equivalent of the center-surround receptive field is implemented to spatially differentiate the image and thus enhance the detection of edges.

5.4.2 Color

The retina initiates the process of analyzing color in the world. The presence of three spectrally distinct types of cones in the primate retina (L, M, and S) provides the physiological basis for trichromatic vision (R, G, B). The responses of an individual cone do not vary with wavelength, but only with the number of photons absorbed. Thus, one can obtain an identical response from a green-sensitive cone using either green or red light as long as one adjusts the intensities of the two lights to provide equivalent photon capture by the

green-sensitive cone. For this reason, color discrimination requires comparisons between inputs from different classes of cones. Synaptic comparisons between different types of cones produce responses that are color opponent, i.e., cells that respond to one wavelength by depolarizing but to another wavelength by hyperpolarizing. In non-mammalian vertebrates, color opponency is evident in horizontal and bipolar cells (Twig et al. 2003). In mammalian retinas, color opponency is first detected in ganglion cells (Dacey and Packer 2003).

There are red/green and blue/yellow opponent cell types in the retina. These two classes of opponent neurons contribute to the perception of color opponent after-images (e.g., the illusory appearance of red produced after gazing steadily at a field of green). In primate retina, the red/green hue axis of color vision utilizes separate retinal circuits and ganglion cell types from the blue/yellow axis. Red/green opponency in primate P-type ganglion cells arises from the selective segregation to center and surround of M and L cone inputs via distinct bipolar cells (Dacey and Packer 2003). Via specialized S cone bipolar cells, S cones provide the input for responses to blue light in the receptive field center of blue ON bistratified and blue OFF-type ganglion cells. Responses to yellow light in the receptive field surround are generated from a sum of L and M cone signals.

5.4.3 Directional Selectivity

Some ganglion cells are excited by stimuli moving along one axis of the receptive field (e.g., upward) but show little response to stimuli moving in the opposite direction (e.g. downward). ON directionally-selective retinal ganglion cells cluster along three preferred axes of motion that correspond to the three axes

of the semicircular canals (Wei and Feller 2011; Borst and Euler 2011). These cells project to the accessory optic system in the midbrain where they drive optokinetic reflex eye movements that, together with vestibuloocular reflexes, can correct for mismatches between head and eye motion (“retinal slip”) that blur the image on the retina. While directional selective cells mediate eye reflex control, conscious motion perception appears to be largely computed in visual cortex by comparing temporal delays between spatially offset receptors.

ON directionally-selective ganglion cells receive synaptic inputs from bipolar cells and symmetrically radiating starburst amacrine cells (Vaney et al. 2012). While still not fully understood, it appears that the directional selectivity in ganglion cells arises from multiple mechanisms including asymmetric inhibition from starburst amacrine cells involving differences in the number of GABAergic synapses in different parts of the cell (Briggman et al. 2011) and asymmetric excitation from bipolar cells.

5.4.4 Rod Pathways

Scotopic or low-light vision is mediated by rod photoreceptors. The mammalian retina has a duplex organization in which rods communicate with ganglion cells using a largely separate circuit from cones. The primary rod circuit begins with rods communicating with ON-type rod bipolar cells that, unlike cone bipolar cells, do not contact ganglion cells directly but instead contact AII amacrine cells. AII amacrine cells form gap junctions with cone ON bipolar cell terminals and inhibitory, sign-inverting glycinergic synapses with cone OFF bipolar cells. Output from AII amacrine cells, therefore, feeds rod signals into cone bipolar cells driving both ON and OFF center ganglion cells. AII amacrine cells provide the primary pathway for rod signals at very low light levels, but at higher light levels there is also a contribution from direct rod inputs into OFF bipolar cells and the transmission of rod signals through gap junctions into neighboring cones (Bloomfield and Dacheux 2001).

There is tremendous synaptic convergence in the rod pathway with as many as 75,000 rods converging onto a single ganglion cell. By contrast, cones show much less convergence, with a 1:1 connection from cones to midrange bipolar cells to midrange ganglion cells in the foveal center. Limited convergence in the cone pathway is important for the high visual acuity mediated by cone circuits. On the other hand, the large convergence of signals in the rod pathway facilitates the perception of very dim flashes of light. The ability of individual rods to respond to a single photon of light combined with convergence allows us to perceive the absorption of as few as a dozen photons within 100 ms (Field et al. 2005).

5.5 Retinal Pigment Epithelial Cells

The RPE is a monolayer epithelium of hexagonal cells whose name reflects the facts that it contains melanin pigment granules and forms the outermost layer of the retina. The apical processes of RPE cells ensheath outer segments of rods and cones and each RPE cell contacts ~20–30 rods (Strauss 2005; Marmor and Wolfensberger 1998).

Melanin granules are concentrated in the apical processes and cytoplasm of RPE cells but are nearly absent from basal cytoplasm. By absorbing stray photons of light that have passed through photoreceptor outer segments, melanin granules improve the optical isolation of individual photoreceptors.

Neighboring RPE cells are connected by tight junctions that help to create the blood/retinal barrier separating the neurosensory retina from fenestrated capillaries in the choroid. The basement membrane of RPE, together with the adjacent basement membrane of the choroid, forms a structure known as Bruch’s membrane. RPE cells possess a number of organic and ion transporters to help move polar molecules across the blood retinal barrier. These include transporters for amino acids, folate, ascorbic acid, myo-inositol, organic anions, glucose and lactate.

Photoreceptors continually synthesize new phototransduction proteins for incorporation into newly formed outer segment discs. As new discs are formed at the base of the outer segment, older discs are phagocytosed by RPE cells at the tip of the outer segment. Each outer segment disc is formed and shed in ~2 weeks. The phagocytosis of discs occurs in circadian bursts (rods at the end of night, cones at the end of day).

The bleached, all-trans form of the chromophore retinal cannot be converted back into photosensitive 11-cis-retinal within photoreceptor cells but requires participation of the RPE or, for cones, of Müller cells (Lamb and Pugh 2004; Wang and Kefalov 2011). Following the conversion of 11-cis-retinal to all-trans-retinal by light, all-trans-retinal is rapidly converted to all-trans-retinol (all-trans-vitamin A) in the photoreceptor outer segment. All-trans-retinol is then transported out of the photoreceptor cell, through the interstitial space, and into adjacent RPE or Müller cells via a process involving interstitial retinoid binding proteins (IRBP). The regeneration of cone pigment molecules by Müller cells is described in the next section. Upon entering RPE cells, retinol is bound to cellular retinol binding protein. All-trans-retinol is converted to 11-cis-retinol by retinol isomerase (RPE65) and 11-cis-retinol is converted back to 11-cis-retinal by 11-cis-retinol dehydrogenase. 11-cis-retinal is transferred to cellular retinal binding protein (CRALBP) for transport to the RPE cell surface. Finally, regenerated 11-cis-retinal is transported back to photoreceptors via IRBP. By depriving photoreceptors of chromophore, mutations of visual cycle proteins (e.g., retinol

isomerase [RPE65], 11-cis-retinol dehydrogenase, CRalBP, and IRBP) can lead to photoreceptor degeneration.

The interstitial space between RPE and photoreceptors contains a sticky interphotoreceptor matrix consisting of glycoproteins, proteoglycans, and hyaluronic acid that helps the retina adhere to the back of the eye. Retinal adhesion is also promoted by extrusion of water from the RPE to choroid. Basolateral Cl channels and apical Na/K/2Cl transporters are particularly important for water transport out of the RPE. Because photoreceptors are not physically bound to the RPE, the retina can detach from the RPE with a strong blow to the eye, fluid build-up behind the retina (rhegmatogenous detachment), or traction from overlying cells in proliferative vitreoretinopathy. Detachment of photoreceptor cells from the adjacent RPE prevents recycling of the photopigment which blocks phototransduction by depriving opsin of sufficient chromophore.

One byproduct of photoisomerization in photoreceptor outer segments is N-retinylidene-N-retinylethanolamine (A2E). A2E is ingested by RPE cells when they phagocytose outer segments. However, A2E cannot be enzymatically degraded by RPE cells and thus accumulates in these cells, where it becomes a major component of lipofuscin granules (Sparrow and Boulton 2005). Stargardt's macular degeneration involves a defect in the ABCR transporter that increases accumulation of A2E suggesting it may play a role in macular degeneration. The damage to the RPE associated with A2E accumulation may result from stimulation of apoptosis in A2E-laden RPE cells produced by exposure to blue light.

A major risk factor for age-related macular degeneration (ARMD) is the presence of drusen deposits (Velez-Montoya et al. 2014). White drusen spots visible with an ophthalmoscope are formed by deposits between the RPE and Bruch's membrane. Drusen formation involves inflammatory reactions and RPE cells overlying drusen often show signs of impending cell death. Studies on genetic factors contributing to ARMD have identified mutations in immune complement-related genes as important risk factors (Edwards et al. 2005; Haines et al. 2005; Klein et al. 2005; Gold et al. 2006). This has led to the suggestion that mutations that promote overactive inflammatory responses may contribute to drusen formation leading to RPE cell damage and ARMD.

5.6 Glia

The predominant retinal glial cell is the Müller cell. Müller cells are radial glia that span the retina from the OLM to ILM and ensheath virtually every cell in the retina. They play a number of important roles in maintaining homeostasis (Newman and Reichenbach 1996). For example, Müller cells are a primary storage depot for glycogen in the retina that

can provide metabolites (e.g., lactic acid) to neurons during times of metabolic stress. Müller cells also help to remove and redistribute metabolic waste products. They exhibit high levels of glutathione that can help protect the retina from oxidative stress. Müller cells also possess Na/HCO₃ co-transport mechanisms and carbonic anhydrase to stabilize pH levels in the retina.

Another important role of Müller cells is the spatial redistribution of K⁺ from regions of high concentration to regions of low concentration in order to maintain a stable extracellular K⁺ concentration of about 3 mM (Kofuji and Newman 2004). When neuronal depolarization causes K⁺ levels to increase in the IPL and OPL, excess K⁺ ions enter Müller cells processes. K⁺ influx in the plexiform layers is accompanied by a simultaneous efflux through K⁺ channels clustered at the vitreal surface and along blood vessels. Conversely, a reduction in K⁺ accompanying neuronal hyperpolarization is accompanied by an efflux of K⁺ out of Müller cells into the plexiform layers. The current flowing through radially oriented Müller cells as a result of this spatial buffering of K⁺ produces measurable trans-retinal potentials, such as slow PIII of the electroretinogram. It was once believed that Müller cell K⁺ currents were the predominant mechanism responsible for the ERG b-wave, but more recent studies suggest that the b-wave primarily reflects ON bipolar cell responses.

Müller cells are major sites for the uptake and removal of neurotransmitters, most notably glutamate and GABA. Glutamate transport into neurons is smaller and slower than transport into Müller cells and thus uptake into Müller cells is the principal mechanism responsible for the initial removal of extracellular glutamate following synaptic activation (Pow 2001). In addition to neurotransmitter transporters, Müller cells possess neurotransmitter receptors and can release neuroactive substances (e.g., ATP) (Newman 2004). Thus, activity of retinal neurons can influence Müller cells and Müller cell activity can in turn influence adjacent neurons.

As described in the previous section, RPE cells are required for regenerating 11-cis-retinal from all-trans-retinal derived from rods. However, regeneration of the bleached photopigment from cones is largely performed by Müller cells (Wang and Kefalov 2011). All-trans-retinol is transported into Müller cells where it is converted into the 11-cis isomer and then transported back into cones where it is oxidized to 11-cis-retinal. Regeneration of cone pigment in Müller cells is generally similar to regeneration in RPE but uses a different isomerase (isomerase II in Müller cells vs. RPE65 in RPE cells) and Müller cells export the alcohol, 11-cis-retinol, rather than the aldehyde, 11-cis-retinal.

In addition to Müller cells, the retina contains three other types of glial cells: astrocytes, oligodendrocytes and microglia. Retinal astrocytes are located primarily in the

nerve fiber layer and oligodendrocytes form the myelin sheath of axons in the optic nerve. Hematopoetically-derived microglia are small stellate cells that, when quiescent, associate with inner retinal blood vessels.

Infection or damage to the retina stimulates Müller cell gliosis (Garcia and Vecino 2003) and migration of microglia to the injured area to assist in phagocytosing debris from dying cells. Microglia and Müller cells both release cytokines in response to injury and the release of pro-inflammatory cytokines can sometimes exacerbate cell damage during retinal disease. Cytokines released by Müller cells include vascular endothelial growth factor and transforming growth factor beta, which promote neovascularization, as well as basic fibroblastic growth factor. At least in some species, Müller cells respond to injury and cytokines by dedifferentiating into progenitor cells that can give rise to neurons (Fischer and Bongini 2010).

5.7 Blood Supply

Blood is supplied to the retina by the central retinal artery and choroidal blood vessels (Oyster 1999). The central retinal artery arises from the ophthalmic artery, which in turn branches off the internal carotid artery. Upon entering the retina, the central retinal artery branches into deep capillary beds in the INL and superficial capillary beds in the ganglion cell layer. Endothelial cells of retinal capillaries are joined by tight junctions, contributing to the blood/retinal barrier. There is little or no autonomic regulation of the retinal circulation; blood flow through these capillaries is instead controlled primarily by autoregulation (Wangsa-Wirawan and Linsenmeier 2003). Retinal capillaries drain into the central retinal vein.

Choroidal vessels derive from posterior and short posterior ciliary arteries that, like the central retinal artery, branch off from the ophthalmic artery. The choroidal circulation forms a dense bed of fenestrated capillaries, known as the choriocapillaris, just beneath the basolateral surface of the RPE. The flow rate through the choriocapillaris is among the highest in the body and the arterio-venous drop in PO_2 is minimal. This high flow rate supplies the energetically demanding photoreceptors with large amounts of oxygen and maintains the retina at a constant temperature despite changing levels of radiant energy focused onto the back of the eye. Oxygen for the photoreceptors comes primarily from the choroid. The high level of oxygen consumption by photoreceptor cells in darkness produces a PO_2 of zero at the level of the inner segments in mammalian retina (Linsenmeier 1986). Under these conditions, there is no oxygen available for the remainder of the retina from the choroid. Other retinal neurons instead receive their oxygen from the retinal circulation. (By contrast with mammals, many cold-blood vertebrates

lack retinal capillaries and rely on the choroid to supply the entire retina with oxygen and other nutrition.) While capable of autoregulation (Kiel and Shepherd 1992), blood flow in the choriocapillaris is also regulated by autonomic inputs. Choroidal capillaries drain into four vortex veins, one from each quadrant of the eye.

5.8 Review Questions

1. Briefly summarize the major steps in phototransduction by rods.
2. What are the calcium-dependent steps in light adaptation?
3. What are the major physiological features of magnocellular and parvocellular retinal ganglion cells in the primate retina?
4. In mammalian retina, which capillary beds supply oxygen to the photoreceptors and which to the remaining parts of the retina?
5. For an ON-type ganglion cell, describe how the center-surround organization of visual receptive fields improves edge detection.
6. Summarize the key roles of the RPE.
7. Describe the rod pathway used in scotopic vision.
8. Describe the key enzymatic steps in the visual cycle converting bleached all-trans-retinal back into light-sensitive 11-cis-retinal in RPE cells.
9. Describe the spatial redistribution of K^+ by Müller cells.
10. Summarize the enzymatic mechanisms by which phototransduction is terminated after photo-stimulation.
11. Which of the following is the predominant glial cell type in the retina?
 - a. Astrocyte
 - b. Müller cell
 - c. Microglia
 - d. Schwann cell
 - e. RPE cell
12. In which layer of the retina do bipolar cell terminals contact ganglion cell dendrites?
 - a. Outer nuclear layer
 - b. Outer plexiform layer
 - c. Inner nuclear layer
 - d. Inner plexiform layer
 - e. Ganglion cell layer
13. Which of the following statements is true about the fovea?
 - a. The fovea contains both rods and cones
 - b. The fovea is the region of the retina where ganglion cell axons exit the eye.
 - c. The fovea is the region of the retina responsible for the highest acuity vision in humans and primates.
 - d. The location of the fovea on the retina is not a fixed anatomical feature but varies with focus.

- e. At the center of the fovea, light must first pass through ganglion, amacrine, horizontal and bipolar cells before reaching photoreceptor outer segments.
14. Upon which chromosome are genes for M cone pigments located?
 - a. *X chromosome*
 - b. Y chromosome
 - c. Chromosome 3
 - d. Chromosome 7
 - e. Chromosome 10
15. Which of the following statements is true about ON and OFF type retinal bipolar cells?
 - a. ON bipolar cells possess KA/AMPA receptors and OFF bipolar cells possess NMDA receptors.
 - b. ON bipolar cells possess NMDA receptors and OFF bipolar cells possess mGluR6.
 - c. ON bipolar cells possess mGluR6 and OFF bipolar cells possess NMDA receptors
 - d. *ON bipolar cells possess mGluR6 and OFF bipolar cells possess KA/AMPA receptors*
 - e. ON bipolar cells possess KA/AMPA receptors and OFF bipolar cells possess mGluR6
16. Which of the following is NOT considered to be a major role of Müller cells?
 - a. Spatial redistribution of potassium
 - b. Neurotransmitter uptake and removal
 - c. Homeostatic maintenance of retinal pH levels
 - d. Glycogen storage
 - e. *Promoting retinal adhesion to the back of the eye*
17. Which of the following is NOT considered to be a major role of RPE cells?
 - a. *Phagocytosis of injured cells*
 - b. Formation of blood-retinal barrier
 - c. Absorption of stray light
 - d. Photopigment recycling
 - e. Phagocytosis of outer segment discs
18. Which of the following proteins is a major constituent of synaptic ribbons?
 - a. Retinol dehydrogenase
 - b. Melanin
 - c. *Ribeye*
 - d. RGS/G β 5
 - e. Bestrophin
19. Which cell types are responsible for converting all-trans vitamin A into the 11-cis form needed for phototransduction?
 - a. Rods and cones
 - b. *Müller cells and RPE cells*
 - c. Choroid
 - d. Horizontal cells and bipolar cells
 - e. Astrocytes and microglia
20. What is the light-sensitive molecule found in intrinsically photo-sensitive retinal ganglion cells of mammalian retina?

- a. *Melanopsin*
- b. Transducin
- c. Rhodopsin
- d. Cryptochrome
- e. Peropsin

5.9 Answers

1. Absorption of a photon stimulates a conformational change in the chromophore, 11-cis-retinal (vitamin A aldehyde), so that it assumes an all-trans configuration (all-trans-retinal). This conformational change in the chromophore causes a conformational change in the G-protein-coupled receptor, rhodopsin, so that it achieves its active conformation, meta-rhodopsin. Activated meta-rhodopsin activates the G protein alpha subunit, transducin, which in turn stimulates cGMP-specific phosphodiesterase to cleave cGMP into GMP. This reduction in [cGMP] causes cGMP-gated non-selective cation channels to close. Closure of these channels reduces the influx of Na⁺ (and Ca²⁺) and thus hyperpolarizes the rod.
2. In response to light, the influx of Ca²⁺ into outer segments through cGMP-gated channels diminishes but its efflux by the Na⁺/Ca²⁺ exchanger continues. This causes the concentration of Ca²⁺ in the outer segment to diminish in light. The reduction in Ca²⁺ levels has two major effects on phototransduction: (1) Decreased [Ca²⁺] enhances the activity of guanylyl cyclase in rods and cones stimulating the production of cGMP. The resulting increase in cGMP levels opens cGMP-gated channels causing photoreceptor cells to depolarize. (2) Decreased intracellular [Ca²⁺] increases the affinity of cGMP for cGMP-gated cation channels in cones and thus enhances the opening of these channels. The membrane depolarization that results from re-opening of cGMP-gated cation channels restores the operating range of the photoreceptor cell by allowing it to hyperpolarize in response to another flash of light.
3. Magnocellular ganglion cells have large cell bodies and large dendritic arborizations resulting in large receptive fields. Because of their large receptive fields, M cells do not contribute to fine feature analysis. Instead, their output is primarily related to motion and other changes in illumination.
 Parvocellular ganglion cells have small cell bodies with small dendritic arborizations resulting in small receptive fields. P cells are also wavelength-selective. P cell output thus contributes to fine feature analysis and color vision.
4. Choroidal capillaries provide oxygen to photoreceptors. Retinal capillaries in the INL and ganglion cell layer provide oxygen to the remainder of the retina.

5. The center-surround arrangement of the receptive field is due to the presence of an annular inhibitory region surrounding a central excitatory region. A small spot of light illuminating only the excitatory center strongly excites the cell whereas an annulus of light illuminating the inhibitory surround strongly inhibits it. By contrast, stimulation of both the excitatory center and inhibitory surround by full field illumination produces relatively small changes in ganglion cell output. A bar or edge of light illuminating the entire excitatory center along with small portions of the inhibitory surround will evoke a stronger response than full field illumination, but not as strong as a spot of light illuminating just the excitatory center. The net result of this center-surround receptive field arrangement is that cells respond more strongly to spots, bars and edges than to full field illumination
6. Directional selective ganglion cells are excited by stimuli moving along one axis of the receptive field but show little response to stimuli moving in the opposite direction. The directional selectivity in ganglion cells results from the fact that cholinergic excitation from starburst amacrine cells occurs earlier than GABAergic inhibition with visual stimuli moving in the preferred direction, but GABAergic inhibition precedes excitation with stimuli moving in the opposite, non-preferred direction. Because the preceding GABAergic inhibition dampens the cell's responses to movement in the non-preferred, directional selective ganglion cells respond more strongly to movement in the preferred direction.
7. Rods contact ON-type rod bipolar cells. These rod ON bipolar cells do not contact ganglion cells directly but instead contact AII amacrine cells. AII amacrine cells form gap junctions with cone ON bipolar cell terminals as well as inhibitory, sign-inverting glycinergic synapses with cone OFF bipolar cells. Thus, AII amacrine cells feed rod signals into ON and OFF cone bipolar cells driving ON and OFF center ganglion cells, respectively.
8. After the bleaching of 11-cis-retinal to all-trans-retinal by light, all-trans-retinal converts rapidly to all-trans-retinol (all-trans-vitamin A) in the photoreceptor outer segment. All-trans-retinol is transported out of photoreceptor cells, through the interstitial space, and into the RPE through a process involving interstitial retinoid binding proteins (IRBP). In the RPE, all-trans-retinol is converted to 11-cis-retinol by retinol isomerase (RPE65) and 11-cis-retinol is converted back to 11-cis-retinal (11-cis-vitamin A aldehyde) by 11-cis-retinol dehydrogenase. Regenerated 11-cis-retinal is transported back to photoreceptors via IRBP.
9. When K^+ levels increase in the IPL and OPL due to neuronal depolarization, excess K^+ ions enter K^+ channels localized to Müller cells processes. This K^+ influx in the

plexiform layers is accompanied by a simultaneous efflux through K^+ channels clustered at the vitreal surface and along blood vessels. Conversely, a reduction in K^+ accompanying neuronal hyperpolarization is accompanied by an efflux of K^+ out of Müller cells into the plexiform layers.

10. Inactivation of the light-activated form of rhodopsin, meta-rhodopsin, begins with its phosphorylation by rhodopsin kinase. Phosphorylation of meta-rhodopsin by rhodopsin kinase allows arrestin to bind and arrest further enzymatic activity. Light-activated transducin is shut down by the intrinsic GTPase activity of transducin that converts GTP into GDP. The G protein, transducin, is de-activated by converting GTP into GDP. The intrinsic GTPase activity of transducin is accelerated by binding to an accessory protein, RGS/G β 5.

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Abstract

The hippocampus is a symmetrical structure located inside the medial temporal lobe on both sides of the human brain. In cross-sections, the hippocampus consists of two interlocking sheets of cortex with three distinct sub-regions: the dentate gyrus, the hippocampus proper (CA1–CA3) and the subiculum. The hippocampus has a much defined laminar structure with layers visible where rows of pyramidal cells are arranged. A striking feature of hippocampus is its connection circuitry. The connections within the hippocampus generally follow this laminar format and are largely unidirectional. They form well-characterized closed loops that originate mainly in the adjacent entorhinal cortex. Thus information flow through the hippocampus proceeds from dentate gyrus to CA3 to CA1 to the subiculum, forming the principal trisynaptic circuit. Together with the adjacent amygdala and entorhinal cortex, the hippocampus forms the central axis of the limbic system and plays an important role in spatial learning and awareness, navigation, and episodic/event memory. In addition, the hippocampus plays a role in neuroimmunomodulation.

Keywords

CA1 • CA3 • Dentate gyrus • Hippocampus • Long-term potentiation • Mossy fibers • Neuroimmunomodulation • Neurotransmitter • Perforant path • Schaffer collaterals • Spatial memory • Synapse • Synaptic transmission

6.1 Introduction

The hippocampus is a brain structure located inside the temporal lobe. It forms a part of the limbic system and plays an important role in the formation, consolidation and retrieval of episodic memories. It has been shown that repetitive activation of excitatory synapses in the hippocampus causes an increase in synaptic strength that last for hours or days designated as long-term potentiation (LTP). It is widely believed

that LTP provides an important key to understand the cellular and molecular mechanisms by which memories are formed and stored. Sensory information enters the hippocampus mainly through the perforant pathway consisting of the axons of neurons in layers II and III of the entorhinal cortex. The perforant path axons terminate on the dendrites of the dentate gyrus granular cells. Then information flows through the hippocampus from the dentate gyrus to the CA3, the CA1 and the subiculum, forming hippocampal intrinsic trisynaptic circuit. In addition to its “traditional” role in learning and memory, the hippocampus is also involved in neuroimmunomodulation. Lesion of hippocampus alters neuroimmunomodulation and neuronal functions in the hippocampus are modulated by a variety of immune active molecules.

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6.2 Anatomy of the Hippocampus

The hippocampus, so named because its shape vaguely resembles that of a seahorse, is a curved sheet of cortex folded into the medial surface of the temporal lobe. In transverse sections from rodent brain, the hippocampus has the appearance of two interlocking Cs with three distinct sub-fields: the dentate gyrus, the hippocampus proper (cornu ammonis, CA) and the subiculum (Fig. 6.1), these together is termed hippocampal formation. While hippocampus proper generally includes only CA1–CA3; the CA4 is frequently called the hilus and considered part of the dentate gyrus (Fig. 6.1). The hippocampus of primates and humans occupies less of the telencephalon in proportion to cerebral cortex than lower hierarchy animals does, reflecting it is among the phylogenetically oldest parts of the brain.

The anatomy of the hippocampus has been studied intensively in rodents since Cajal (1911) published his famous drawing illustrating the main cells, connections and flow of impulse traffic in the hippocampal formation (Fig. 6.1), followed by a consecutive of physiological, biochemical and axonal tracing studies, making it one of the most studied and best known structures in the brain (Raisman et al. 1966; Swanson and Cowan 1975; Swanson et al. 1981; Frotscher 1985). Its cytoarchitecture, neural circuit, main afferents and efferents are summarized in Fig. 6.2. Its predominant intrinsic circuit is characterized as three-order projections from dentate granular cells to CA3 pyramidal cells and then from the later to the CA1 (also including CA2) and subiculum neurons (Fig. 6.2).

6.2.1 The Dentate Gyrus

6.2.1.1 Cytoarchitecture

The dentate gyrus is a sharply folded trilayered cortex (molecular, granule cell and polymorphic layers from outer gyrus to hilus) that forms a cap over the free edge of Ammon's horn (C or V shape in rodents). The granule cells are principal neurons in the dentate gyrus which are small (about 10 μm in diameter) and densely packed in cell layer (Fig. 6.1). Their dendrites extend perpendicularly to the cell layer and into the molecular layer where they receive synaptic inputs mainly from perforant afferents (Figs. 6.1 and 6.2). The axons of granule cells are called mossy fibers and extend into the polymorphic layer in the hilus that is a transition area between the dentate gyrus and hippocampus proper. The mossy fibers synapse onto mossy cells and basket interneurons in the hilar area, the former give rise to excitatory recurrent projections to the granule cells and the later may have more than five types mostly with inhibitory feedback to the granule cells (Fig. 6.2).

Interestingly, the dentate gyrus is one of the few brain regions where neurogenesis takes place. Neurogenesis is thought to play a role in the formation of new memories. It has been shown an increased neurogenesis in response to both antidepressants and physical exercise, implying that neurogenesis may improve symptoms of depression (Yamashima et al. 2007).

6.2.1.2 Fiberarchitecture

Afferents to the dentate gyrus—The perforant path: The major afferents to the dentate gyrus are from the entorhinal cortex (but also perirhinal cortex, among others) by way of a

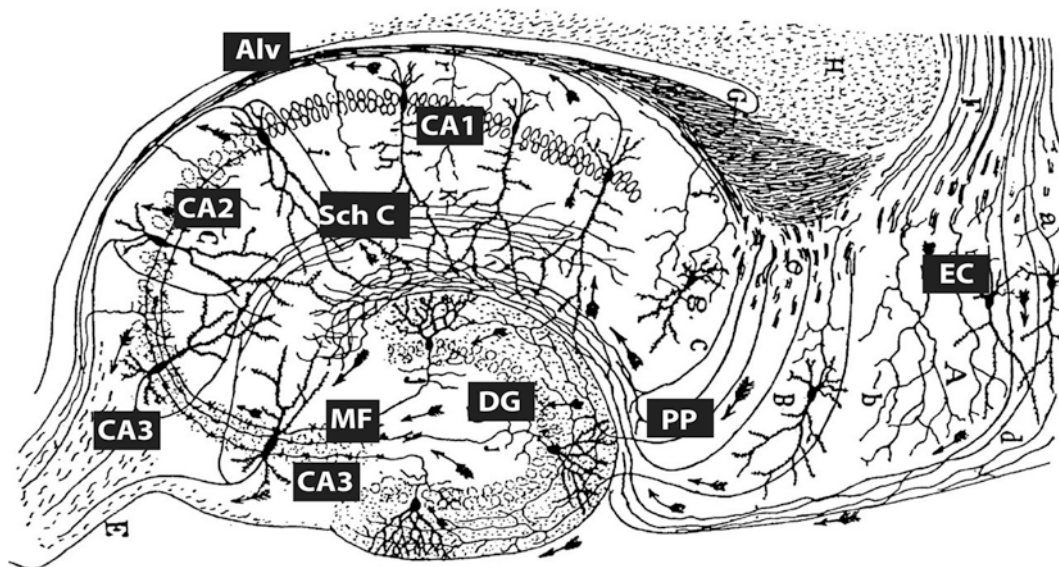


Fig. 6.1 Schematic drawing by Cajal (1911) of the main cells, connections and flow of impulse traffic in the hippocampus

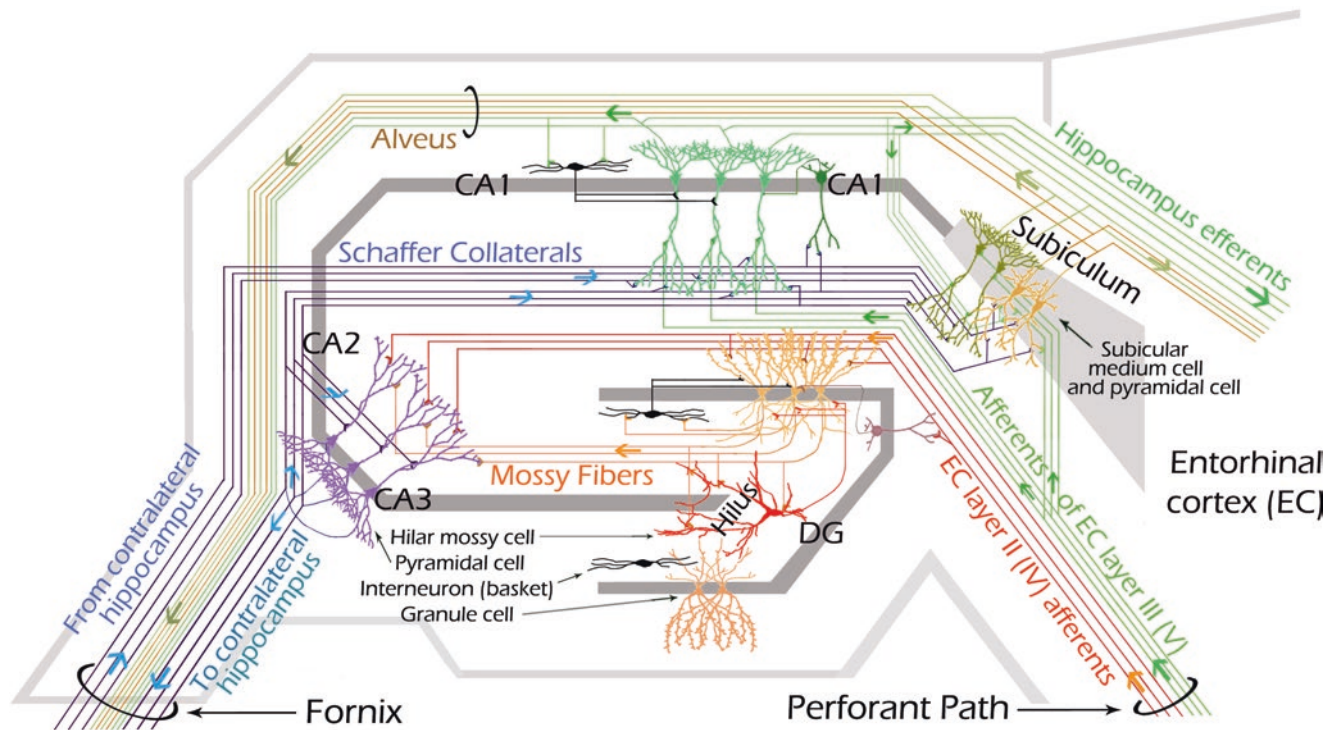


Fig. 6.2 Schematic drawing illustrating cytoarchitecture and fiberarchitecture of the hippocampus formation

fiber system called the perforant path and terminate in outer two thirds of the dentate molecular layer (equivalent to stratum lacunosum and molecular) (Lorente de No 1934). The dentate neurons also relay the massive projections from entorhinal cortex to the CA3 pyramidal cells. Recurrent projections onto dentate neurons are conducted through hilar mossy cells and local basket interneurons (Figs. 6.1 and 6.2).

It was through the perforant pathway that LTP was first discovered (Bliss and Gardner-Medwin 1973). Approximately 60–70% of the total dendritic fields of individual granule cells are taken up with the perforant input (Desmond and Levy 1982). Other afferents to the dentate gyrus originate from brain stem raphe nuclei (Moore and Halaris 1975), the locus coeruleus (Room et al. 1981) and hypothalamic supra-mammillary nucleus (Wyss et al. 1979).

Efferents from the dentate gyrus—The mossy fibers: The dentate granule cells project predominantly to the CA3 region and less heavily to the hilus through mossy fibers. The mossy fiber terminals are large (3–6 μm) and make asymmetric synaptic contact on the dendritic shaft and dendritic spines (thorny excrescence) of the CA3 pyramidal cells. A single CA3 pyramidal cell can be innervated by more than one granule cells. This pathway has been studied extensively as a model for the functional roles of kainate receptors in synaptic plasticity. For instance, LTP is *N*-methyl-D-aspartate (NMDA) receptor-independent in this pathway, but it appears to involve pre-synaptic kainate receptors. The mossy fibers are glutamatergic, however they have also been shown to be

immunoreactive for γ -aminobutyric acid (GABA) and opiate peptides (dynorphin and enkephalin) (Commons and Milner 1996; Gutierrez and Heinemann 2006; Walker et al. 2002).

Neural tract tracing electron microscopy studies on mossy fibers has disclosed that fibers terminating onto the hilar mossy cells are branched from long projecting mossy fibers to the CA3 region (Blackstad and Kjaerheim 1961).

6.2.2 The Hippocampus Proper (CA1–CA3 Fields)

6.2.2.1 Cytoarchitecture

Hippocampus proper is a U-shaped fold of cortex composed of CA1–CA3 fields (Fig. 6.1) extending from subiculum to the hilus of the dentate gyrus. Adjacent to the subiculum is a field of tightly packed medium-sized cells, which Lorente de No (1934) named the CA1. Next to CA1 are the fields CA2 and CA3 containing large, less densely packed cells. CA2 pyramidal cells can be distinguished from CA3 pyramidal cells in Golgi preparations by the absence of characteristic thorny spines on the proximal apical dendrites.

Hippocampal pyramidal cells generally have dendrite trees with apical and basal dendrites; the former heavily branches in the molecular layer (equivalent to stratum lacunosum and molecular) and the latter gives off branches between polymorphic and pyramidal cell layers (equivalent to stratum oriens and pyramidal from outer border to center;

Fig. 6.2). The axons generally originate from or near the basal dendrite and travel into the alveus where it may bifurcate. In the CA1 field, one branched axon travels from CA1 to the subicular area and the other toward the fimbria through alveus bundle (Fig. 6.2). In the CA3 region, the pyramidal axons give rise to three branches in polymorphic layer and all of them are termed as Schaffer collaterals.

6.2.2.2 Fiberarchitecture

Afferents to cornu ammonis areas: The CA3 pyramidal cells receive robust projections from the dentate gyrus through mossy fibers, and accept less heavily afferent innervations from layer II (slightly from layer IV) of the entorhinal cortex. The CA3 neurons are also innervated by recurrent projections from themselves through Schaffer collaterals (Figs. 6.1 and 6.2).

The CA2 and CA1 pyramidal cells are predominantly innervated by bilateral CA3 neurons through Schaffer collaterals from both ipsi- and contralateral sides (Fig. 6.2). In the CA1 field, the pyramidal cells also receive projections from the layer III (slightly from layer V) of the entorhinal cortex (Fig. 6.2). The dendrite trees of CA1 neurons in both molecular and polymorphic layers are heavily innervated by CA3 neuronal axons. This Schaffer collateral to CA1 pathway is widely studied for NMDA receptor-dependent LTP and long-term depression (LTD).

Recurrent feedback innervations onto the CA1 pyramidal neurons are carried out through local interneurons and some small pyramidal cells (Fig. 6.2). The CA1 also receives a fairly substantial input from the amygdaloid complex, and a few of noradrenergic and serotonergic projections.

Efferents of hippocampus proper: The well-known efferents of the CA3 pyramidal cells are Schaffer collaterals projecting to both ipsi- and contralateral hippocampus. These efferent collaterals also give rise to recurrent innervations to the CA3 neurons (Fig. 6.2). The efferent fibers from CA1 neurons travel through polymorphic layer (equivalent to stratum oriens) and bifurcate into three bundles of branches, one group join alveus toward fimbria, the other branches project onto the subiculum cells and another group branches merge into the efferent bundle toward entorhinal cortex (Fig. 6.2). In addition, CA1 neuronal axons in the alveus again give rise to collaterals to local interneurons that feedback project to the CA1 pyramidal cells (Fig. 6.2).

The septal nucleus is the major extrahippocampal target of the pyramidal projections from all CA fields. Axons from both CA1 and CA3 terminate in the septal nucleus. The fibers from dorsal hippocampal project to dorsomedial areas of the lateral septal nucleus, while progressively more ventral parts of the hippocampus terminate in correspondingly more lateroventral bands in the lateral septal nucleus (Meibach and Siegel 1977a). Some branches of the CA1 axons terminate sparsely in olfactory bulb (de Olmos et al. 1978) and prefrontal cortex (Swanson 1981).

6.2.3 The Subicular complex

The subiculum curves anteriorly and laterally to wrap around the posterior extension of the dentate gyrus. It borders the medial entorhinal cortex and field of CA1 (Figs. 6.1 and 6.2). The subiculum can be divided into three distinct cytoarchitectural areas. The parasubiculum borders the medial entorhinal cortex and contains moderately packed medium-sized cells. The presubiculum is between parasubiculum and subiculum, which is characterized by densely packed small cells. The subiculum proper borders to the field of CA1 and has a loosely packed pyramidal cell layer and a wide molecular layer (Figs. 6.1 and 6.2).

Hippocampal intrinsic afferents to the subiculum originate from the CA1 neurons and from bilateral CA3 pyramidal cells through the Schaffer collaterals (Swanson et al. 1978). The extra-hippocampal projections to the subiculum mostly originate from layer II (slightly from layer IV) of the entorhinal cortex, and less often from the raphe nuclei (Conrad et al. 1974), locus coeruleus (Jones and Moore 1977; Haring and Davis 1985), amygdaloid nuclei (Krettek and Price 1977), septal nucleus (Meibach and Siegel 1977b) and perirhinal cortex (Kosel et al. 1983).

The targeted regions for subicular projections include, but are not limited to, thalamic nuclei (Aggleton et al. 2005), reunions nucleus (Herkenham 1978), mammillary body of the hypothalamus (Aggleton et al. 2005) and amygdaloid nucleus (Kishi et al. 2006). The parasubiculum and presubiculum project heavily to the anterior thalamic nuclei (Sikes et al. 1977; Cohen and Eichenbaum 1993).

6.3 Role of the Hippocampus in Learning and Memory

6.3.1 Memory Functions of the Hippocampus

The role of the hippocampus in learning and memory has been the focus of neuroscience studies since Scoville and Milner reported the case of Henry Molaison (HM) who had severe anterograde amnesia following bilateral medial temporal lobe resection (Scoville and Milner 1957). The pattern of impaired and spared memory functions displayed by HM prompted a now commonly acknowledged characterization of the temporal lobe amnesic syndrome. It is widely believed that damage to the hippocampus disrupts declarative memory processes and more specifically episodic memory functions (Tulving 2001; Tulving and Markowitsch 1998). Declarative memories have been further divided into episodic and semantic memories. Episodic memory is concerned with conscious recall of specific episodes, and semantic memory with the storage of factual information. The memory functions that are spared in temporal lobe

lesions have been classified as nondeclarative or procedural memories (Squire 1992; Cohen and Eichenbaum 1993). Nondeclarative or procedural memory processes are thought to operate automatically and do not include information about where or when learning experience took place. The pattern of memory deficits in humans with temporal lobe damage prompted the question, what role did the hippocampus play in memory?

The precise role of the hippocampus in learning memory remains to be determined. Accumulating evidence suggest that hippocampus has an essential role in the formation of new memories about experienced events. Damage to the hippocampus usually results in profound difficulties in forming new memories (anterograde amnesia), and normally also affects access to memories prior to the damage (retrograde amnesia). Although the retrograde effect normally extends some years prior to the brain damage, in some cases older memories remain. This sparing of older memories leads to the idea that consolidation over time involves the transfer of memories out of the hippocampus to other parts of the brain. However, it is difficult to test the sparing of older memories experimentally. Also, in some cases of retrograde amnesia, the sparing appears to affect memories formed decades before the damage to the hippocampus occurred, so its role in maintaining these older memories remains controversial.

Animal studies indicate that the hippocampus plays a role in storing and processing spatial information. In rodents, the firing rate of hippocampal neurons was found to correlate to the location of the animal in a test environment and these cells are referred to as place cells (O'Keefe and Conway 1978; O'Keefe and Dostrovsky 1971). There are many thousands of different place cells, which can be activated in response to a location in a particular environment. Different place cells have different place fields, which are not fixed in absolute space and are relative to spatial cues. The discovery of place cells led to the idea that the hippocampus might act as a cognitive map—a neural representation of the layout of the environment (O'Keefe and Nadel 1978). There has been considerable support for the cognitive mapping theory from lesion and unit recording studies. It has been reported that rats with hippocampal lesions exhibit impairment in learning as detected in radial arm (Jarrard 1983), T-maze (Bannerman et al. 2001) and Morris water maze, where lesioned rats had poor performance in finding the hidden platform (Morris et al. 1986; Morris et al. 1982). Indeed, neuroimaging studies in humans revealed that the hippocampus becomes active during spatial navigation, suggesting that the hippocampus in humans contributes to the encoding and retrieval of spatial information. This finding corresponds well with the results in animal studies, which show a significant deficit in spatial navigation resulting from hippocampal damage.

6.3.2 Synaptic Mechanisms of Memory

The nature of the physiological basis of learning and memory remains an enigma in neurobiology. The assumption that information is stored in the brain as changes in synaptic efficacy was initially proposed by Ramon y Cajal and later refined by Hebb (1949). This assumption was not tested until the early 1970s when Timothy Bliss and Terje LØmo made an important discovery that brief high frequency electrical stimulation of an excitatory pathway to the hippocampus produced a long-lasting enhancement in the strength of the stimulated synapses. This effect is now known as LTP.

LTP is expressed as a persistent increase in the size of the evoked synaptic response recorded from single cells or group of cells. It can be induced by high frequency stimulation (HFS, typically 100 Hz) or other types of stimulation, such theta-burst stimulation. These stimulus paradigms resemble the synchronized firing patterns at similar frequencies that occur in the hippocampus during learning (Otto et al. 1991), making them useful experimental means to generate “learning activities” in the hippocampus. Over the past 30 years, LTP has been intensively studied because it is the leading experimental model for the synaptic changes that may underlie learning and memory.

6.3.2.1 Basic Properties of LTP

Although LTP was first demonstrated at the perforant path synapses on the granule cells in the dentate gyrus (Bliss and Gardner-Medwin 1973), the majority of experiments on understanding the mechanisms of LTP have been performed on the Schaffer collateral/commissural synapses on the CA1 pyramidal cells in the hippocampus.

LTP in the hippocampus has three basic properties: cooperativity, associativity and input-specificity (Bliss and Collingridge 1993). Cooperativity refers to the fact that long-lasting synaptic enhancement following HFS increases with the number of stimulated afferents (McNaughton et al. 1978). Threshold stimulus intensity during HFS is required for synaptic enhancement. ‘Weak’ HFS, which activates relatively few fibers, did not produce LTP, whereas strong stimulation at the same frequency and for the same duration produced LTP. Associativity means that a weak input (small number of stimulated afferents) can be potentiated if it is active at the same time as a strong tetanus (large number of stimulated afferents) to a separate but convergent input (Bliss and Collingridge 1993). This associativity has often been viewed as a cellular analog of associative or classic conditioning (Malenka and Nicoll 1999). Another basic property of LTP is its input-specificity. When LTP is induced by repetitive stimulation the increase in synaptic strength usually does not occur in other synapses (on the same cell) that are not active at the time of repetitive stimulation (Bliss and Collingridge 1993). This property increases the storage capacity of individual neurons (Malenka and Nicoll 1999).

One remarkable feature of LTP is that it can be induced by a brief HFS, lasting less than or equal to a second and consisting of stimulation frequency well within the range of normal axon discharging. Longevity is an additional feature of LTP. Once induced, LTP can persist for many hours in brain slices *in vitro* or in the anaesthetized animal, and for days or weeks (possible even a lifetime) in the freely moving animal.

6.3.2.2 Mechanisms of Hippocampal LTP

It is well accepted that activation of postsynaptic NMDA receptors, a subtype of glutamate receptors, is required for the induction of LTP in the hippocampus. The key role that the NMDA receptors play in LTP induction relies on the voltage-dependent block of its channel by Mg^{2+} (Ascher and Nowak 1988). In this way the NMDA receptor channel complex behaves as a molecular detector for LTP induction. To trigger the induction of LTP, two events must occur simultaneously: the cell membrane must be sufficiently depolarized to expel Mg^{2+} from NMDA channels at the same time L-glutamate binds to NMDA receptors and promotes the opening of these receptor-ligand-gated ion channels. The membrane depolarization can be achieved by repetitive tetanic stimulation of synapses or by directly depolarizing the cell while continuing low frequency stimulation of synapses (Gustafsson et al. 1987). At Schaffer collateral—CA1 pyramidal cell synapses, Na^+ ions passing through the α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors are responsible for this membrane depolarization. When the membrane depolarization is sufficient and reaches a certain level, it expels Mg^{2+} from the NMDA receptor channel, allowing Ca^{2+} as well as Na^+ to enter the cell. The influx of Ca^{2+} through NMDA receptor channel raises intracellular Ca^{2+} and triggers the induction of LTP.

Considerable evidence now links this rise in postsynaptic Ca^{2+} concentration to the induction of LTP. The most compelling evidence in support of this model comes from experimental results wherein LTP induction can be blocked by pharmacological inhibition of NMDA receptors (Collingridge et al. 1983), or prevented by the injection of a Ca^{2+} chelator into the postsynaptic neuron (Yang et al. 1999; Lynch et al. 1983). Ca^{2+} imaging studies have demonstrated that tetanic stimulation directly increases Ca^{2+} within dendrites and spines as a result of NMDA receptor activation (Regehr and Tank 1990; Perkel et al. 1993; Yuste and Denk 1995). Although NMDA receptors are the primary source of Ca^{2+} entry into the dendrites and spines, activation of dendritic voltage-gated Ca^{2+} channels and Ca^{2+} release from intracellular stores also elevate Ca^{2+} levels and contribute to the induction of LTP. However, the mechanisms underlying the Ca^{2+} channel-dependent LTP may differ from NMDA receptor-dependent LTP (Malenka and Nicoll 1999).

6.3.2.3 Expression of LTP

It has long been a challenge for neurobiologists to identify whether the increase in synaptic strength is mediated primarily through a pre- or postsynaptic mechanism. Available experimental results indicate that the increase in the synaptic strength could be either presynaptic or postsynaptic or both, or through an extrasynaptic mechanism, such as reduction in uptake of glutamate by glial cells resulting in an elevated concentration of glutamate at synaptic cleft. Evidence for pre-synaptic mechanisms comes from experiments measuring the overflow of radiolabeled or endogenous L-glutamate in hippocampus before and after the induction of LTP (Bliss et al. 1986). In addition, quantal analysis of synaptic transmission reveals the proportion of synaptic failures decreases after the induction of LTP (Kullmann and Siegelbaum 1995; Malenka and Nicoll 1999). As synaptic failures represent the failure of neurotransmitter release or silent synapses, it was concluded that the induction of LTP is the consequence of an increase in the probability of neurotransmitter release. Evidence supporting this conclusion came from the finding that the variation around the mean of excitatory postsynaptic currents (EPSCs) decreased during LTP (Kullmann and Siegelbaum 1995; Malenka and Nicoll 1999). If the probability of neurotransmitter release increases during LTP, then the quantal content will, on average, increase and the coefficient of variation will decrease, because the coefficient of variation (SD/mean) is inversely proportional to the quantal content (Malenka and Nicoll 1999).

Experimental results from other studies have shown a post-synaptic mechanism for LTP induction. First, paired-pulse facilitation (PPF) is not altered after the induction of LTP and this has been interpreted as evidence for a post-synaptic facilitation of LTP. PPF occurs when two pre-synaptic stimuli are delivered with a short interval (50–200 ms) and is thought to result from residual Ca^{2+} in the pre-synaptic terminal following the first stimulus, enhancing release during the second stimulus (Malenka and Nicoll 1999). Various manipulations known to increase transmitter release do cause a decrease in the facilitation ratio, because release is already enhanced to near saturation during the first stimulus (Manabe et al. 1993). The failure in the alteration of PPF ratio suggests a post-synaptic locus of LTP expression.

A number of studies have shown that during LTP the AMPA receptor component of the EPSC is selectively enhanced, with little or no change in the component of NMDA receptors (Larkman and Jack 1995), though both receptors are frequently co-localized at individual synapses. Pharmacological modulation of transmitter release affects AMPA and NMDA components equally, arguing for a selective postsynaptic alteration in either the density or properties of AMPA receptors (Kullmann and Siegelbaum 1995).

6.4 Neuroimmunomodulation via Hippocampus

One of the important recent advances in understanding the biological basis of neurodegenerative disorders is the recognition that there is extensive communication between the central nervous system (CNS) and the immune system. Initial evidence that the immune system may communicate with the CNS was provided by Besedovsky and colleagues (1977), who observed that activation of the immune system was accompanied by changes in hypothalamic, autonomic and endocrine processes. The existence of neural-immune interactions is now supported by abundant evidence showing that the immune system communicates with the CNS through immunotransmitters (primarily cytokines) leading to direct CNS activation (Berkenbosch et al. 1987; Sapolsky et al. 1987) or by release of CNS-derived cytokines, and that the CNS regulates the immune system via neurotransmitters, hormones and neuropeptides. It has been shown that cells of immune system can synthesize and release several immunomodulatory hormones, neuropeptides and catecholamines (Blalock 1989, 1994). For example, lymphocytes and macrophages produce the endogenous opioid peptides, norepinephrine, and epinephrine (Lolait et al. 1984; Harbour et al. 1987; Engler et al. 2005). A recent study (Rivest 2003) shows that the CNS responds to systemic bacterial infection with innate immune reaction without pathogen's direct access to the brain. Whether caused by a microbe, trauma, toxic metabolite, autoimmunity, or part of a wide degenerative process, activation of the immune system results in changes in the activity of discrete populations of brain neurons, including hippocampal neurons. Accumulating evidence indicates that these mechanisms are relevant for the course of infectious, inflammatory, autoimmune and neoplastic diseases.

As resident innate immune cells in the brain, microglia behaves like a dual-edged sword. On one hand, they release neurotrophic factors to promote neuronal development and growth and, on the other hand, they produce neurotoxic substances resulting in neuronal dysfunction and injury (Block et al. 2007; Perry et al. 2010). Microglia also functions as a CNS sensor and detects neural injury, inflammation and/or infection (Perry et al. 2010; Block et al. 2007; Perry and Holmes 2014). Elevated levels of circulating pro-inflammatory cytokines and over-activation of microglia can result in the CNS neuronal injury. It has been shown that microglia density in the hippocampus is relatively higher than telencephalon cortex, diencephalon, brainstem and cerebellum (Lawson et al. 1990), suggesting a potential occurrence of neuroimmunomodulatory activities in the hippocampus.

6.4.1 Immunomodulation of Neuronal Functions in Hippocampus

Hippocampal neuronal activities have been examined thoroughly in studies on neuroendocrine, autonomic and cognitive function, as well as psychomotor behavior. Circumstantial evidence indicates that hippocampal physiology can be modulated by the immune system (Jankowsky and Patterson 1999; Jankowsky et al. 2000). This modulation could be achieved through proinflammatory cytokines including, but not limited to, interleukin-1 beta (IL-1 β), IL-2, IL-6, tumor necrosis factor alpha (TNF- α) and interferon gamma (INF- γ) (Wrona 2006). Indeed, a number of cytokines, including the aforementioned ones, are expressed in the hippocampus and alteration of their expression levels can affect hippocampal functions. Studies have shown that inflammatory cytokines, released in response to the detection of foreign substances (antigens), influence ion channel activities, intracellular Ca²⁺ homeostasis, membrane potentials, and suppress or enhance the induction of LTP in the hippocampus (Koller et al. 1997). Extensive experimental results have implicated IL-1 among the other cytokines as a likely candidate for a key immunotransmitter, communicating immunological activation to the brain including the hippocampus (Besedovsky et al. 1975; Besedovsky et al. 1986). It is worth to point out that cytokines rarely work in isolation. For instance, the release of IL-1 β is usually associated with the release of the other pro-inflammatory cytokines, such as TNF- α and IL-6, which are indeed expressed in the hippocampus.

IL-1 β and its receptors are expressed in the hippocampus (Farrar et al. 1987; Ban et al. 1991; Cunningham and De Souza 1993). High density of binding sites for IL-1 β has been detected in the hippocampus, with highest density in the dentate gyrus (Takao et al. 1990). It has been shown that peripheral immune activation by lipopolysaccharide (LPS) up-regulates IL-1 β mRNA expression and increases IL-1 β protein in the hippocampus (Laye et al. 1994; Nguyen et al. 1998). This suggests that immune activation may modulate hippocampal function via releasing immune active molecules, such as IL-1 β . As hippocampus is a brain region involved in learning and memory, the immune associated upregulation of IL-1 β mRNA and protein expression may interrupt hippocampal functions such as learning and memory. Indeed, IL-1 β suppresses the induction of LTP in the CA1 and CA3 areas of the hippocampus as well as in the dentate gyrus (Bellinger et al. 1993; Katsuki et al. 1990; O'Connor and Coogan 1999; Cunningham et al. 1996; Xiong et al. 2000), while having no significant effects on excitatory postsynaptic potential (EPSP) evoked by low frequency stimulation. The IL-1 β -mediated suppression of LTP is antagonized by an IL-1 β receptor antagonist, suggesting that

IL-1 β inhibits LTP through IL-1 β receptors. In addition to IL-1, IL-2 had similar effects on LTP in hippocampus. Application of recombinant IL-2 inhibited the induction of both short-term potentiation (STP) and LTP. It also inhibited post-tetanic potentiation (PTP) and LTP maintenance without affecting basal synaptic transmission (Tancredi et al. 1990). Moreover, IL-2 deficiency results in altered hippocampal cytoarchitecture (Beck et al. 2005). LTP was also suppressed by TNF- α in both the CA1 region (Tancredi et al. 1992) and the dentate gyrus (Cunningham et al. 1996). In the CA1, the induction of LTP was inhibited by TNF- α if the tetanic stimulation was given at least 50 min after TNF- α application. In contrast to IL-1 and IL-2, TNF- α increased basal synaptic transmission in the CA1 region of the slices acutely exposed to TNF- α (Cunningham et al. 1996) but not in the dentate gyrus. The underlying mechanisms, by which TNF- α increased basal synaptic transmission, have not been determined. Brief treatment of hippocampal slices with TNF- α did not influence LTP, while long-lasting application (>50 min) of TNF- α inhibited LTP.

Cytokines and chemokines are the messengers and regulators between neurons and glial cells communications (Harrison et al. 1998; Ransohoff 2009). It has been shown that fractalkine (CX3CL1) is the cytokine expressed predominantly by neurons and its receptor—CX3CR1 is expressed in microglia (Ransohoff and Stevens 2011). This CX3CL1 and CX3CR1 axis becomes a typical example of neural-immune communications and plays a pivotal role in neuronal modulation of microglia activity (Bachstetter et al. 2011). Studies by immunofluorescent staining have shown that CX3CL1 is highly expressed in pyramidal neurons in CA1~CA3 regions of hippocampus (Bachstetter et al. 2011; Sheridan et al. 2014), a brain structure associated with learning and memory. It has been demonstrated that high frequency stimulation induces robust LTP in the hippocampus and LTP has been considered as a cellular/synaptic mechanism for learning and memory (Tulving and Markowitsch 1998; Tulving 2001; Otto et al. 1991). Thus, alteration of CX3CL1 and CX3CR1 axis may impact the induction of LTP and LTP-associated learning and memory (Maggi et al. 2009; Rogers et al. 2011; Sheridan et al. 2014).

6.4.2 Lesion of Hippocampus Affects Neuroimmunomodulation

It has been shown that during immune challenge the hippocampus exhibits time-dependent changes in neurotransmitter levels and that an intact hippocampus is essential for the normal humoral immunity for the primary immune response in rats (Devi et al. 2004). Lesions of the dorsal hippocampus were found to produce a transient increase in splenocytes and thymocytes, as well as increased T-cell mitogen responses

(Brooks et al. 1982). Lesions of the hippocampus were also found to cause differential effects on humoral immunity depending on the lesions of different subfields of the hippocampus (Pan and Long 1993). Axotomy of afferent fibers within the molecular layer of the dentate gyrus caused activation of neural-immune elements in the slice cultures (Coltman and Ide 1996). In addition, electrical stimulation of hippocampus increased the number of neutrophils and phagocytic index while also decreasing the number of lymphocytes and plasma corticosterone level in rats (Devi et al. 1993). Lesions in hippocampus induced by kainic acid resulted in elevated antibody production including IgM and IgG (Nance et al. 1987). Taken together, these studies have clearly shown that lesions (or stimulation) in hippocampus affect immune functions.

6.5 Review Questions

1. Where is the hippocampus located in the human brain?
2. What are the three major subfields in the hippocampus?
3. What are the principal cells in the dentate gyrus and the CA1 field?
4. The main input to the dentate gyrus originates from which part of brain and via which fiber path?
5. Mossy fibers originate from which part of the hippocampus and synapse predominantly onto the neurons of which field(s)?
6. Describe sources of afferent fibers to the CA1 region.
7. Briefly describe synaptic information flow through the hippocampus
8. Damage to the hippocampus disrupts what type(s) of memory processes?
9. Describe how long-term potentiation (LTP) is expressed and why LTP is considered as synaptic base for learning and memory?
10. Lesion of hippocampus affects neuroimmunomodulation?

6.6 Answers

1. It is located in the medial temporal lobe of the brain.
2. The three major subfields in the hippocampus are dentate gyrus, CA3 and CA1.
3. The principal cells in the dentate gyrus and CA1 are granule cells and pyramidal cells, respectively.
4. The main input to the dentate gyrus originates from entorhinal cortex via perforant pathway.
5. The Mossy fibers stem from granule cells in the dentate gyrus and their axonal fibers (the mossy fiber pathway) project predominantly to the CA3 neurons.
6. The major afferent fibers to the CA1 pyramidal cells come predominantly from bilateral CA3 neurons through Schaffer collaterals from both ipsi- and contralateral sides.

The CA1 pyramidal cells also receive projections from the layer III (slightly from layer V) of the entorhinal cortex.

7. Information enters the hippocampus mainly through the perforant pathway consisting of the axons of neurons in layers II and III of the entorhinal cortex. The perforant path axons terminate on the dendrites of the dentate gyrus granular cells. Then information flows through the hippocampus from the dentate gyrus to the CA3, the CA1, forming an intrinsic tri-synaptic circuit in the hippocampus.
8. Damage to the hippocampus disrupts declarative memory processes and more specifically episodic memory functions.
9. LTP is expressed as a persistent increase in the size of the evoked synaptic response recorded from single cells or group of cells. It can be induced by high frequency stimulation (HFS, typically 100 Hz) or other types of stimulation, such theta-burst stimulation. These stimulus paradigms resemble the synchronized firing patterns at similar frequencies that occur in the hippocampus during learning, making them useful experimental means to generate "learning activities" in the hippocampus.
10. Increasing evidence indicate that hippocampus is involved in neuroimmunomodulation. Lesions of the hippocampus cause differential effects on neuroimmunomodulation depending on the site of lesion. Lesions of the dorsal hippocampus produce a transient increase in splenocytes and thymocytes, as well as increased T-cell mitogen responses. Lesions in hippocampus induced by kainic acid result in elevated antibody production including IgM and IgG. In addition, electrical stimulation of hippocampus increases the number of neutrophils and phagocytic index while also decreasing the number of lymphocytes and plasma corticosterone level in rats. The aforementioned clearly show that lesions (or stimulation) in hippocampus affect immune functions.

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Abstract

The complex organization of the brain into neural networks allows for rapid adaption and plasticity necessary for learning and memory. Recent evidence supports the notion that in neurodegenerative disorders behavioral alterations are associated with network dysfunction. The objective of this chapter is to provide an overview of the structure and organization of the nervous system into networks relevant to the understanding of the pathogenesis of Alzheimer's disease, Parkinson's disease, HIV neurocognitive impairment and other neurodegenerative and neuroinflammatory disorders. The chapter also provides a summary of the nervous system unique circulatory organization and interactions between neural and vascular activity that accounts for the mechanisms regulating the trafficking of immune cells from peripheral tissues. This chapter provides the structural framework for the better understanding of these interactions under physiological conditions and in neurological diseases.

Keywords

Brainstem • Cerebrum • Cerebellum • Connectivity • Microcirculation • Neuroanatomy • Synapses • Trafficking

7.1 Introduction

The understanding of the gross and fine structure of the brain is of fundamental importance to elucidate how the nervous system works, how it interacts with other peripheral organs and tissues and how it responds to external stimuli. The functions and activity of the nervous system are not only regulated by its intrinsic wiring but also by interactions with cellular components of peripheral tissues. Of significant importance are the interactions between the nervous system with the immune and endocrine systems. While for the former, interactions involve trafficking of immune and hematopoietic cells into the brain; the latter interactions are primarily of a chemical nature, mediated by hormones and growth factors that reach the nervous system via the circulation.

In this context, the main objective of this chapter will be to provide an overview to the structure and organization of the nervous system that is relevant to the understanding of the unique interactions between the nervous and the immune system and to elucidate the pathogenesis of Alzheimer's disease (AD), Parkinson's disease (PD), HIV encephalitis (HIVE), and other neurodegenerative and neuroinflammatory disorders (Ringheim and Conant 2004; Gendelman 2002; Ho et al. 2005).

The central nervous system (CNS) has been traditionally considered an immunologically privileged site; however, it should be viewed as an immunological specialized region. In fact, cells from the immune system such as lymphocytes and macrophages constantly circulate through the nervous system under physiological conditions. Interactions between these trafficking immune cells and neuronal and glial components of the nervous system are critical in maintaining the stability and functional activity of selected neuronal circuitries involved in memory formation, sleep, and regulation of hormonal production (Avital et al. 2003; Opp and Toh 2003). For example, a recent study showed that trafficking of T-cells into

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the adult hippocampus contributes to maintenance of neurogenesis and learning (Ziv et al. 2006). Remarkably, recent evidence shows that not only does the immune system regulate neuronal function but also conversely neuronal activity regulates the immune response. For example, recent studies have shown that cholinergic neurotransmission is capable of inhibiting pro-inflammatory cytokine release and protects against systemic inflammation (Pavlov and Tracey 2005).

Therefore, immunological reactions in the nervous system are not exclusively related to pathological conditions such as viral infections, autoimmune diseases, and inflammatory disorders (Owens et al. 2005; Eskandari et al. 2003) but also may be involved in regulation of neural homeostasis under physiological conditions and stress (Buller 2003). In fact immune reactions that occur in the nervous system take a unique character which is probably determined by the very specialized local anatomy. Important anatomical characteristics of the nervous system that determine the unique nature of the neuro-immune reactions include the relative lack of lymphatic drainage, the lack of endogenous antigen presenting cells, and the selective permeability of the blood brain barrier (BBB).

The distribution and patterns of migration of neuroimmune cells are regulated by genetically encoded programs, patterns of connectivity in anatomical regions and blood flow. Moreover, and as it will be described later, there are endogenously derived neuroimmune cells such as the astrocytes and microglia as well as cells derived from the peripheral circulation such as lymphocytes and macrophages. There are three routes for leukocyte entry into the nervous system: circulation through the subarachnoid space which is mediated by P-selectin, migration across the choroid plexus which is mediated by PECAM, and extravasation from post-capillary venules mediated by P-selectin, lymphocyte adhesion molecule (LFA-1) and intercellular adhesion molecule (ICAM) (Engelhardt and Ransohoff 2005).

In summary, the nervous system has a unique circulatory organization based on the flow of the cerebrospinal fluid (CSF), the microvasculature, the BBB and the regulation of the blood flow by the neural activity that provides a series of mechanisms to selectively regulate interactions between the nervous system and trafficking cells from peripheral tissues. This chapter provides the structural framework for the better understanding of these interactions under physiological conditions and in neurological diseases.

7.2 Gross Anatomical Structure of the Brain

7.2.1 General Organization of the Central Nervous System

7.2.1.1 Introduction

The nervous system is divided into the CNS and the peripheral nervous system (PNS). The CNS is composed of the

brain and spinal cord and a triple membranous covering denominated meninges. The outer membrane is the dura, the intermediate arachnoid, and the innermost pial membrane. The complexity of the CNS, with its millions of neurons and connections can be summarized to six major divisions: (1) cerebral hemispheres, (2) diencephalon, (3) midbrain, (4) pons and cerebellum, (5) medulla and (6) spinal cord (Martin 1989) (Fig. 7.1). The PNS consists of nerves connected to the brain and spinal cord (cranial and spinal nerves) and their branches within the body (Crossman and Neary 2005). Spinal nerves serving the upper or lower limbs join to form the brachial or lumbar plexus, respectively, within which fibers are distributed into named peripheral nerves. The PNS also includes some groups of peripherally located nerve cell bodies that are located within ganglia (e.g. dorsal root ganglia). Neurons that detect changes in, and control the activity of, the internal organs are denominated autonomic nervous system (ANS). Its components are present in both the central and peripheral nervous systems. The ANS is divided into two anatomically and functionally distinct components called the sympathetic and parasympathetic divisions, which generally have antagonistic effects on the structure that they innervate. The ANS innervates smooth muscle, myocardium and secretory glands and it is an important player of the homeostatic mechanisms that regulate the internal environment of the body.

7.2.1.2 Cerebral Hemispheres

The cerebral hemispheres are divided into two parts by the interhemispheric (sagittal) fissure. Interconnecting the two hemispheres is the corpus callosum (Martin 1989) (Fig. 7.1). The cerebral hemispheres have four major parts, which are the cerebral cortex, basal ganglia (or striatum), hippocampal formation, and amygdala (Fig. 7.2). The human cerebral cortex is a highly convoluted structure. The elevated convolutions are known as gyri and the folds that separate the gyri are called sulci. The cerebral cortex includes the frontal, parietal, temporal, and occipital regions (Fig. 7.1). In these regions the cerebral cortex usually has six layers. The frontal and parietal lobes are separated by the central sulcus (Rolandic sulcus) which separates two functional regions of the cortex namely the primary motor cortex which is located in the precentral gyrus and the primary somatosensory cortex which is distributed in the postcentral gyrus (Fig. 7.1). The central sulcus extends from the longitudinal fissure along the midline ventrally almost into the lateral cerebral sulcus (Sylvian sulcus) (Fig. 7.1). The frontal lobe, the largest of the cerebral lobes, extends from the central sulcus to the frontal pole. The function of the precentral gyrus is to integrate motor functions from different brain regions. The neurons are organized in a somatotopic manner, which means that different parts of the precentral gyrus are associated with distinct parts of the body both anatomically and functionally. Immediately anterior to the premotor cortex there are three parallel gyri, the superior

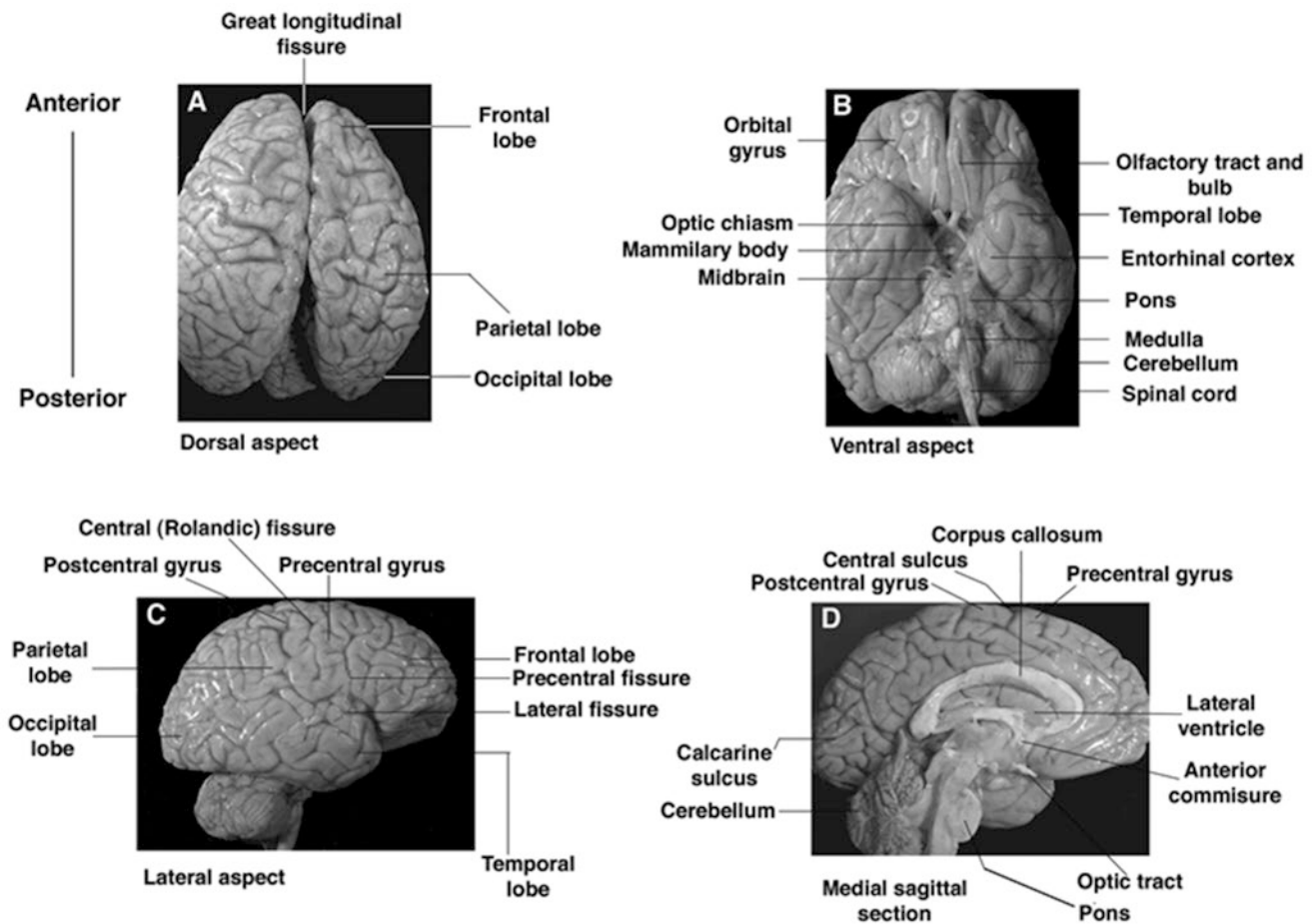


Fig. 7.1 Gross external structures of the brain

middle and inferior frontal gyri. These areas involve processing of executive functions such as abstraction, thinking, cognition, language and emotion. In patients with AD and frontotemporal dementia (FTD), these areas are heavily damaged resulting in the characteristic profile of cognitive impairment typical of these patients. Part of the inferior frontal cortex (left side) includes Broca's motor speech area, an area important in the formulation of motor components of speech. The parietal lobes include the somatosensory cortex within the postcentral gyri (Fig. 7.1). The remainder of the parietal lobe is divided into a superior and inferior lobes separated by the interparietal sulcus. The supramarginal gyrus and the angular gyrus divide the inferior parietal lobe, these regions receive input from the auditory and visual cortex and are involved in integration and discrimination of perception. Ventral to these gyri and extending into the temporal cortex is Wernicke's area; this structure is involved in language comprehension. While injury to Broca's area results in broken language, damage to Wernicke region results in difficulty understanding language.

The temporal cortex is situated inferior to the lateral sulcus and is divided into superior middle and inferior temporal gyri (Fig. 7.2). On the inner aspect of the superior temporal cortex are the gyri of Heschl, which is the primary auditory region. The inferior portion of the temporal lobe is involved in vision function and parts of the medial temporal lobe are involved in olfactory functions. The temporal lobe also includes the hippocampus and parahippocampal cortex. The hippocampus and amygdala are the second and third components of the cerebral hemispheres and are located under the cortical surface (Fig. 7.2). The hippocampus is involved in memory formation while the amygdala modulates the action of the autonomic nervous system, hormone release and emotions. These two structures are part of the limbic system which includes the cingulate cortex as well as part of the diencephalon and midbrain. Together the limbic system plays a central role in the regulation of cognitive functions and mood. The occipital cortex or striate cortex is involved in integration of visual information.

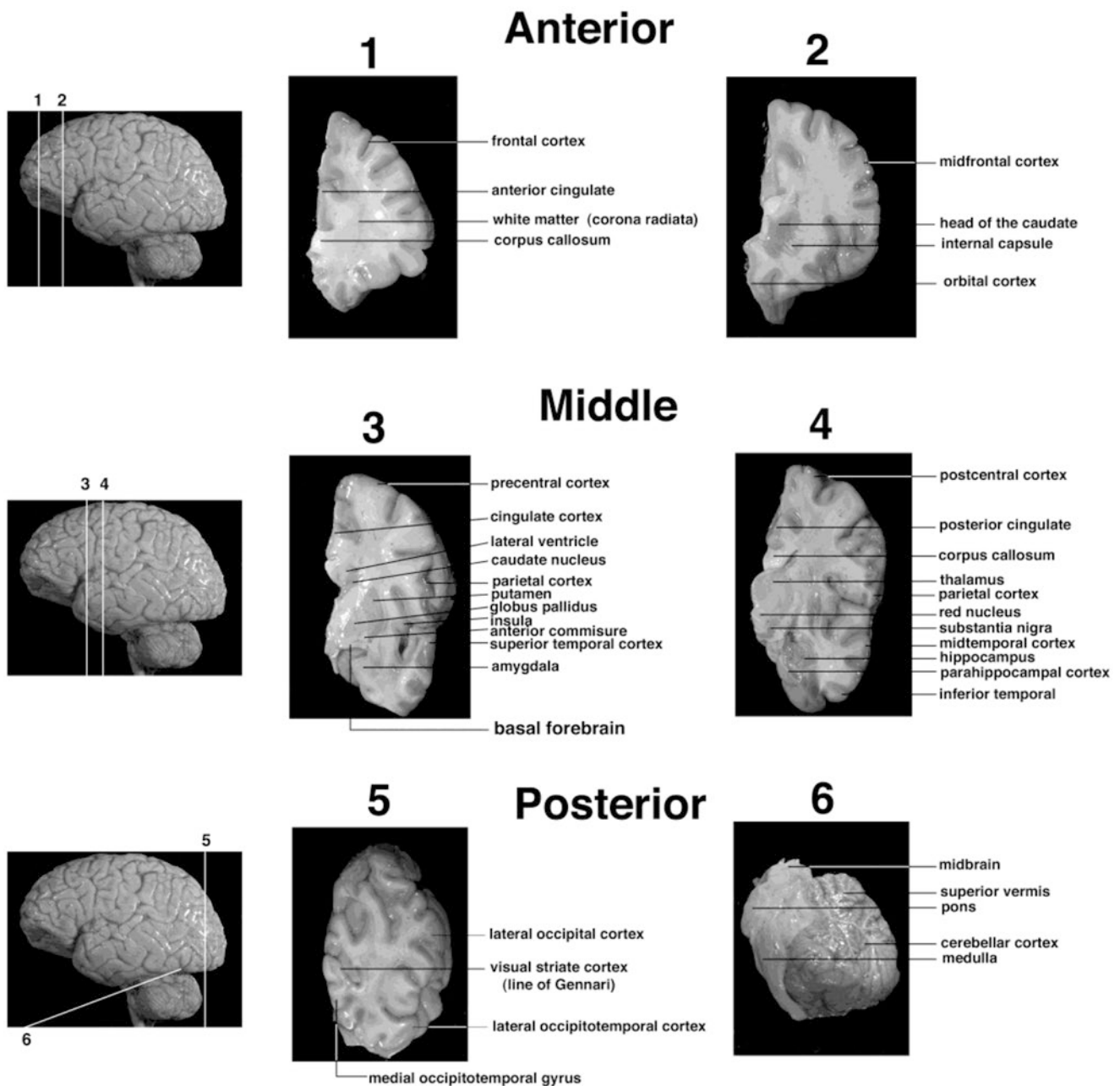


Fig. 7.2 Gross internal structures of the central nervous system

The second major component of the cerebral hemisphere is the basal ganglia, which includes the caudate, putamen and globus pallidus (Fig. 7.2). A white matter tract, denominated anterior commissure, divides the inferior aspect of the putamen and globus pallidus and separates the major cholinergic center in the brain also known as nucleus Basalis of Meynert or substantia innominata (Fig. 7.2). The loss of cholinergic input in patients with AD is related to degeneration of this region, which is dependent on NGF for survival. The caudate and putamen are divided by the anterior capsule. The basal ganglia participate in control of movement but also have a

role in behavior. In patients with Huntington's disease, this brain structure is severely affected.

7.2.1.3 The Diencephalon

Rostral to the brainstem lies the forebrain, consisting of the diencephalon and cerebral hemispheres (Crossman and Neary 2005). The two sides of the diencephalon are separated by the lumen of the third ventricle, whose lateral walls they constitute (Fig. 7.2). The diencephalon consists of four main subdivisions in a dorsoventral direction: the epithalamus, thalamus, subthalamus, and hypothalamus (Fig. 7.2).

The epithalamus is small and its most recognizable component is the pineal gland, which lies in the midline immediately rostral to the superior colliculi of the midbrain. The thalamus is by far the largest component of the diencephalon and it forms much of the lateral wall of the third ventricle. The thalamus plays an important role in sensory, motor and cognitive functions and has extensive connections with layers IV and V of the cerebral cortex. The thalamus is a central structure for relaying information to the cerebral hemispheres (Fig. 7.2). The thalamus includes a large complex of nuclei that includes three principal nuclear masses (anterior, medial, lateral) divided by the internal medullary lamina. Within the internal medullary lamina lies the intralaminar nuclei. On the lateral aspect of the thalamus, the reticular nucleus can be found. The hypothalamus forms the lower part of the walls and floor of the third ventricle (Fig. 7.2). It is a highly complex and important region because of its involvement in many systems, most notably the neuroendocrine system, the limbic system, and the ANS. From the ventral aspect of the hypothalamus in the midline emerges the infundibulum or pituitary stalk, to which is attached the pituitary gland. The hypothalamus integrates the functions of the ANS and endocrine hormone release from the pituitary gland. The subthalamus is located under the thalamus and dorsal lateral to the hypothalamus it contains the subthalamic nucleus and the zona incerta. The subthalamic nucleus is connected to the globus pallidus and substantia nigra and is important in the control of movement. This circuitry is often affected in patients with PD and is amenable to surgical manipulation.

7.2.1.4 The Brainstem

The midbrain, pons, cerebellum, and medulla constitute the brainstem (Fig. 7.2). The brainstem has three general functions. The first is to receive sensory information from cranial structures and to control muscles of the head. This function of the brainstem is similar to that of the spinal cord. The cranial nerves are constituents of the PNS that provide the sensory and motor innervation of the head and therefore are analogous to the spinal nerves. The second is related to the fact that the brainstem contains neural circuits that transmit information from the spinal cord to higher brain regions and back. Finally, the integrated actions of the medulla, pons, and midbrain regulate the levels of awareness and arousal. This function is mediated by a diffuse collection of structures in the brainstem, denominated the reticular formation. In addition to these general functions, the various divisions of the brainstem provide specific sensory and motor functions. The medulla and the pons play a role in the vital regulation of the blood pressure and respiratory functions. The midbrain, also known as the mesencephalon, is divided into dorsal and ventral parts at the level of the aqueduct (Fig. 7.2). The dorsal part is known as the tectum and includes the colliculi. The most ven-

tral part of the midbrain tegmentum contains the substantia nigra, which consists of pars compacta and pars reticularis. The substantia nigra contains melanin pigmented neurons that produce dopamine. Degeneration of the substantia nigra is characteristic of patients with PD and results in disconnection between the midbrain and the caudo-putamen region. Other important structures contained in the midbrain are the oculomotor nucleus, the red nucleus, the periaqueductal grey and the medial lemniscus. Furthermore, the branches of the third cranial nerve emerge from the midbrain.

The pons (Fig. 7.2) is further divided into ventral and dorsal sections. The ventral section contains the pontocerebellar fibers and the pontine nucleus. Corticospinal fibers run longitudinally. Other important structures in the pons include the locus ceruleus, which is main source of adrenergic fibers in the CNS. The neurons in the locus ceruleus are pigmented and produce epinephrine and norepinephrine. This brainstem structure is often affected in patients with AD, PD and depression. The medulla is divided into a caudal, mid and rostral portion. The caudal portion includes the nucleus of the spinal tract of the trigeminal nerve. The mid portion of the medulla includes the nucleus gracilis and cuneatus as well as the decussation of the pyramids. The rostral medullary portion includes the inferior olivary nucleus which connects with the cerebellum. Damage to the pons and medulla is almost always life threatening.

The cerebellum regulates body movements, and may do so by controlling the timing of skeletal muscle contractions. The cerebellum and pons are considered together because they develop from the same portion of the embryonic brain. The cerebellum is attached to the brainstem by a large mass of nerve fibers that lie lateral to the fourth ventricle on either side (Fig. 7.2). The cerebellum is divided into three sections by the inferior, middle, and superior cerebellar peduncles. These contain nerve fibers between the medulla, pons, and midbrain, respectively, and the cerebellum. The largest and most prominent is the middle cerebellar peduncle. The cerebellum consists of an outer layer of grey matter, the cerebellar cortex, surrounding a core of white matter. The cortical surface is highly convoluted to form a regular pattern of narrow, parallel folds or folia. The cerebellar cortex contains three layers the outer or molecular, the Purkinje cells, and the inner granular layer. The cerebellar white matter consists of nerve fibers running to and from the cerebellar cortex. The white matter has a characteristic branching, tree-like arrangement in section, as its ramifications reach towards the surface. The cerebellum is involved with the coordination of movement.

7.2.1.5 The Spinal Cord

The spinal cord has the simplest organization of all six major divisions. It participates in the control of limb and trunk musculature, in visceral functions, and in the processing of

sensory information from these structures. Also, it is a conduit for the flow of information to and from the brain. The spinal cord is the only portion of the central nervous system that has a clear external segmental organization, reminiscent of its embryonic and phylogenetic origins. The spinal cord is divided into the central thoracic lumbar and sacral segments. While in the cerebrum the gray matter is in the cortex and the white in the core, in the spinal cord the gray matter is central and the white is peripheral.

At cross section, the gray matter is the cord from the anterior and posterior columns, which contains the motoneurons and sensory neurons, respectively. An important feature of each spinal cord segment is the presence of a pair of roots (or associated branches or rootlets) called the dorsal and ventral roots. The dorsal roots contain sensory axons whereas the ventral roots contain motor axons. These sensory and motor axons, which are part of the peripheral nervous system, become mixed in the spinal nerves en route to their peripheral targets. The spinal nerves, which are also components of the peripheral nervous system, transmit sensory information to the spinal cord and motor commands to the muscles and viscera.

7.2.2 Internal Organization of the Central Nervous System

7.2.2.1 Projection and Connections in the Brain

The cerebral hemisphere and diencephalon have a more complex organization than that of the brainstem and spinal cord (Martin 1989). The thalamus relays information from subcortical structures to the cerebral cortex via two different functional classes of nuclei; namely, those that are for relay and those that are for diffuse projection. Three of the four anatomical divisions of the thalamus serve relay functions (anterior, medial, and lateral nuclei) and one is a diffuse projection nuclei (intralaminar). Thalamic neurons send the axons to the cerebral cortex via the internal capsule, as do cortical neurons that project to subcortical sites. There are two major somatosensory pathways: the dorsal column of the medial lemniscal system, which mediates tactile, and vibration, and the anterolateral system, which mediates pain and temperature sense.

There are three other major somatosensory cortical areas, which include the primary, secondary, and tertiary somatosensory cortical areas and are somatotopically organized. The secondary somatosensory cortex and the posterior parietal cortex receive their projections from the primary somatosensory cortex and the posterior insular cortex receives input from the secondary somatosensory cortex. Corticocortical projections as well as callasol connections are made by neurons of layers two and three. Descending projections to the striatum, brainstem, and spinal cord originate from neurons in

layer five, while projections to the thalamus originate from neurons in layer six.

Another important source of connectivity is that of the limbic system which includes the cingulate, hippocampus, and amygdala. The hippocampal formation is integrated by an infolding of the inferomedial part of the temporal lobe into the lateral ventricle along the choroid fissure. The dentate gyrus is distributed in between the parahippocampal gyrus and the hippocampus and contains a layer of granular neurons and the molecular layer, which receives connection from the entorhinal cortex and the parahippocampal gyrus. The hippocampal formation receives projections from the inferior temporal cortex via the entorhinal cortex and from contralateral fibers via the fornix. Efferent fibers merge on the ventricular surface of the hippocampus as the fimbria. The limbic system includes intrinsic as well as extrinsic connectivity. Connections between the entorhinal cortex and the molecular layer of the dentate gyrus (perforant pathway), Mossy fibers, CA1–CA4, and subiculum integrate the intrinsic connections. These structures are often affected in AD and are responsible for the short-term memory loss in this condition. The extrinsic limbic connections are between the cingulate gyrus, the hippocampal formation, the amygdala, and the septum, which in turn connect with the hypothalamus. Overall the limbic system network should be considered as functional units that include the prefrontal cortex, cingulate cortex, amygdaloid nucleus, limbic thalamus, nucleus accumbens, anterior hypothalamus, and raphe nucleus (Morgane et al. 2005).

7.2.2.2 Laminar Organization of the Cerebral Cortex

The cerebral cortex is the structure to which the dorsal column-medial lemniscal system projects and the origin of the corticospinal tract. It has a characteristic structure with neurons that are organized into layers. Different cortical regions contain characteristically different numbers of cell layers (Fig. 7.3). Most of the cerebral cortex contains at least six cell layers, and this cortex is termed the isocortex (Fig. 7.3). Because the isocortex dominates the cerebral cortex of phylogenetically higher vertebrates, it is also termed neocortex. In contrast to the isocortex, the allocortex contains fewer than six layers. Although present in higher vertebrates, the allocortex dominates the cortex of phylogenetically more primitive vertebrates. The phylogenetically oldest type of allocortex, the archicortex, constitutes the hippocampal formation and contains three cell layers, which are the molecular, granular, and pyramidal. The paleocortex, thought to be a more advanced allocortex, is associated with areas that mediate olfactory function. The neocortex comprises the major sensory, motor, and association areas. Regions of neocortex that serve different functions have a different microscopic anatomy. Areas that regulate sensation

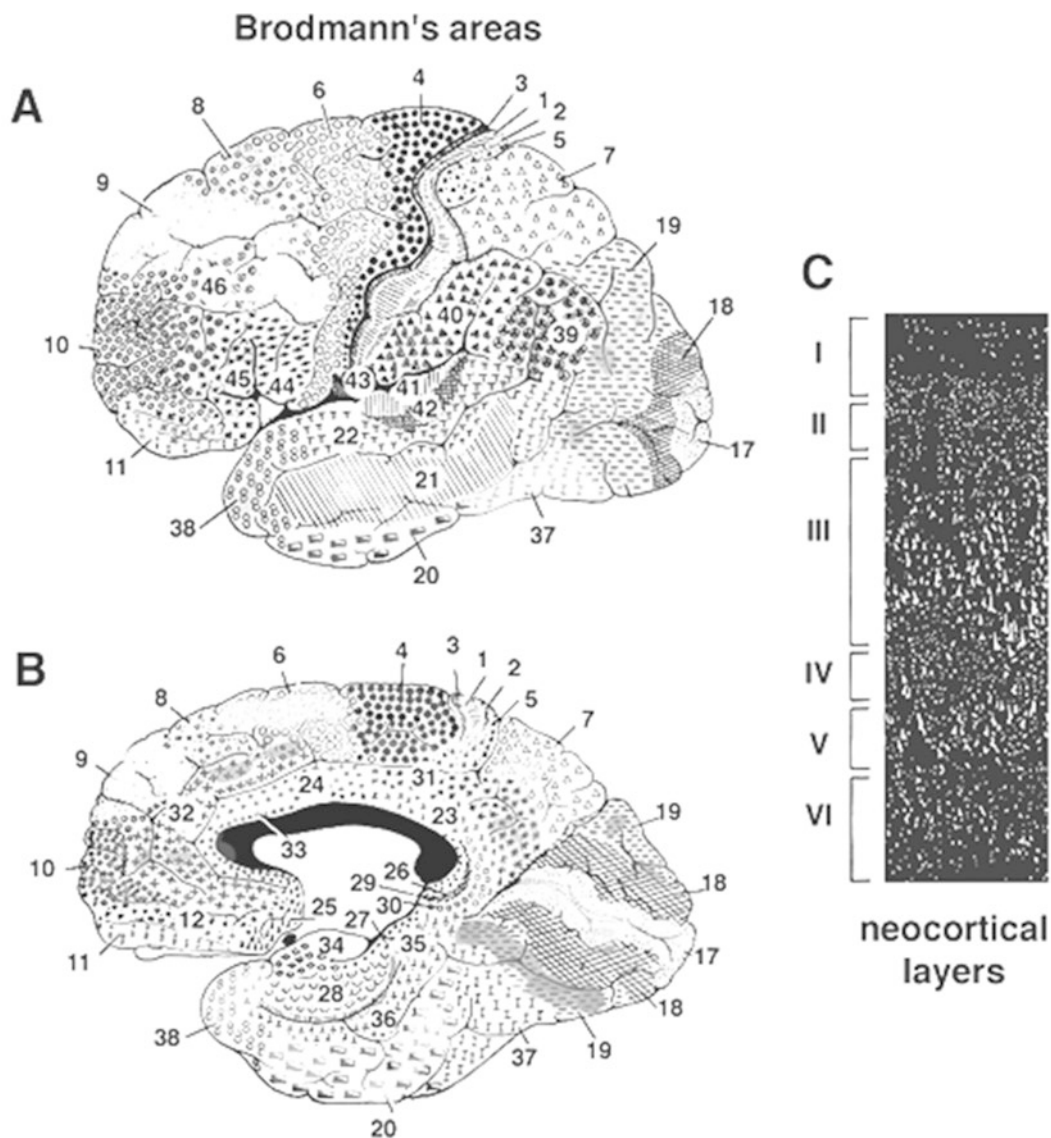


Fig. 7.3 Diagrammatic representation of the neocortical layers and Brodmann areas

have a well-developed layer IV. This is the layer to which most thalamic neurons from the sensory relay nuclei project. The primary visual cortex has this morphology. In contrast, the primary motor cortex has a thin layer IV and a thick layer V. Layer V contains the pyramidal neurons that project to the spinal cord, via the corticospinal tract. Association areas of the cerebral cortex, such as prefrontal and parietal association cortex have a morphology that is intermediate between those of sensory cortex and motor cortex. In summary, based primarily on differences in the thickness of cortical layers and on the sizes and shapes of neurons there are two types of cortex. The neocortex (or isocortex) has six layers; the allocortex has fewer than six layers and includes the archicortex of the hippocampus and the paleocortex of the olfactory regions.

7.2.2.3 Neuronal Subtypes and Patterns of Interconnectivity

The activity of the CNS depends on the complex patterns of connectivity among neurons and associated glial cells (Fig. 7.4). The neurons are composed of a neuronal cell body, axons and dendrites (Fig. 7.4a). The axons have a terminal end that constitutes the presynaptic site of the synapse. The dendrites have an apical site and multiple branches and spines. The connections among neurons are denominated synapses. Synapses occur between axons and dendrites, axons and cell bodies, and axons and axons. Neurons are excitatory and inhibitory. Excitatory neurons produce glutamate while inhibitory neurons produce GABA. Other neurotransmitters include acetylcholine as well as neuropeptides. Excitatory neurons are usually pyramidal (Fig. 7.4a) and mul-

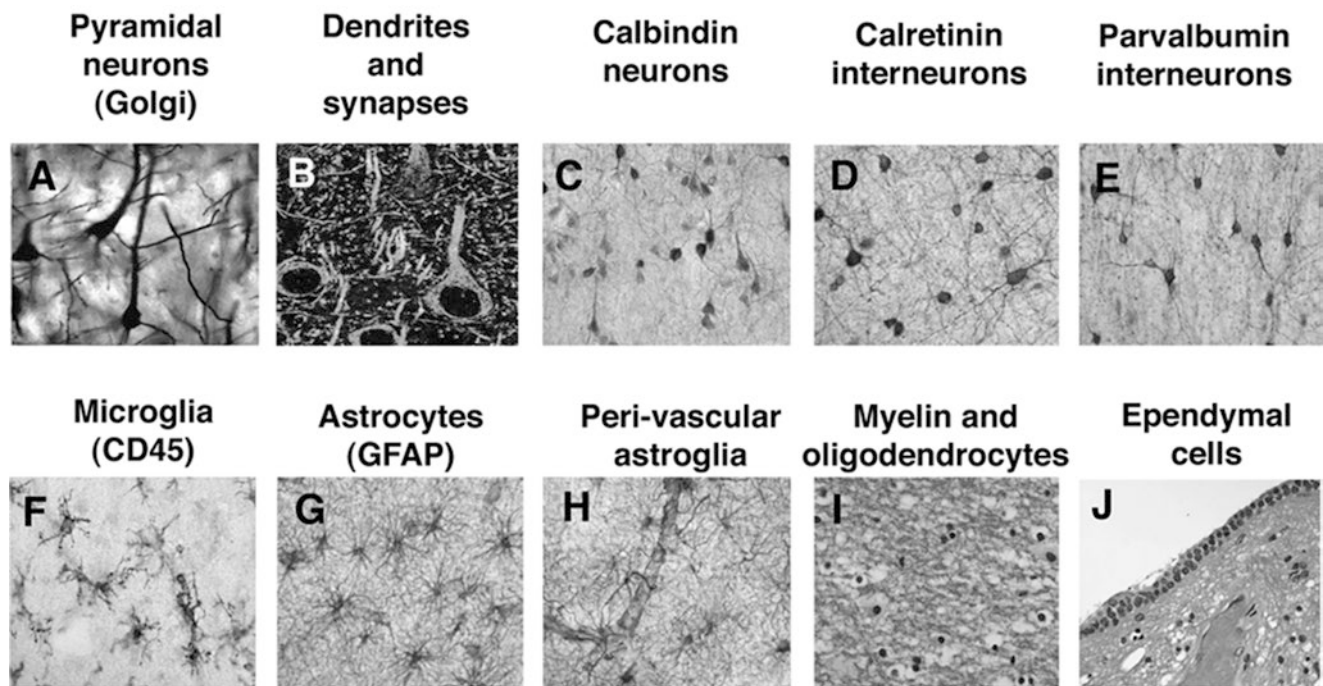


Fig. 7.4 Cellular components of the central nervous system. (a) Neurons impregnated with Golgi silver satin, (c) calbindin immunoreactive interneurons in the neocortex, (g) astrocytes immunoreactive

with an antibody against GFAP, (h) peri-vascular astrocytes components of the BBB, (i) oligodendrocytes and white matter tracts stained with luxol fast blue, (f) ependymal cells around the periventricular zone

tipolar. Inhibitory neurons, also known as interneurons, are bipolar or pseudo unipolar and contain calcium binding proteins such as calbindin (Fig. 7.4b), parvalbumin and calretinin. Types of interneurons include the Martinotti cells, chandelier cells, double bouquet cells, giant basket cells, Cajal Retzius cells and bipolar cells. These sensory neurons receive information through dendrites and transmit that information to the ICNS via axon terminals (Siegel and Sapru 2006). Retinal bipolar cells, sensory cells of the cochlear, and vestibular ganglia are included in this category.

Pseudo-unipolar neurons have a single process that arises from the cell body and divides into two branches. One of these branches projects to the periphery, while the other projects to the CNS. Each branch has the structural and functional characteristics of an axon. Information collected from the terminals of the peripheral branch is transmitted to the CNS via the terminals of the other branch. Unipolar neurons are relatively rare in vertebrates. In these neurons, dendrites arise from the apical part of the cell body and axons form the base where the dendrites are located. Neurons can also be divided into principal or projecting neurons also known as type I or Golgi type I neurons. Principal neurons (e.g., motor neurons in the ventral horn of the spinal cord) have very long axons and form long fiber tracts in the brain and the spinal cord. Intrinsic neurons, also known as type II or Golgi type II neurons, have very short axons. These neurons are interneurons and are considered to have inhibitory function. They are abundant in the cerebral and cerebellar cortex.

7.3 Cerebrovascular Circulation

7.3.1 Blood Supply to the Central Nervous System

The cerebral hemispheres and diencephalon receive blood from the anterior and posterior circulations (Fig. 7.5). The cerebral cortex receives its blood supply from the three cerebral arteries: the anterior and middle cerebral arteries, which are part of the anterior circulation, and the posterior cerebral artery, which is part of the posterior circulation. The diencephalon, basal ganglia, and internal capsule are supplied from branches of the internal carotid artery, the three cerebral arteries, and the posterior communicating artery (Crossman and Neary 2005). The anterior and posterior systems are interconnected by two networks of arteries: (1) the circle of Willis, which is formed by the three cerebral arteries, the posterior communicating artery, and the anterior communicating artery, and (2) terminal branches of the cerebral arteries, which anastomose on the superior convexity of the cerebral cortex (Fig. 7.5). The arterial supply of the cerebral cortex is provided by the distal branches of the anterior, middle, and posterior cerebral arteries. These branches are often termed “cortical” branches (Lee 1995). The anterior cerebral artery originates at the division of the internal carotid artery, and courses within the interhemispheric fissure and around the rostral and dorsal surfaces of the corpus callosum.

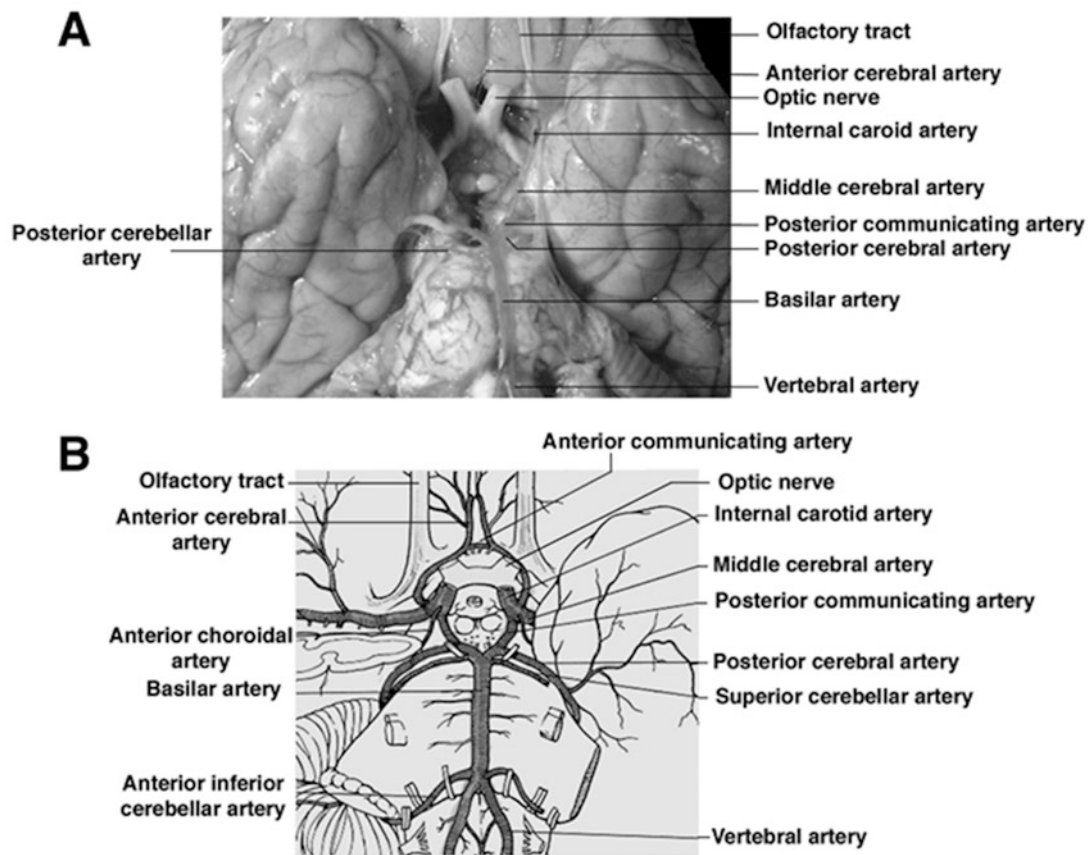


Fig. 7.5 Gross external appearance of the circle of Willis and subarachnoid vessels

The middle cerebral artery, which originates at the division of the internal carotid artery passes through the lateral sulcus (Sylvian fissure) en route to the lateral convexity of the cerebral hemisphere, to which it supplies blood. The middle cerebral artery travels along the surface of the insular cortex, over the inner surface of the frontal, temporal, and parietal lobes, and appears on the lateral convexity. The posterior cerebral arteries originate at the bifurcation of the basilar artery, and each one passes around the lateral margin of the midbrain. The posterior cerebral artery supplies the occipital lobe and portions of the medial and inferior temporal lobe. The arterial supply of the spinal cord is derived from the vertebral arteries and the radicular arteries. The brain is supplied by the internal carotid arteries (the anterior circulation) and the vertebral arteries, which join at the pontomedullary junction to form the basilar artery (collectively termed the posterior circulation). The brainstem is supplied by the posterior system. The medulla receives blood from branches of the vertebral arteries as well as from the spinal arteries and the posterior inferior cerebellar artery (PICA). The pons is supplied by paramedian and short circumferential branches of the basilar artery. Two major long circumferential branches are the anterior inferior cerebellar artery (AICA) and the superior cerebellar artery. The midbrain receives its arterial supply pri-

marily from the posterior cerebral artery as well as from the basilar artery. The venous drainage of the spinal cord drains directly to the systemic circulation. By contrast, veins draining the cerebral hemispheres and brain stem drain into the dural sinuses. Cerebrospinal fluid also drains into the dural sinuses through unidirectional valves termed arachnoid villi.

7.3.2 Immune Cell Trafficking Through the Cerebral Vascular Network

The subarachnoid vessels penetrate the cortical structures and branch to produce the microvascular network which is in turn surrounded by the cellular components of the BBB through which immune cells may need to traffic (Fig. 7.5b). The capillary endothelium of the BBB contains tight junctions that control the movement of leukocytes. In addition these cells are surrounded by a basement membrane and the processes of astroglial cells. The routes for trafficking of leukocytes into the nervous system are through the choroid plexus, subarachnoid space, and perivascular space (Ransohoff et al. 2003). In the first route, leukocytes migrate from the blood to CSF across the choroid plexus. In this pathway of migration, leukocytes travel across the fenestrated endothelium of the

choroid-plexus, migrate through the stromal core villi, interact with epithelial cells of the choroid plexus and enter the CSF at its site of formation. This pathway is likely to be one route by which immune cells enter the CSF under physiological conditions using P-selectin and PECAM.

For the second route, leukocytes reach the CNS from blood to subarachnoid space. In this pathway, leukocytes travel from the internal carotid artery, across postcapillary venules at the pial surface of the brain into the subarachnoid space and the Virchow-Robin perivascular spaces. There, they might encounter cells of the monocyte/myeloid lineage that are competent for antigen presentation. The perivascular regions, where there is direct communication with the CSF compartment, are considered probable sites of lymphocyte-APC interaction and therefore, of immune surveillance of CNS. This pathway is also dependent on P-selectin.

The third pathway involves leukocyte immigration from blood to parenchymal perivascular space. In this third pathway, immune cells can enter the parenchyma directly, passing from internal carotids through the branching vascular tree of arterioles and capillaries and finally extravasating through postcapillary venules. In this case, leukocytes are required to cross the BBB and the endothelial basal lamina. Trafficking of activated lymphocytes across a resting cerebrovascular endothelium is a low-efficient event. This process is dependent on P-selectin, LFA-1 and ICAM (Greenwood et al. 2002).

7.4 Glial Cell Types

The supporting cells located in the CNS are called neuroglia or glial cells. They are relatively nonexcitable and more abundant. Neuroglia has been classified into the following groups: astrocytes, oligodendrocytes, microglia, and ependymal cells (Siegel and Sapru 2006) (Fig. 7.4). Astrocytes are the largest and have a stellate (star-shaped) appearance because their processes extend in all directions (Fig. 7.4g). Their nuclei are ovoid and centrally located. The astrocytes provide support for the neurons, a barrier against the spread of transmitters from synapses, and insulation to prevent the electrical activity of one neuron from affecting the activity of neighboring neurons. Some transmitters (for example, glutamate and γ -aminobutyric acid [GABA]), when released from nerve terminals in the CNS, are taken up by astrocytes, thus terminating their action. The neurotransmitters taken up by astrocytes are processed for recycling. When extracellular K^+ increases in the brain due to local neural activity, astrocytes take up K^+ via membrane channels and help to dissipate K^+ over a large area because they have an extensive network of processes. Astrocytes are further divided into the following subgroups: protoplasmic astrocytes, fibrous astrocytes, and Muller cells. Protoplasmic astrocytes are cells present in the gray matter in close association with neurons. Because of their close association with the neurons, they are

considered satellite cells and serve as metabolic intermediaries for neurons. They give out thicker and shorter processes, which branch profusely. Several of their processes terminate in expansion called end-feet. The neuronal cell bodies, dendrites, and some axons are covered with end-feet joined together to form a limiting membrane on the inner surface of the pia mater (glial limiting membrane) and outer surface of blood vessels (called perivascular lining membrane). The perivascular end-feet may serve as passage for the transfer of nutrients from the blood vessels to the neurons across the BBB (Fig. 7.4h). Abutting of processes of protoplasmic astrocytes on the capillaries as perivascular end-feet is one of the anatomical features of the blood-brain barrier. Fibrous astrocytes are found primarily in the white matter between nerve fibers. Several thin, long, and smooth processes arise from the cell body; these processes show little branching. Fibrous astrocytes function to repair damaged tissue, which may result in scar formation. Muller Cells are modified astrocytes present in the retina.

The oligodendrocytes are involved in the myelination process (Fig. 7.4i). The oligodendrocytes present in the gray matter are called perineurial oligodendrocytes. Oligodendrocytes are smaller than astrocytes and have fewer and shorter branches. Their cytoplasm contains the usual organelles (e.g., ribosomes, mitochondria, and microtubules), but they do not contain neurofilaments. In the white matter, oligodendrocytes are located in rows along myelinated fibers and are known as interfascicular oligodendrocytes.

The microglia are the smallest of the glial cells and are involved in phagocytosis and neuroinflammatory response in the CNS (Fig. 7.4f). These cells are probably derived from monocytes from the bone marrow. They usually have a few short branching processes with thorn-like endings. These processes arising from the cell body give off numerous spine-like projections. They are scattered throughout the nervous system. When the CNS is injured, the microglia become enlarged, mobile, and phagocytic. Ependymal cells consist of three types of cells: ependymocytes, tanycytes, and choroidal epithelial cells. Ependymocytes are cuboidal or columnar cells (Fig. 7.4g) that form a single layer of lining in the brain ventricles and the central canal of the spinal cord. They possess microvilli and cilia. The presence of microvilli indicates that these cells may have some absorptive function. The movement of their cilia facilitates the flow of the CSF. Tanycytes are specialized ependymal cells that are found in the floor of the third ventricle, and their processes extend into the brain tissue where they are juxtaposed to blood vessels and neurons. Tanycytes have been implicated in the transport of hormones from the CSF to capillaries of the portal system and from hypothalamic neurons to the CSF. Choroidal epithelial cells are modified ependymal cells. They are present in the choroid plexus and are involved in the production and secretion CSF. They have tight junctions that prevent the CSF from spreading to the adjacent tissue.

7.5 Brain Regions Linked to Neurodegeneration and Other Neurological Diseases

As the life expectancy of the human population continues to increase, the possibility of developing neuroinflammatory and neurodegenerative diseases has increased considerably during the past 50 years. Of the neurodegenerative disorders, Alzheimer's disease continues to be the leading cause of dementia in the aging population. Traditionally, neurodegenerative disorders have been defined as conditions in which there is selective loss of neurons within specific regions of the brain accompanied by astrogliosis. However, in the past 20 years, we have learned that the pathological process leading to the disfunction of selected circuitries in the brain initiates with damage to the synapses rather than with the loss of neurons. In fact, neuronal loss is a late event that is probably preceded by damage to axons and dendrites followed by shrinkage of the neuronal cell body and abnormal accumulation of filamentous proteins.

Therefore, the revised concept of neurodegeneration suggests that neuronal injury initiates at the synaptic junction and propagates throughout selected circuitries leading to neuronal dysfunction, which resolves in the classical clinical symptoms characteristic to each of the neurodegenerative disorders (Hashimoto and Masliah 2003). For example, in Alzheimer's disease, early damage to the synapses between the entorhinal cortex and the molecular layer of the dentate gyrus (perforant pathway) results in the short term memory deficits characteristic of this dementing disorder. Later on disconnection of the cortico-cortico fibers in the frontal, parietal, and temporal cortex results in more severe memory deficits, alterations in executive functions, and abstraction. Degeneration of connections between the nucleus basalis of Meynert and the neocortex results in attention and memory deficits usually associated with loss of cholinergic neurons. Other circuitries and neuronal populations are also affected in Alzheimer's disease, illustrating the complexity of these disorders and the fact that the concept of single population is affected needs to be expanded to multiple populations. This is the case with several other disorders including Parkinson's disease, where degeneration is not limited to the dopaminergic system but also involves the limbic system, the raphe nucleus, the insula, and other systems.

In response to injury, neurons produce adhesion molecules and trophic factors that recruit astroglial and microglial cells to participate in the process of repair. In addition, the microvasculature and other glial systems might also participate in the process. Thus, neurodegeneration is accompanied by astrogliosis, microgliosis, and microvascular remodeling. While astroglial cells initially produce trophic factors and cytokines that aid in tissue repair, eventually these factors could amplify the inflammatory response by increasing vascular permeability resulting in microglial

activation, which in turn might lead to the production of more proinflammatory cytokines and chemokines. A critical balance between the repair and proinflammatory factors often determines the future rate and progression of the degenerative process.

The understanding of the mechanisms of neurodegeneration and inflammatory response in these neurological conditions has undergone a tremendous progress in the past 10 years. It is now generally accepted that small soluble misfolded protein aggregates denominated oligomers are responsible for the injury. So for example, in Alzheimer's disease, A-beta protein oligomers might damage the synapses in the limbic system while in Parkinson's disease; α -synuclein oligomers damage the axons in the striatum and cortical regions. While significant progress has been made in understanding the fundamental mechanisms for the neuronal injury, less is known about the reasons for the selective neuronal vulnerability characteristic to these neurological conditions.

7.6 Summary

The understanding of the gross and fine structure of the brain is of fundamental importance to elucidate how the nervous system works, how it interacts with other peripheral organs and tissues and how it responds to external stimuli. The main objective of this chapter will be to provide an overview to the structure and organization of the nervous system that is relevant to the understanding of the unique interactions between the nervous and the immune system and to elucidate the pathogenesis of Alzheimer's disease (AD), Parkinson's disease (PD), HIV encephalitis (HIVE), and other neurodegenerative and neuroinflammatory disorders. The nervous system has a unique circulatory organization based on the flow of the cerebrospinal fluid (CSF), the microvasculature, the BBB and the regulation of the blood flow by the neural activity that provides a series of mechanisms to selectively regulate interactions between the nervous system and trafficking cells from peripheral tissues. This chapter provides the structural framework for the better understanding of these interactions under physiological conditions and in neurological diseases and includes sections of gross anatomical structure of the brain, projections, cellular variety, and cerebrovascular circulation.

7.7 Review Questions

1. Interconnecting the two hemispheres is the
 - (a) *Corpus callosum*
 - (b) Anterior commissure
 - (c) Rolandic fissure
 - (d) Sylvian fissure
 - (e) Precentral gyrus

2. The Central fissure divides the

- (a) Visual cortex
- (b) *Sensory and motor cortex*
- (c) Limbic system
- (d) Brain stem
- (e) Cerebellum

3. Correlate the brain regions with the neurotransmitter

(a)	Acetyl choline	Neocortex, hippocampus (a)
(b)	Glutamate	Locus ceruleus (c)
(c)	Norepinephrine	S. Nigra (d)
(d)	Dopamine	Basal forebrain (b)

4. Correlate the brain region with the disease

(a)	Parkinson's disease	Neocortex, hippocampus, basal forebrain (b)
(b)	Alzheimer's disease	Basal Ganglia (c)
(c)	Huntington's disease	S. Nigra (a)
(d)	Aphasia	Broca's area (d)

5. The primary auditory cortex or gyri of Heschl is located in the

- (a) Frontal lobe
- (b) Parietal lobe
- (c) Occipital lobe
- (d) *Temporal lobe*
- (e) Brainstem

6. The middle cerebral artery irrigates the

- (a) *Fronto-temporal cortex*
- (b) Visual cortex
- (c) Brainstem
- (d) Cerebellum

7. Neurons that produce GABA are also known as

- (a) Pyramidal cells
- (b) *Inhibitory interneurons*
- (c) Microglia
- (d) Astrocytes

8. The perforant pathway connects the

- (a) Entorhinal cortex with the forebrain
- (b) *Hippocampus with the hypothalamus*
- (c) Entorhinal cortex with hippocampus
- (d) Cerebellum and brainstem
- (e) Hippocampus and midbrain

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Abstract

Since the discovery of inflammasomes more than a decade ago, there has been tremendous development in the understanding of inflammasomes as central nervous system (CNS) disease determinants. The cytokines, IL-1 β and IL-18, are implicated in both acute and chronic neuro-inflammatory disorders. The cells of macrophage lineage represent the powerhouse of inflammasome activation in the CNS, although other cells including astrocytes and neurons exhibit inflammasome activation in stimulus and species restricted manner. The outcomes of inflammasome activation in the CNS are wide ranging encompassing both protective and pathogenic consequences depending on the individual PAMP/DAMP, inflammasome, cell type and species. The activation of inflammasomes is a complex sequence of events that lends itself to opportunities for modulation at multiple steps and demands further exploration. However, with the current understanding of molecular mechanisms underlying the effects of inflammasome inhibitors, it is feasible to target inflammasome activation at an early stage to delay neurological disease onset and progression. A major concern remains as to whether targeting at the level of inflammasome or blocking the cytokines will be beneficial for control of neuropathogenesis. Given an early prediction of disease, targeting upstream at the level of inflammasome induction would optimize outcomes by not only limiting levels of cytokine release into the brain micro-environment, but also by reducing cell death due to pyroptosis. As new therapies are developed targeting the sensor, adaptor and effector components of inflammasomes, the prospect of regulating inflammasome activation at multiple stages may become a clinical reality, permitting improved outcomes for inflammasome-driven neurological diseases.

Keywords

Cytokines • Inflammasome • Interleukin-1 • Neuroinflammation • Pyroptosis

8.1 Introduction

8.1.1 Discovery of Inflammasomes

Inflammation in the central nervous system (CNS), termed neuroinflammation, is an integral aspect of many neurological diseases. The CNS has its own immune cells that

directly mediate innate immune actions and also contribute to adaptive immunity in the CNS. Innate immune responses are initiated by host recognition of pathogen-associated molecular patterns (PAMPs) expressed on microbial pathogens (e.g., bacteria, viruses, parasites) or by danger-associated molecular patterns (DAMPs) produced by host cells (e.g., ATP, DNA, uric acid). These molecules serve as ligands for pattern-recognition receptors (PRRs) expressed on cells of innate immune system (Bryant and Fitzgerald 2009). Inflammasomes are cytosolic protein scaffolds and represent pivotal components of innate immune system. In response to PAMPs' or DAMPs' ligation by PRRs, inflammasomes

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assemble into multiprotein complexes providing platforms for proteolytic cleavage and subsequent release of two pro-inflammatory cytokines, interleukin (IL)-1 beta (IL-1 β) and IL-18 as immune defenses. It has been established that several protein-protein interactions are required for inflammasome assembly. Although IL-1 α binds to IL-1 receptors like IL-1 β , it does not undergo proteolytic cleavage to enable activation and release. IL-1 β is present in cells as an inactive precursor (pro-IL-1 β) and requires cleavage (at Asp116) for conversion into mature active form, which subsequently drives host responses to injury and infection. This cleavage is mediated by inflammasome regulation of caspase-1, also known as IL-1 β -converting enzyme (ICE) (Dagenais et al. 2012; Martinon et al. 2002). Pro-caspase-1 undergoes autocatalysis during inflammasome activation to yield active caspase-1, which can cleave IL-1 β and IL-18. An important and unique consequence of inflammasome activation is cell death by pyroptosis (fiery death), usually following microbial infection. Pyroptosis is a type of programmed cell death accompanied by swelling and rupture of the cell with extrusion of cellular contents including cytokines into the extracellular space (Bergsbaken et al. 2009).

Inflammasomes were first described in 2002 by Jurg Tschopp's group, as caspase-activating complexes, and since have been identified to be present in macrophages, monocytes, dendritic cells, neutrophils, epithelial cells etc. Nucleotide-binding domain (NB) and leucine-rich repeat (LRR) containing receptors (NLR), also called Nod like receptors, are cytosolic PRRs found in myeloid cells. Before the term 'inflammasome' was coined, mutations in NLR genes were linked to several inflammatory diseases, including systemic acute inflammation due to cold temperature exposure. The intracellular protein implicated in the phenomenon was named 'cryopyrin' since patients developed fever when exposed to cold. Later, inflammasomes were identified as intracellular molecular platforms and key elements in innate immunity. NLR family, pyrin domain containing 3 (NLRP3), also termed NACHT, LRR and PYD domains-containing protein 3 (NALP3), inflammasome was one of the first intracellular PRRs to be identified in humans (Tschopp and Schroder 2010). Since the initial discovery more than a decade ago, inflammasome research has witnessed substantial advancements in understanding and associated applications.

8.1.2 Periodic Fever Syndromes

Periodic Fever Syndromes (PFS) denote a spectrum of hereditary disorders caused by mutations in innate immune-associated proteins leading to disruption of IL-1 related pathways. The PFS are a spectrum of autoinflammatory

diseases resulting from immune activation in an antigen-independent manner. Most of these syndromes (e.g., Familial Mediterranean Fever, Tumor Necrosis Factor (TNF) receptor associated periodic syndrome) present in children with recurrent fevers lasting from few days to few weeks, with symptom free-period in between episodes (Drenth and van der Meer 2001). The term 'autoinflammation', which is primarily linked with innate immunity, was introduced in 1999 and is different from 'autoimmune' diseases which are linked to adaptive immunity (McDermott et al. 1999).

There are several recognized 'gain-of-function' mutations in the *NLRP3* gene that result in over activation of the inflammasome and increased secretion of IL-1 β and IL-18. These disorders are better known as cryopyrin-associated periodic syndromes (CAPS). Symptoms include urticarial rash, fever, musculoskeletal and ocular disorders, progressive deafness, etc. The specific mutation determines the ensuing severity of disease. Genetic testing is required for precise diagnosis of CAPS. Treatment strategies include IL-1 β blockade to control inflammation (Giat and Lidar 2014).

Another disorder, Deficiency of IL-1 Receptor Antagonist (DIRA), is caused by 'loss-of-function' mutation in the IL-1 receptor antagonist gene, *IL1RN*. This gene encodes for an endogenously expressed antagonist of IL-1 signaling, therefore, the mutation leads to blocking of IL-1 signaling. Systemic inflammation often occurs within first few weeks of birth. Failure to diagnose and treat can lead to severe inflammatory response and multiorgan failure (Jesus and Goldbach-Mansky 2014). However, there are other monogenic 'hereditary fever syndromes', which are not caused by mutations in IL-1 pathways, but are indirectly mediated by IL-1 activation.

8.2 Molecular Structure of Inflammasomes

Inflammasome assembly occurs in the cytosol and is comprised primarily of three components: a sensor component, an adaptor component and an effector component. The sensor components are the most upstream and first molecules to detect danger signals and subsequently recruit the adaptors. Inflammasomes are named after the sensor components forming that particular inflammasome (van de Veerdonk et al. 2011). Two known classes of sensor molecules have been defined: NOD-like receptors (NLRs) and AIM2-like receptors (ALRs). In early 2000s, the NLR gene family was discovered in humans and it is now well established that NLR family comprises innate immune genes in species ranging from sea urchins to humans (Ting et al. 2008). In the human genome, 22 NLRs are encoded but to date only NLRP1, NLRP2, NLRP3, NLRP6, NLRP7 and NLRP12 have been reported to participate in inflammasome assembly. The sensor components of inflamma-

somes have effector domains such as pyrin domain (PYD), caspase recruitment domain (CARD) or nucleotide-binding and oligomerization domain NACHT and a leucine-rich repeats (LRR) domain (Lu et al. 2014a).

The most common adaptor component is formed by apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC). The inflammasomes can be 'ASC-dependent' or ASC-independent. In 'ASC-dependent' inflammasomes, two-step polymerization process results in inflammasome assembly through N-terminal PYD and C-terminal CARD of ASC. First, the sensor protein oligomerizes to form a cluster of PYD, which serves to recruit monomeric ASC, which in turn serves as a platform for the CARD to cluster and to recruit the caspase-1 filament by a homotypic CARD/CARD interaction (Lu et al. 2014a). Thus, ASC acts as a core protein that is essential for translating stimulation of inflammasome to its activation. However, in 'ASC-independent' inflammasomes this function is mediated by CARD/CARD interactions. The classic example is NAIP/NLRC4/caspase 1 inflammasome where CARD of NLRC4 (NLR family CARD domain-containing protein 4) nucleates caspase-1 for activation. Another ASC-independent inflammasome is formed by mouse NLRP1b, which lacks the N-terminal PYD required for ASC recruitment.

The effector component is formed by caspases including caspase-1 (caspase-11 in mouse) as well as caspases-4 and -5 in humans. These caspases are characterized by the presence of CARD domain at the N-terminus which is essential for interaction with the adaptor component. (The 'c' in 'caspase' refers to cysteine protease and 'aspase' refers to the ability to cleave after aspartic acid.) All caspases are produced in cells as catalytically inactive zymogens and require proteolytic cleavage before activation (Martinon and Tschopp 2007). Caspase-1, first described in 1989, was identified as enzyme able to cleave pro-IL-1 β and pro-IL-18. IL-33 is also reported to be cleaved by caspase-1 although the location and consequences of IL-33 proteolysis remain unclear (Zhao and Hu 2010).

Some of the better characterized inflammasomes include NLRP1, NLRP3, NLRP6, NLRC4 and AIM2. NLRP1 is formed by ASC, NACHT and LRR as the adaptors and pyrin domain containing protein 1 (NLRP1) as sensor component. It acts like a cytosolic toll like receptor (TLR) and activates caspase-1 to induce inflammation through IL-1 β . NLRP3 (also called NALP3 or cryopyrin) is the best known inflammasome and is activated by multiple PAMPs and DAMPs. Bacterial pathogens, prokaryotic mRNA, ER stress, extracellular ATP are some of NLRP3 inducers (Sollberger et al. 2014). NLRC4 is required exclusively for bacterial-induced caspase-1 activation and subsequent inflammation. ASC is required for caspase-1-mediated and NLRC4-dependent activation of pro-IL-1 β and proIL-18, but not for caspase-1-

dependent cell death. AIM2 is another inflammasome which binds to double stranded DNA and plays an important role against viral infections.

8.3 Inflammasomes in the Healthy Central Nervous System

Inflammasome-mediated processes are vital for immune responses against microbial infections. Inflammasome assembly is highly regulated at multiple steps to prevent its aberrant activation in immunologically unchallenged conditions. The inflammasome components are auto-inhibited by various domains, which form first check-point to restrict activation. Secondly, the compartmentalization of individual inflammasome components in different cellular organelles limits the interaction and provides efficient mechanism of regulation of inflammasome activation. For example, ASC is found in mitochondria and nucleus whereas NLRP3 is located in endoplasmic reticulum (Misawa et al. 2013). Upon stimulation with ligands, the components assemble in cytosol and lead to downstream production of inflammatory cytokines.

Microglia, neurons, oligodendrocytes and astrocytes comprise the principal cell types of central nervous system (CNS); all of these cells can exert immune effects depending on the circumstances although neurons and oligodendrocytes are not usually regarded as immune effector cells. Inflammasome-mediated inflammatory pathways have been implicated in different CNS disorders with overexpression of pro-inflammatory cytokines (e.g., IL-1 β and IL-18) linked to neuroinflammation. However, basal level of cytokine expression is essential for normal physiological function of the brain and cytokine deficiencies can be detrimental just as their overexpression is widely seen to be pathogenic. Cytokines are recognized to participate in fundamental molecular and cellular mechanisms linked to learning, memory and cognition under physiological conditions (McAfoose and Baune 2009). Therefore, it is of utmost importance to understand the inflammasome in the healthy CNS. Neurocognitive function in humans is influenced by IL-1 β through its actions on long term potentiation (LTP). LTP is an indication of the strengthening of synapses to produce a long lasting increase in signal transmission between two neurons and is associated with learning and memory. To some extent, there is cell-specific inflammasome expression in the CNS, with NLRP3 found predominantly in microglia and macrophages, NLRP2 in astrocytes and AIM2 and NLRP1 in neurons; although cell type expression is likely dependent on the individual species. In healthy physiological conditions, IL-1 β is required to maintain an optimum LTP balance, thereby positively correlating with learning and memory (Ross et al. 2003). This effect is primarily observed in hippocampus, the area of brain associated with declarative memory function.

The brain has been classified as first-tier tissue (based on a three tier classification), which constitutively expresses inflammasomes, primarily NLRP1 and NLRP3. According to this model, first tier tissues (including brain, blood and thymus) initiate inflammation more rapidly than second and third tier tissues (Yin et al. 2009).

Inflammasomes in different CNS cell types: In the CNS, inflammasomes are expressed ubiquitously in microglia, trafficking macrophages, astrocytes and perhaps in a species-specific manner in neurons. Microglia are the resident tissue macrophages of the brain originating from distinct progenitor cells rather than hematopoietic macrophages, which traffic into the brain post-natally. The NLRP3 inflammasome is activated in microglia and macrophages by various priming PAMP or DAMP signals e.g., lipopolysaccharide (LPS), muramyl dipeptide (MDP), adenosine-triphosphate (ATP). Microglial NLRP3 activation was shown by priming signals derived from macrophages infected with mycobacteria (Lee et al. 2013). Primary microglia respond to the same innate stimuli as hematopoietic macrophages but often with a different magnitude of response than macrophages. Conversely, microglial responses are more persistent due to lack of negative regulation on pro-IL-1 β expression (Burm et al. 2015). Microglia are the primary producers of IL-1 β , following NLRP3 upregulation, in a rat depression model. Microglial NLRP3 inflammasome activation is increasingly seen as a mediator of IL-1 β -related neuroinflammation during chronic stress (Pan et al. 2014). In addition to NLRP3, AIM2 plays a critical role in regulating IL-1 β release and cell survival during acute CNS bacterial infection by *Staphylococcus aureus* (Hanamsagar et al. 2014). Flagellated bacteria, *Legionella pneumophila*, infect and replicate in macrophages and invade brain. During bacterial infection by *L. pneumophila*, primary microglial cells responded via activation of NLRC4 inflammasome (Jamilloux et al. 2013).

Astrocytes are resident brain cells functioning in synaptic formation and provide support and protection to neurons. Astrocytes can also respond to diverse stimuli and are major contributors of chemokines and cytokines, including IL-1 β . Elevated levels of IL-1 β have been detected in astrocytes in neurodegenerative disorders like Alzheimer's disease (AD) and HIV infection. Palmitate, the most abundant saturated fatty acid in the diet, can activate astrocytes eventually causing neuronal damage. The involvement of NLRC4 and ASC has been shown in the inflammatory response to palmitate by astrocytes, associated with AD. Expression of both NLRC4 and ASC is elevated in AD patients (Liu and Chan 2014). In a Parkinson's disease model, intracellular oxidative stress in astrocytes exacerbated neuroinflammation to dopaminergic neurons via the activation of NLRP3 inflammasome in astrocytes (Lu et al. 2014b). It was recently demonstrated that human astrocytes express a novel NLRP2 inflammasome complex, activated by ATP. The adaptor protein in NLRP2

complex is ASC with caspase-1 as the effector protein. NLRP2 interacts with P2X7 receptor and the pannexin 1 channel for the downstream activation of caspase-1 and IL-1 β production. Astrocytic NLRP2, thus, is an important component during CNS inflammatory responses (Minkiewicz et al. 2013).

Neurons can also sense danger stimuli associated with mechanical, thermal or chemical nature. They express TLRs and are known to express inflammasomes like NLRP3 and AIM2. However, the study of activation of inflammasomes in neurons is at its beginning stage and needs further exploration (Santoni et al. 2015).

Thus, multiple inflammasomes exist in the CNS and are functional in different neural cell types which are activated depending upon nature of stimuli. The overall mechanism is complicated by the simultaneous activation of multiple inflammasomes, in either similar or different cell types.

8.4 Inflammasomes in Acute Neurological Disease

Stroke, bacterial meningitis and traumatic brain injury (TBI) represent prototypic acute neurological disorders with inflammatory activation implicated in the disease process. Inflammasome activation in acute and chronic neurological disorders is summarized in Table 8.1.

Stroke: Stroke is a common cause of death, implicated in 10% of deaths worldwide and often leads to long-term disability in rest of the cases. Stroke is characterized by reduced blood flow in focal areas of the CNS leading to neurological disability. It may be caused either by thrombotic or embolic occlusion of a cerebral artery (ischemic stroke) or by rupture of a cerebral blood vessel (intracerebral or subarachnoid hemorrhage). Amongst the two types, ischemic stroke is most common and occurs in 80% of patients with stroke (Fann et al. 2013). Inflammation during stroke begins with secretion of pro-inflammatory cytokines (e.g. TNF- α , IL-1 β and IL-18) by activated glial cells in response to ischemia and perhaps hypoxia. This primary response is followed by secondary infiltration of neutrophils and monocytes from the vascular circulation, which also secrete additional cytokines and cytotoxic agents e.g., reactive oxygen species (ROS), inducible nitric oxide synthase (iNOS) and matrix metalloproteinases (MMPs). Activated MMPs can disrupt the blood-brain barrier (BBB) leading to hemorrhage, brain edema and eventually neuronal cell death. This secondary damage is referred to as ischemic reperfusion injury. During ischemic stroke, NLRP1 and NLRP3 inflammasomes are major sensors for cellular damage detection. However the precise stimuli for activation of these inflammasomes are unknown. One of the major drivers of inflammasome activation during ischemic stroke is lower cytosolic ATP. Lower ATP/AMP (adenosine monophosphate) ratio

Table 8.1 Inflammasome activation in neurological diseases

Disease	Inflammasome/trigger	Cell type	References
Ischemic stroke	NLRP1/ATP	Neurons	Abulafia et al. (2009) and Fann et al. (2013)
	NLRP3/H ⁺ ions		
Traumatic brain injury	NLRP1	Neurons	de Rivero Vaccari et al. (2008) and Liu et al. (2013)
	NLRC4		
	AIM2		
Bacterial meningitis	NLRP3/Cathepsin B	Macrophages, microglia	Hanamsagar et al. (2014) and Hoegen et al. (2011)
West Nile virus encephalitis	NLRP3	Neurons, microglia	Kumar et al. (2013) and Ramos et al. (2012)
NeuroAIDS/HAND	NLRP3/viral proteins	Microglia, astrocytes	Walsh et al. (2014)
Alzheimer disease	NLRC4/palmitate	Microglia, astrocytes, neurons	Heneka et al. (2013) and Tan et al. (2013)
	NLRP3/A β		
	NLRP1		
Parkinson's disease	NLRP3/ROS	Macrophages, astrocytes	Codolo et al. (2013)
Multiple sclerosis	NLRP3/ATP	Macrophages, microglia	Furlan et al. (1999) and Inoue et al. (2012)
Prion disease	NLRP3/PrP fibrils	Macrophages, microglia	Hafner-Bratkovic et al. (2012)

activates AMPK (AMP-activated protein kinase), which eventually activates NLRP1 receptor. Interestingly, AMPK activation alone is not enough to drive NLRP1 inflammasome activation, but lower ATP is a co-requirement, suggesting that ATP in normal cellular conditions inhibits NLRP1 receptor (Liao and Mogridge 2013).

Extracellular or intracellular acidosis leads to activation of the NLRP3 inflammasome in glial cells. Extracellular acidosis may result from the release of H⁺ ions by necrotic cells, whereas intracellular acidosis could result from reduced oxygen availability and accumulation of lactic acid resulting from anaerobic glycolysis. Another important factor for NLRP3 activation during stroke is cytosolic high Ca²⁺ and reduced K⁺ concentration. Lower ATP results in abnormal functioning of Na⁺/K⁺-ATPase pump, resulting in imbalance in ionic concentrations and eventually NLRP1 and NLRP3 inflammasome activation (Ding et al. 2000; Rajamaki et al. 2013; Munoz-Planillo et al. 2013).

Mitochondria are a significant source of ROS during stroke. Disturbed electron transport chain produces ROS in cytoplasm which activates NLRP3 inflammasome. Following NLRP3 activation, ASC on mitochondria translocates into perinuclear space, which brings ASC closer to NLRP3 and thus NLRP3 can receive mitochondria-derived signals such as ROS (Misawa et al. 2013). Mice models of cerebral ischemia have shown the evidence of inflammasome activity. The activation of NLRP1 and NLRP3, during stroke, mediates caspase-1 induced apoptosis and pyroptosis in neuronal and glial cells. Binding of IL-1 β to IL-1 receptor 1 (IL-1R1) in cells of injured cerebral tissue results in neurotoxicity; however, initial response is meant to be neuroprotective (Zhang et al. 2014; Denes et al. 2010).

Current treatments in stroke consist of administration of recombinant tissue plasminogen activator for removal of the blood clot. However, a major obstacle is the 3–4.5 h treatment window after which the treatment is less efficacious and possibly dangerous. Therefore, targeting inflammasome signaling is emerging as new therapeutic strategy for controlling neuronal damage during stroke. The potential targets include inflammasome components (NLRPs, ASC and caspase-1), signaling pathways (NF- κ B and MAPK), plasma membrane receptors/channels (P2X7 receptors, pannexin 1 and K⁺ channels), cytokines (IL-1 β and IL-18) and cytokine receptors (i.e. IL-1R1 and IL-18R) involved in inflammasome signaling (Fann et al. 2013).

Meningitis: Meningitis refers to acute inflammation of meninges or membranes of the brain and spinal cord. The meninges are comprised of three membranes including the outermost dura mater, middle arachnoid mater and the innermost and most delicate, pia mater. The dura mater is thick and durable membrane attached to the skull whereas the pia mater forms contact with the brain and spinal cord. Meningitis may be caused by various infectious pathogens including bacterial or viral infections. Bacterial meningitis is often caused by *Streptococcus pneumoniae* and carries a mortality rate of 16–37%, with approximately half of the survivors suffering from neurological disabilities (Hoogman et al. 2007). It occurs as a result of poor host defenses resulting in pathogens reaching the subarachnoid space in meninges.

Inflammasomes have been extensively implicated in bacterial meningitis. This is because inflammasomes are known to be activated by microbial PAMPs and endogenous DAMPs such as ATP, oxygen radicals and imbalance in cellular ion concentrations. Cerebrospinal fluid (CSF) of patients with

pneumococcal meningitis shows evidence of upregulation of caspase-1 activity and cytokines IL-1 β and IL-18 (Hoegen et al. 2011). A murine model of meningitis was developed by intranasal inoculation of pneumococci with hyaluronidase. Correlation of elevated IL-1 β and IL-18 levels with disease severity and complications has been reported (Zwijnenburg et al. 2001). Interestingly, ASC and NLRP3 knockout mice exhibited decreased bacterial loads; however, NLRP3 expression was protective against brain damage. NLRP3 knockout mice showed increased leukocyte infiltration and cerebral hemorrhage following bacterial infection with worse outcomes (Geldhoff et al. 2013). Inflammasome activation may be detrimental in some chronic conditions but it is likely intended to be protective in acute inflammation. Indeed, it was shown that IL-1 receptor knockout mice were more susceptible to developing meningitis after intranasal infection with *S. pneumoniae*. Higher mortality and enhanced growth of pneumococci in IL-1R $^{-/-}$ mice compared with wild-type mice has been reported (Zwijnenburg et al. 2003). These studies emphasized on the beneficial effects of IL-1 β induction and expression in bacterial clearance during host immune responses.

In bacterial meningitis, protease activity works upstream of NLRP3 inflammasome activation. It is proposed that lysosomal damage releases lysosomal proteases such as cathepsin B into the cytoplasm. These proteases activate NLRP3 either by catalyzing degradation of an NLRP3 inhibitor or cleavage of a substrate releasing NLRP3 ligand into the cytoplasm. Nigericin, one of the important activators of NLRP3, also acts through lysosomal release of cathepsin B and subsequent caspase-1 activation (Hoegen et al. 2011). Meningitis has high mortality rate and alternative treatment regimens are necessary because of spread of penicillin-resistant *S. pneumoniae* (Fiore et al. 2000). Enhanced inflammasome signaling during meningitis might be a promising therapeutic strategy in the future.

Traumatic brain injury: Traumatic brain injury (TBI) is a complex injury leading to long-term disabilities. It occurs when a sudden trauma causes mild, moderate or severe damage to the brain. Some of the moderate symptoms include headache, dizziness and blurred vision but can also result in epileptic seizures, loss of motor control and co-ordination as well as memory impairment. A robust inflammatory response is generated in the injured tissue immediately after TBI. The complexity of this post-traumatic response includes the activation of resident glial cells; microglia, and astrocytes along with infiltration of blood leukocytes. Acute CNS injury models show recruitment of inflammatory cells, elevated pro-inflammatory cytokines at both gene expression and protein levels along with inflammasome-related signaling cascades (Woodcock and Morganti-Kossmann 2013).

Caspase-1 is activated after both spinal cord injury (SCI) and TBI. The expression levels of NLRP1 inflammasome

were unchanged but ASC and caspase-1 protein levels were elevated in CSF of patients with TBI. Expression of NLRP3 inflammasome in cerebral cortex was found post injury in a rat TBI model (Liu et al. 2013). An interesting experimental observation in TBI is inflammasome activation in neurons. It was shown that NLRP1 was activated in rat neurons and constituted important inflammatory response following SCI (de Rivero Vaccari et al. 2008). A recent report shows that neurons respond to injury by activation of AIM2 inflammasome and oligomerization of ASC. Neurons then undergo pyroptosis following caspase-1 activation, formation of discrete pores in plasma membrane and release of IL-1 β (Adamczak et al. 2014). Thus, inflammatory innate immune responses, especially in the post-injury period, are important therapeutic targets for controlling neuronal damage during TBI.

8.5 Inflammasomes in Chronic Neurological Disease

Prolonged activation of inflammasomes in the brain leads to neuroinflammation which is pathogenic in many chronic neurological disorders. HIV/AIDS associated brain disease (NeuroAIDS), Alzheimer Disease (AD) and Multiple Sclerosis (MS) are chronic neurological diseases in which inflammasome-driven pathways are key aspects of the disease process. Specifically, the NLRP3 inflammasome has been reported to be activated in a number of neuroinflammatory disease scenarios including NeuroAIDS, MS and AD (Walsh et al. 2014).

NeuroAIDS: Human Immunodeficiency Virus (HIV) enters into the brain very early during the infection. The persistence of HIV infection in the brain represents a challenge to the researchers attempting to fully eradicate HIV infection. TNF- α and IL-1 β are the cytokines that have been most consistently implicated as participants during CNS HIV infection (Tyor et al. 1993; Wesselingh et al. 1997; Xing et al. 2009a, b). These are central regulatory molecules that are directly activated by HIV-1 infection and are responsible for driving the inflammatory response through their effects on immune cell activation and recruitment. In addition to the further recruitment of immune cells into the brain, several of these molecules have been reported to exert effects on local cell types, such as astrocytes and neurons, to promote neuropathology (Bezzi et al. 2001; Zhang et al. 2003). Despite the characterization of an inflammatory response to HIV in the brain, there is still relatively little known about the innate immune sensing of the virus or virus-infected cells at the molecular level. Current studies on HIV's interaction with the innate immune system have focused on the sensing of viral genomic products and activation of antiviral interferon (IFN) responses. In the periphery, HIV-1 elicits a strong Type 1 interferon response from specialist plasmacytoid dendritic

cells (pDC) (Lepelley et al. 2011). In these cells, virus is endocytosed and sensed by TLR7 and TLR9 which bind to viral RNA (Beignon et al. 2005). Type 1 interferon responses have been reported in the context of CNS HIV infection (Polyak et al. 2013). The release of both TNF- α and IL-1 β in response to whole virus has been replicated using individual viral proteins such as viral envelope protein gp120/gp160 and HIV trans-activating protein Tat (Cheung et al. 2008; Ben Haij et al. 2013; Jin et al. 2012) and the viral protein R (Jones et al. 2007). Recent data from our laboratory show that IL-1 β , IL-18 and caspase-1 are induced in brains of HIV-1 infected individuals, specifically in microglia. Induction of pro-IL-1 β occurred within 4 h of microglial infection with HIV, by NLRP3 dependent mechanism. Adapter protein ASC translocated from nucleus to cytoplasm, pointing towards assembly of inflammasomes, within few hours following HIV infection. The study highlighted that targeting the inflammasome activation in brain might represent a therapeutic intervention in HIV-associated neurocognitive disorder (HAND) (Walsh et al. 2014).

Alzheimer Disease: Alzheimer disease (AD) is clinically characterized by extracellular accumulation of amyloid- β (A β) in senile plaques together with phosphorylated neurofilaments comprised of the tau protein. It is a chronic neurodegenerative disease that accounts for more than 50% of dementia cases. The early symptoms include short-term memory loss which then progresses into speech impairment, disorientation and memory loss with progressive neurocognitive decline (Burns and Iliffe 2009).

The causes of sporadic age-related AD (the most common type) are not well defined but both genetic and environmental determinants have been associated with AD. The accumulation of A β activates NLRP3 inflammasome in resident microglia with associated neuroinflammation. Multiple inflammatory cytokines and chemokines are reported to be activated in brain and CSF from AD patients and IL-1 β is one of the prominent cytokines amongst those (Tan et al. 2013). The mechanism of NLRP3 activation during AD involves the following steps: a) increased A β phagocytosis by microglia b) lysosomal breakdown in microglia c) release of cathepsin B d) NLRP3 activation followed by caspase-1 activation and IL-1 β release. NLRP3 activation is positively correlated with neuropathology in transgenic mice expressing A β . These findings were corroborated with findings of decreased A β deposition and better spatial memory in NLRP3 knockout mice (Heneka et al. 2013). One of the important observations was the restriction of NLRP3 activation in plaque-associated microglia, which emphasized the direct relation between A β and NLRP3 activation. It also suggested that NLRP3 activation was linked to disease progression in AD. Neurons undergo excitotoxic death that is modulated by IL-1 β signaling. IL-1 β -mediated neuronal cell death in AD is mediated by mitogen-activated protein kinase (MAPK) pathway and

by iNOS production in hippocampal neurons (Barone et al. 2001). Further investigations are required to understand the inflammatory signaling pathways involved in AD. The NLRP3 inflammasome might be a future molecular target for therapeutic intervention for AD.

Multiple sclerosis: Multiple Sclerosis (MS) is defined by progressive neurological disability caused by inflammation in the CNS, although the cause of MS remains unknown. Neuroinflammation is accompanied by demyelination and eventual axonal loss (Legroux and Arbour 2015). Immune system pathways (innate and adaptive) are implicated in activating the brain's inflammatory molecular machinery with both adverse and protective outcomes, but what drives these inflammation-mediated neurological outcomes remains uncertain.

Given the established associations between IL-1 β and -18 and neuroinflammation, it is not surprising that substantial evidence implicates inflammasomes in the pathogenesis of MS and associated MS models. Several inflammasome components are known to be upregulated at the mRNA and protein levels in both acute and chronic demyelinating MS lesions, including ASC, caspase-1 (Furlan et al. 1999; Laule et al. 2011), NLRP3 (Hauser et al. 1990), IL-18 and IL-1 β (Broz and Monack 2011). Elevated expression of IL-18, IL-1 β and caspase-1 can also be found in peripheral blood mononuclear cells, serum and CSF from MS patients.

Different groups have investigated the contributions of inflammasome components in models of MS, including experimental autoimmune encephalomyelitis (EAE) and the cuprizone model of demyelination. In EAE, multiple reports indicate that knock-out of key inflammasome components (NLRP3^{-/-}, ASC^{-/-}, and caspase-1^{-/-}) causes an attenuated form of EAE. Knock-out of the key inflammasome cytokine, IL-18, causes a similar phenotype, as does knock-out of the IL-1 β receptor, IL-1R. Both IL-1 β and IL-18 promote Th1, Th17 and humoral immunity; IL-18 is important for IL-17 production by Th17 cells. Pharmacological inhibition of both caspase-1 and the ATP receptor, P2X7 (known to induce inflammasome activation) causes attenuation of disease severity in the EAE model. The inflammasome's key role in demyelination was also reported in the cuprizone model; knock-out of NLRP3, ASC and IL-18 all cause reduced neuroinflammation and demyelination (Guarda et al. 2011). However, other studies found that demyelination can occur in the absence of the inflammasome. For example, NLRP3 knockout exerts variable effects on the pathogenesis of EAE and IL-1 β knock-out does not affect demyelination or neuroinflammation in the cuprizone model. These latter results may reflect heterogeneity in genetic backgrounds of mice such as Nlrp1 allelic variants. This dichotomy also implies that multiple inflammasomes and inflammasome-independent processes may contribute to inflammatory demyelination. Of interest, Type 1 interferons, one of several

MS-associated disease modifying therapies regulate IL-1 β production and inflammasome activation. Thus, inflammasomes are essential to investigate in MS because they provide insight into the cause(s) of MS by deciphering their agonists/signals. They participate in the fundamental mechanisms underlying MS yielding a greater understanding of the disease and they offer immediate 'drug-able' targets for interventions in MS.

8.6 Pharmacological Inhibition of the Inflammasome

Inflammasomes are multi-protein complexes and each component is controlled by various regulatory mechanisms. Thus, inflammasomes are unique candidates for the development of effective anti-inflammatory therapeutics. Targeted therapies with inhibition of inflammasome complexes can be achieved to improve neurological outcomes in particular diseases.

Conventional anti-inflammatory therapies are predicated on inhibiting the effects of IL-1 β using anti-IL-1 β antibodies or recombinant IL-1 receptor antagonists. However, effective anti-IL-18 therapies are currently unavailable (Netea et al. 2015). Blockade of IL-1 signaling to control inflammation was an early approach, which dates back to 1993 with the introduction of anakinra. Anakinra is a recombinant IL-1 receptor antagonist that blocks the activity of both IL-1 α and IL-1 β and was approved for use as a drug by the U.S. Food and Drug Administration (FDA) in 2001. The advantages of anakinra as a drug include safety, short half-life and multiple routes of administration. As a result, it has been extensively used in treatment of broad range of diseases including rheumatoid arthritis, CAPS and Type 2 diabetes (Dinarello et al. 2012). Neutralizing IL-1 β with specific antibodies is another therapeutic strategy for control of IL-1 β -induced inflammation. Canakinumab, a human monoclonal antibody, was approved by the FDA in 2009 because of its specificity against IL-1 β . It is currently in clinical trials for several inflammatory disorders including arthritis, other inflammatory joint diseases, Type 1 diabetes and pulmonary diseases (Chakraborty et al. 2013; Howard et al. 2014). Some of the endogenous inhibitors of inflammasomes are IL-37 and Type 1 interferons. IL-37 is a 30 kDa member of the IL-1 family of ligands and has amino acid residues common with those of IL-18. It can, therefore, bind to IL-18 receptor in a non-competitive manner. IL-37 exerts its anti-inflammatory effects by nuclear translocation followed by inhibition of cytokines such as IL-1 and TNF- α . The inhibitory effects of IL-37 have been confirmed in cells of myeloid lineage such as bone marrow derived macrophages and neutrophils (Moretti et al. 2014). IFN- β is a widely used drug to treat MS and is shown to inhibit NLRP3-mediated brain inflammation

in mouse EAE model. Innate immune cells, such as macrophages and dendritic cells (DCs), exert inhibitory effects through IFN- β , which in turn is exerted by inhibition of T helper 17 (TH17) responses. IFN- β reduces the production of mitochondrial ROS with subsequent inhibition of NLRP3 inflammasome activity. In addition, IFN- β had no effect on progression of NLRP3-independent EAE and could reduce disease progression only in case of NLRP3-dependent EAE. This clearly demonstrated a direct relationship between IFN- β and NLRP3 suppression (Inoue et al. 2012).

Pro-inflammatory cytokines represent key factors mediating macrophage-driven inflammation in ischemic heart disease. Interestingly, moderate consumption of alcohol is associated with reduced risk of coronary disease-associated mortality. Recently, it was established that ethanol inhibits both NLRP3 and AIM2 inflammasome in macrophages, providing a potential molecular mechanism underlying ethanol-induced protection in ischemic heart disease. Ethanol reduced the secretion of IL-1 β by reducing caspase-1 activity but pro-IL-1 β production was unaffected, hence, acting at the level of NLRP3 inflammasome assembly. Ethanol inhibited inflammasome by maintaining lysosomal integrity and preventing the release of cathepsin B. It also reduced oligomerization of ASC, thereby, inhibiting upstream of caspase-1 activation. Ethanol also inhibited AIM2 inflammasome, which was evident as reduced IL-1 β production even in the presence of synthetic double stranded DNA. Ethanol-mediated reduced IL-1 β secretion might explain atheroprotective effects of alcohol and also susceptibility to infections associated with excessive alcohol consumption (Nurmi et al. 2013).

Many small molecule compounds have been identified as inhibitors of inflammasome assembly and activation. Some of these are already in use whereas others are in clinical trials for efficacy and safety.

Glyburide: Glyburide is a commonly used drug for treatment of Type 2 diabetes and belongs to sulfonylurea class of drugs. In pancreatic β cells, glyburide inhibits ATP-sensitive K⁺ channels (K_{ATP}), which regulate glucose-dependent insulin secretion (Ashcroft 2005). With the inhibition of K_{ATP} channels, membrane potential changes resulting in calcium influx. This triggers release of insulin stored in small vesicles inside the pancreatic β cells. In addition, it was the first compound reported to act upstream of NLRP3, thus inhibiting, PAMP- and DAMP-induced caspase-1 activation, IL-1 β secretion and subsequent cell death. However, the inhibitory effect of glyburide on NLRP3 is independent of its action on K_{ATP} channels; for example, glyburide can block NLRP3 even in the absence of K_{ATP} channels (Lamkanfi et al. 2009). It inhibits NLRP3 downstream of the P2X7 receptor. Of note, P2X7 receptor signaling leads to ROS-dependent inflammasome activation (Bartlett et al. 2013).

Although glyburide is an oral medication used in the treatment of Type 2 diabetes, there is also formulation for intravenous

injection, RP-1127, which is in clinical trials for preventing malignant edema in patients with ischemic stroke. It is also being tested against mild, moderate or severe traumatic brain injury for safety and efficacy in preventing hemorrhage and edema (<https://clinicaltrials.gov> ID#NCT01794182, NCT01454154). These studies, if successful, will clearly emphasize the role of inflammasome activity in disease progression and an important therapeutic target.

One of the major limitations of glyburide as an anti-inflammatory compound is that it is effective only when delivered in high-doses, leading to side-effects of hypoglycemia. To address this problem, an intermediate substrate free of the cyclohexylurea moiety in the glyburide synthesis, was developed. Since cyclohexylurea is indispensable for insulin release, absence of this moiety results in a compound possessing the inhibitory activity against NLRP3 but without effects on insulin release. The compound (5-Chloro-2-methoxy-*N*-[2-(4-sulfamoylphenyl) ethyl] benzamide) successfully inhibits NLRP3 in cardiomyocytes and limits infarct size in experimental model of myocardial infarction, without affecting glucose metabolism (Marchetti et al. 2014). It is intriguing that insulin also suppresses IL-1 β expression as well as other cytokines together with inducing expression of PPAR γ , which exerts anti-inflammatory effects (Hyun et al. 2010).

VX-765: The prodrug VX-765 (Vertex Pharmaceuticals, Cambridge, MA) is an orally absorbed compound that is converted to an active metabolite, VRT-043198, by plasma and liver esterases. VRT-043198 is a small molecule and a selective caspase-1 inhibitor. Distinct structural features of caspase-1 have been utilized for designing potent reversible and irreversible inhibitors. The mode of action is based on incorporation of an aspartic acid residue into the active site of caspase-1, which mimics the substrate pro-IL-1 β (MacKenzie et al. 2010). As release of active IL-1 β is dependent on caspase-1 activation by the inflammasome complex, a caspase-1 inhibitor would be expected to lower IL-1 β levels. Indeed, VX-765 reduces the production of IL-1 β and IL-18 both in vitro and in vivo. It has no effect on either cell apoptosis or cell proliferation, when tested in human neuroblastoma cells. This property imparts beneficial effects of usage as an anti-inflammatory drug. In addition, it also reduces the severity of disease in models of rheumatoid arthritis and skin inflammation by controlling inflammatory cytokine production (Wannamaker et al. 2007).

Oral administration of VX-765 results in blockade of active IL-1 β . Chronic stress-induced depression is associated with over production of IL-1 β in murine models. VX-765 reduced serum and hippocampal levels of IL-1 β along with significantly improving depression-like behaviors induced by chronic mild stress (Zhang et al. 2015). VX-765 is currently in clinical trials to assess the reduction in seizure frequency in patients with epilepsy (<https://clinicaltrials.gov> ID#NCT01501383), based on studies showing reduction of

seizures in animals following IL-1 β inhibition by VX-765 (Maroso et al. 2011).

Parthenolide: Parthenolide is a plant lactone extract and has been extensively used as an herbal remedy for treatment of various inflammatory diseases including fever, arthritis and psoriasis. It inhibits the NF- κ B pathway, but in addition, parthenolide is also an inflammasome inhibitor. It acts by directly inhibiting both the protease activity of caspase-1 and ATPase activity of NLRP3. Compared to the NF- κ B inhibitor, BAY 11-7082, which specifically inhibits NLRP3, parthenolide can inhibit multiple inflammasomes including NLRC4-induced caspase-1 activity (Juliana et al. 2010). In a rat model of stroke, parthenolide treatment reduced blood-brain barrier permeability and infarct size along with suppression of caspase-1 expression (Dong et al. 2013).

Cytokine Release Inhibitory Drugs (CRIDs): These are class of sulfonylurea containing compounds that inhibit post-translational processing of IL-1 β . CRID3, a small molecule inhibitor of both NLRP3 and AIM2, was developed which inhibits caspase-1 activation and IL-1 β secretion in response to stimulation of NLRP3 and AIM2 but not NLRC4. CRID3 also prevents AIM2-dependent pyroptosis, as compared to the NLRP3 inhibitors glyburide and parthenolide, which do not inhibit AIM2 activation. CRID3 exerts its effect by inhibiting the formation of ASC complexes, in response to NLRP3 and AIM2 activation (Coll et al. 2011).

Other Inhibitors: Small molecule inhibitors are being developed to improve safety, efficacy and mode of delivery for an effective potential therapy in humans. For example, MCC950 is a potent and selective inhibitor of NLRP3, but not AIM2, NLRC4 or NLRP1 inflammasomes. It suppresses IL-1 β production in vivo, in conjunction with reduction in severity of EAE (Coll et al. 2015). It might be effective in MS, arthritis and other inflammatory disorders because it is readily absorbed from the gut and can potentially cross the blood-brain barrier.

In addition several inflammasomes (e.g. NLRP1, NLRP3 and NLRC4) can be inhibited by arsenical compounds including sodium arsenite and arsenic trioxide. These compounds have been shown to inhibit inflammasome-dependent pyroptosis by modulating cellular environment, which indirectly inactivates caspase-1. Arsenic trioxide is used to treat hematological cancers, and therefore, has a clinical range of efficacy (Maier et al. 2014).

8.7 Review Questions

1. What are inflammasomes? How were they discovered?
2. Describe the role of ASC in inflammasome activation. How does the inflammasome assembly work in the absence of ASC?

3. How is anakinra used as an anti-inflammatory drug? How is it different from small molecule inflammasome inhibitors such as glyburide and VX-765?
4. The activation and assembly of NLRP3 inflammasome in the cytoplasm results in which of the following:
 - (a) pro-IL-1 β is degraded.
 - (b) pro-IL-1 β is synthesized.
 - (c) *pro-caspase-1 is cleaved into active caspase-1, which further cleaves pro-IL-1 β into active IL-1 β .*
 - (d) Blockade of IL-1 receptor and thus reducing the effects of IL-1 β secretion.
5. One of the major drivers of inflammasome activation during ischemic stroke is:
 - (a) IL-1 β and IL-18
 - (b) Lower cytosolic ATP
 - (c) Cytosolic high Ca²⁺ and reduced K⁺ concentration
 - (d) All of the above
 - (e) *b and c*
6. Pyroptosis is characterized by:
 - (a) Programmed cell death mediated by caspase-1
 - (b) Programmed cell death mediated by caspase-4
 - (c) Rupture of plasma membrane with release of intracellular contents
 - (d) *a and b*
 - (e) *b and c*

8.8 Answers

1. Inflammasomes are oligomeric multiprotein complexes that assemble in the cytoplasm in response to danger- or pathogen-associated molecular patterns (DAMPs or PAMPs). Inflammasome assembly and activation leads to caspase-dependent activation of interleukin-1 β (IL-1 β) and IL-18, and pyroptotic cell death. Inflammasomes were discovered during identification of underlying mechanisms of periodic fever syndromes. Inflammasomes were first described in 2002, as caspase-activating complexes. Researchers discovered mutations in NLR family genes, while studying patients who were exquisitely sensitive to cold. The intracellular protein implicated in the phenomenon was named 'cryopyrin' since patients developed fever when exposed to cold. Later, inflammasomes were identified as intracellular molecular platforms and key elements in innate immunity.
2. The inflammasome assembly comprises of a sensor component, an adaptor component and an effector component. The apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) is the most common adaptor protein for most of the inflammasomes (e.g. NLRP1, NLRP3 etc). Following stimulation, sensor component oligomerizes resulting in formation of clusters of N-terminal

pyrin domain (PYD). This is followed by recruitment of monomeric ASC, which in turn serves as a platform for the caspase recruitment domain (CARD) to cluster and recruit the caspase-1 filament by a homotypic CARD/CARD interaction. Thus, ASC acts as a core protein that acts as a bridge between stimulation of inflammasome and its activation. However, few other inflammasomes (e.g. NLRC4) do not require ASC. This function is mediated by CARD/CARD interactions. The CARD domain of sensor component directly nucleates caspase-1 for activation.

3. Pro-inflammatory cytokine IL-1 β , one of the major products of inflammasome activation, is up-regulated and implicated in many acute and chronic neurological diseases. Anakinra is a recombinant IL-1 receptor antagonist that blocks the activity of both IL-1 α and IL-1 β and was approved for use as a drug by the U.S. Food and Drug Administration (FDA) in 2001. Because of advantages like safety and efficacy, it has been used in treatment of broad range of diseases including rheumatoid arthritis and Type 2 diabetes. Glyburide acts upstream of NLRP3 and inhibits inflammasome assembly. VX-765 inhibits caspase-1 activation which results in reduced IL-1 β production. Therefore, the important difference between anakinra and small molecule inflammasome inhibitors is the mechanism and the site of action.

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Abstract

Leucine rich repeat kinase 2 (LRRK2) is a large protein with a GTPase and kinase domain and several protein-interacting domains. Mutations in the LRRK2 gene, specifically the enzymatic domains, are one of the most common causes of autosomal dominant Parkinson's disease (PD). LRRK2 has been reported as a regulator of many cellular pathways including inflammatory signaling, cytoskeletal maintenance, and autophagy. LRRK2 expression is enriched in cells of the immune system (CD4+ and CD8+ T cells, CD14+ monocytes, and CD19+ B cells), but its function in the immune system and its effects on age-related immunosenescence are as yet unknown. Mutations or polymorphisms in the LRRK2 gene are also associated with several other immunological diseases, including Crohn's disease and increased risk for leprosy infection, indicating that LRRK2 plays an important role in proper immune function. Inhibition of the LRRK2 kinase activity has been proposed as a potential new therapeutic for PD, but specificity and blood brain barrier permeability of the compounds still remain a problem. With widespread expression in both the immune and nervous systems, LRRK2 remains an interesting, yet challenging, pharmacological target. Through the development of more specific LRRK2 kinase inhibitors, the possibility of a disease-modifying therapy for a progressive condition that hasn't seen a new treatment in over 60 years remains a bright spot on the horizon.

Keywords

Immune cell • Immunity • Inflammation • Kinase inhibition • Leucine-rich repeat kinase 2

9.1 Introduction

Parkinson's disease (PD) is a progressive age-related movement disorder characterized pathologically by degeneration in dopaminergic neurons of the substantia nigra and formation of neuronal inclusions of aggregated α -synuclein called Lewy bodies. Although the disease has been extensively studied for decades, the etiology of the disease remains unclear and the development of new treatments and therapeutics has been slow-paced. Mutations in the leucine-rich

repeat kinase 2 (LRRK2) protein were identified in 2004 as a potential marker for PD (Paisan-Ruiz et al. 2004; Zimprich et al. 2004). The identification of the link between LRRK2 mutations and PD led to an exciting new pathway for researchers to pursue. Because clinical presentation of LRRK2-associated PD and idiopathic PD is very similar (Aasly et al. 2005; Thaler et al. 2009; Haugarvoll et al. 2008; Kumari and Tan 2009), the study of LRRK2 function is likely to give insight into sporadic PD etiology. Up to this point, studies involving LRRK2 and its associated mutations have focused on their effects on neuronal function. Within the past few years, it has become clear that increased inflammation, both in the brain and the periphery, is associated with the pathology of the disease (Russo et al. 2014; Pradhan and Andreasson 2013; Neumann et al. 2009; McGeer et al.

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1988; Whitton 2007). The enriched expression of LRRK2 in both innate and adaptive immune cells and its designation as a member of the immune-regulating receptor-interacting protein kinase (RIPK) family (Meylan and Tschopp 2005; Zhang et al. 2010) places LRRK2 in a unique position as a potential regulator of inflammatory and immune responses that influences risk for age-related degeneration.

9.2 LRRK2 Structure and Function

In 2002, the *PARK8* locus on chromosome 12 was identified in a study involving a large Japanese family with many generations affected with PD (Funayama et al. 2002). Two years later, two labs independently cloned and identified mutations in the *PARK8* gene encoding LRRK2, as causes for dominantly inherited PD (Paisan-Ruiz et al. 2004; Zimprich et al. 2004). LRRK2, also known as dardarin (from the Basque word ‘dardara’, for tremor), is a large protein, containing 51 exons and 2527 amino acids (~286 kDa), with several different functional and protein-interacting domains (Mata et al. 2006; Funayama et al. 2002). Enzymatic domains include a ROC (Ras of complex) GTPase domain (Guo et al. 2007) and a serine/threonine kinase domain (Gilsbach et al. 2012). Several protein-interacting domains including a leucine-rich repeat (LRR) domain, a C-terminal WD40 repeat domain, and armadillo and ankyrin repeat domains also comprise the LRRK2 protein.

The kinase domain is a serine/threonine kinase whose physiological substrate has not been definitively established; however, potential substrates include autophosphorylation sites, ERM (ezrin/radixin/moesin) proteins, mitogen activated protein kinase (MAPK), eukaryotic initiation factor 4E (eIF4E)-binding protein (4E-BP), and 14-3-3 proteins (Drolet et al. 2011). There have been over 20 autophosphorylation sites found on LRRK2 including both serine and threonine residues (Kamikawaji et al. 2009; Li et al. 2010; Greggio et al. 2009; Gloeckner et al. 2006; Webber et al. 2011). There is currently much debate over which sites are “bona fide” autophosphorylation sites; however, the primary candidate sites cluster to the GTPase domain-binding pocket as opposed to the kinase domain (Webber et al. 2011; Greggio et al. 2009; Gloeckner et al. 2006). Most kinases have a conserved DFG (aspartic acid, phenylalanine, and glycine) motif at the hinge region of the kinase activation loop, but LRRK2 has a DYG (aspartic acid, tyrosine, and glycine) motif. Although the significance of this alteration has not been determined, it could contribute to unique structural differences in the kinase domain. The most common LRRK2 mutation G2019S occurs in the glycine of this loop, giving mutant LRRK2 a DFS (aspartic acid, phenylalanine, and serine) loop potentially altering protein dynamics and flexibility (Drolet et al. 2011).

LRRK2 is part of the Roco family of GTPases with a ROC-COR domain: a ROC domain with a C-terminal of Roc (COR) domain directly following it (Bosgraaf and Van Haastert 2003). LRRK2 GTPase function has been confirmed and GTP binding is essential for the regulation of LRRK2 kinase activity (Biosa et al. 2013). There are currently two theories about the GTPase function and its regulatory role. The first involves the GTP-dependent formation of LRRK2 dimers composing the active state. Consistent with this idea, the G2019S mutation appears to increase the amount of LRRK2 in dimer form (Sen et al. 2009). The second theory proposes that LRRK2 acts as Ras-GTPase with an “on-off” switch where GTP binding activates the protein and hydrolysis to GDP inactivates terminating signaling. This model requires tight regulation via GEFs (guanine nucleotide exchange factor) to promote GDP release and new GTP binding thereby activating the protein and GAPs (GTPase activating protein) to promote GTP hydrolysis to GDP turning the protein off. In support of this model, a putative GEF (Haebig et al. 2010) and GAP (Xiong et al. 2012; Stafa et al. 2012) have been proposed although they may not be specific to LRRK2 (Gómez-Suaga et al. 2014). Further studies are needed to parse out the activating mechanism of the GTPase.

LRRK2, but not LRRK1 (a LRRK2 paralogue similar to, but distinctly different from LRRK2) can interact with 14-3-3 proteins (Reyniers et al. 2014). This interaction is mediated by phosphorylation at the Ser⁹¹⁰ and Ser⁹³⁵ sites (Nichols et al. 2010). Pathogenic LRRK2 mutations display reduced phosphorylation at these sites, disrupting 14-3-3 protein binding and leading to accumulation of LRRK2 in the cytoplasm in pools that resemble inclusion bodies (Nichols et al. 2010). It is worth noting that phosphorylation at these two serine sites does not control kinase activity. Inhibiting LRRK2 kinase activity via H-1152 and sunitinib in HEK-293 cells recapitulates the mutant phenotype by decreasing phosphorylation at Ser⁹¹⁰ and Ser⁹³⁵ and disrupting 14-3-3 binding resulting in LRRK2 inclusion bodies (Dzamko et al. 2010). Results from these studies indicate that LRRK2 does not directly autophosphorylate at these sites, but somehow indirectly regulates phosphorylation of these serine residues either by activating a nearby kinase or inhibiting phosphatase activity (Dzamko et al. 2010).

LRRK2 has been linked to regulation of autophagy, neurite outgrowth, vesicular trafficking and cytoskeletal components (Plowey et al. 2008; Gómez-Suaga et al. 2012; Tong et al. 2012; Ramonet et al. 2011). An interesting new model proposes that LRRK2 regulates clearance of vesicles derived from the trans-Golgi network in an autophagy-dependent manner (Beilina et al. 2014). This function is regulated by the phosphorylation of LRRK2 by casein kinase 1 α which promotes recruitment of LRRK2 to the trans-Golgi membrane (Chia et al. 2014). Interaction with the GEF,

ARHGEF7, mediates these effects which is consistent with the ideas stated above, where GEFs mediate GTPase activity which in turn regulate kinase activity.

Given the multiple and highly diverse protein interacting domains, it is not surprising that LRRK2 has also been identified to be involved in the Wnt signaling pathway (Berwick and Harvey 2012, 2014). The Wnt signaling pathway has many cell regulatory functions, both in immune cells discussed further below and in neuronal cells (Staal et al. 2008). LRRK2 interacts with the disheveled family of proteins that are essential to Wnt signaling cascade via the ROC-Cor domain. Experiments also showed that this interaction stabilizes LRRK2 protein expression (Sancho et al. 2009). LRRK2 has also been associated with MAPK signaling (Yang et al. 2013; White et al. 2007) and the transcription factors NFkB (Gardet et al. 2010; Hongge et al. 2014) and nuclear factor of activated T cells (NFAT) (Liu et al. 2011), detailed below. With multiple enzymatic and protein interacting domains, it has been difficult to determine the specific physiologic function of LRRK2 and its relation to PD etiology.

9.3 LRRK2 Expression

LRRK2 expression is ubiquitous. It is expressed throughout brain tissue, including human cortex cerebri, hippocampus, and striatum (Westerlund et al. 2008). Cell specific expression in the brain includes astrocytes, microglia (Miklossy et al. 2006), and neurons (Taymans et al. 2006). Expression in peripheral tissues such as lung, heart, kidney, and immune organs such as thymus, spleen, and lymph nodes has also been reported (Paisan-Ruiz et al. 2004; Zimprich et al. 2004; Miklossy et al. 2006; Westerlund et al. 2008). Full length LRRK2 has been detected via western blot in monocytes, dendritic cells, microglia, B cells and T cells (Hakimi et al. 2011; Gardet et al. 2010). Reports of expression in CD4+ and CD8+ T cells have been inconsistent and contradictory depending on the assay (western blot, real time PCR, and flow cytometry) and reagents used (antibodies, primers). Although it has been reported that LRRK2 mRNA is close to undetectable in CD4+ and CD8+ T cells (Thevenet et al. 2011), the authors have reproducibly shown protein expression via the more reliable flow cytometric analysis and can say with confidence that LRRK2 is expressed in both circulating CD4+ and CD8+ T cells (Tansey et al. 2014). Protein expression is highest in CD14+ monocytes, with an intermediate level in CD19+ B cells and the lowest expression in CD4+ and CD8+ T cells. Most recently, expression has been shown in endothelial cells (Hongge et al. 2014).

In neurons, endothelial cells, and immune cells, LRRK2 localizes mostly to the cytoplasm, with some enrichment at mitochondrial membranes, and presence at cellular mem-

branes following stimulation (West et al. 2005; Schapansky et al. 2014; Berger et al. 2010). Recruitment of LRRK2 to the membrane results in the formation of LRRK2 dimers that have increased kinase activity compared to the monomeric form (Berger et al. 2010). Consistent with previous reports, this increased kinase activity is associated with increased GTP binding (Guo et al. 2007; Berger et al. 2010). Membrane-associated LRRK2 also has decreased levels of phosphorylation compared to cytosolic LRRK2 (Berger et al. 2010). More recently, Schapansky et al. provided evidence suggesting that membrane recruitment of LRRK2 in macrophages is an essential step necessary for the regulatory role of LRRK2 in autophagy (Schapansky et al. 2014).

Although the focus of this chapter is on immune cells, the reader is directed to reviews that highlight the role of LRRK2 in neuronal cells and its function in the brain. Gomez-Suaga et al. writes an in depth review of the neurobiology of LRRK2 and its role in autophagy and synaptic alterations (Gómez-Suaga et al. 2014), Blesa et al. discusses dopaminergic cell vulnerability in the context of LRRK2 animal models of PD (Blesa and Przedborski 2014), Martin et al. provides an overview of LRRK2 pathobiology and neuronal dysfunction (Martin et al. 2014), and Rudenko et al. details the direct toxic effect LRRK2 can have on neurons in addition to the shortening of neurite outgrowths associated with mutations (Rudenko and Cookson 2014).

9.4 LRRK2 Mutations, Polymorphisms, and Disease

There are multiple mutations and normal genetic variations in the LRRK2 locus that have been identified and associated with disease (Brice 2005); however, six of these are known to be the most common pathogenic mutations in PD (Rudenko and Cookson 2014). Four of these, R1441G, R1441C, R1441H, and Y1699C, are located in the ROC-COR GTPase domain, and two, G2019S and I2020T are in the kinase domain (Rideout and Stefanis 2014; Greggio and Cookson 2009). Mutations in LRRK2 account for 1–2 % of all PD cases, but are particularly prevalent in the Ashkenazi Jewish population (40 %) and North African Arab population (39 %) (Thaler et al. 2009). The mutation penetrance ranges from 28 % at 54 years of age to 74 % at 79 years of age. In terms of clinical presentation and pathology, LRRK2-associated PD most closely resembles sporadic PD, making it an important model to study the underlying cause of sporadic PD (Aasly et al. 2005; Thaler et al. 2009; Haugarvoll et al. 2008; Kumari and Tan 2009). Because LRRK2 mutations generally cause late-onset PD, LRRK2 is thought to be a susceptibility gene that increases an individual's risk with age.

The R1441G and R1441C mutations, located in the ROC GTPase domain, result in decreased GTPase activity (Guo et al. 2007; Lewis et al. 2007). These mutations also exhibit neuronal toxicity. In an R1441C knock-in mouse model, although there was no dopaminergic neuron loss, the mice had impaired dopaminergic neurotransmission and D2 dopamine receptor dysfunction (Tong et al. 2009). Another mutation, Y1699C is in the COR domain and also results in a decrease in GTPase activity (Gotthardt et al. 2008). This mutation also disrupts Wnt signaling by weakening interactions between LRRK2 and disheveled (DVL) proteins while the R1441G and R1441C mutations strengthen the interaction (Sancho et al. 2009).

The most abundant mutation, G2019S, is located in the kinase domain and results in enhanced kinase activity (West et al. 2005; Greggio et al. 2006) due to stabilization of the kinase activation loop (Gilsbach and Kortholt 2014). Neuronal cell death induced by mutations in LRRK2 has been shown to be kinase dependent (Greggio et al. 2006; Smith et al. 2006). The kinase domain has homology with mitogen activated kinase kinase kinase (MAPKKK) proteins, a series of proteins essential to the MAPK signaling pathway. MAPK signaling is a pathway that begins at the membrane and ultimately results in activation of transcription factors that affect gene expression (Yang et al. 2013). Studies addressing signal transduction of the MAPK pathway in leukocytes found decreased phosphorylation of Src, HSP27, and JNK in both patients with G2019S PD and idiopathic PD compared to healthy controls (White et al. 2007). Although not directly correlated with LRRK2 kinase activity, this indicates a potential dysregulation of MAPK signaling that could contribute to the development of disease regardless of mutation. The similar pathology seen in both the G2019S PD and idiopathic PD is consistent with the clinical features of G2019S PD closely resembling those of idiopathic PD (Aasly et al. 2005; Thaler et al. 2009; Haugarvoll et al. 2008; Kumari and Tan 2009), reinforcing the notion that elucidation of the molecular mechanism underlying the dominantly inherited LRRK2 mutations will shed light on the causes of idiopathic PD.

In a study evaluating both protein and mRNA expression patterns in idiopathic PD and G2019S patients, it was found that there are no differences in neuronal mRNA distribution (using in situ hybridization) between idiopathic PD and G2019S subjects, with widespread neuronal LRRK2 mRNA expression seen in the neocortical regions, brainstem, locus coeruleus, hippocampus, and dopaminergic neurons of the substantia nigra. Consistent expression was also seen in vascular smooth muscle cells. Using qPCR, the authors discovered that total mRNA expression was decreased in idiopathic PD cases in the parietal cortex, cerebellum, amygdala, frontal cortex, and cingulate gyrus; however, there were no alterations in protein levels (by western blot analyses) between subjects (Sharma et al. 2011).

The I2020T mutation in LRRK2 is also located in the kinase domain, but its effect on kinase activity remains controversial. There are multiple studies reporting increased kinase activity (Gloeckner et al. 2006; West et al. 2007; Imai et al. 2008), one decreased activity (Jaleel et al. 2007), and several reports of no change (Anand et al. 2009; Luzón-Toro et al. 2007; Greggio and Cookson 2009). More recently, it has been reported that I2020T does increase kinase activity via stabilization of the kinase activation loop, similar to the mechanism of the G2019S mutation (Ray et al. 2014). The development of improved and consistent kinase assays for LRRK2 is needed to fully understand how these mutations alter enzymatic activity.

In addition to mutations, single nucleotide polymorphisms (SNPs) in LRRK2 have also been associated with disease risk. In 2008, SNPs in LRRK2 were identified as a susceptibility factor for the chronic inflammatory condition Crohn's Disease by a meta-genome wide association study (GWAS) (Barrett et al. 2008). Liu et al. identified a potential mechanism for LRRK2 in Crohn's disease via negative regulation of the (NFAT). Mice deficient in LRRK2 show increased translocation of NFAT into the nucleus and NFAT activity. LRRK2 knock-out mice also had exacerbated colitis consistent with a role for LRRK2 as a negative regulator important in controlling inflammatory bowel disease (Liu et al. 2011). LRRK2 SNPs have also been associated with susceptibility to leprosy in the Han Chinese population (Zhang et al. 2009; Wang et al. 2015). There is a protective variant of LRRK2 (M2397T) that appears to increase NFAT activity in HEK293 cells following LPS stimulation, indicating that increasing the immune response is a necessary mechanism to control *M. leprae* infection. Through the study of myriad mutations and SNPs, LRRK2 has been identified as a regulator of MAPK signaling, a negative regulator of NFAT, and thus, we have gained insight into the kinase domain and its activity. With this new and exciting information, the link between LRRK2 and immune regulation becomes stronger.

9.5 LRRK2 Regulation of the Immune System

The immune system is a highly complex and tightly regulated system responsible for protecting the body against infection, cancer, and other cellular damage. When an immune cell recognizes a foreign antigen the cell enters an activated state; a series of signaling cascades (NFAT, NFkB, Wnt, etc.) begins that results in the upregulation of pro-inflammatory cytokines, adhesion molecules, and further activation and differentiation of innate and adaptive immune cells. This pro-inflammatory state results in altered cell trafficking, differentiation, and mRNA and protein expression,

all of which are part of an orchestrated response aimed at elimination or neutralization of the invading pathogen.

LRRK2 is a member of the receptor interacting protein (RIP) kinase family, which are proteins that detect and respond to cellular stress by regulating cell death and activation of the immune system (Meylan and Tschopp 2005; Zhang et al. 2010). In various cell types, LRRK2 expression has been reported to increase in response to the pro-inflammatory signals IFN- γ (Thevenet et al. 2011; Gardet et al. 2010; Kuss et al. 2014), LPS (Hakimi et al. 2011; Moehle et al. 2012), and IL-1 β (Hongge et al. 2014). Increases after IFN- γ stimulation have been observed in CD14+ macrophages, CD3+ T cells, and CD19+ B cells (Thevenet et al. 2011; Gardet et al. 2010; Kuss et al. 2014). Also, IFN- γ stimulation was shown to increase LRRK2 mRNA and protein expression specifically in the non-classical CD14+CD16+ monocyte population (Thevenet et al. 2011). Inhibition of LRRK2 with multiple inhibitors results in decreased CD14, CD16, and MHC-II expression indicating that LRRK2 is playing a significant role in the activation of monocytes via IFN- γ (Thevenet et al. 2011). Recently, it was reported that increased expression of LRRK2 in monocytes following IFN- γ stimulation is via extracellular signal-related kinase 5 (ERK5) signaling (Kuss et al. 2014).

G2019S LRRK2 has also been linked to increased phosphorylation of ERK1/2 and dysregulation of basal autophagy through this pathway (Bravo-San Pedro et al. 2013; Reinhardt et al. 2013). LRRK2 mRNA levels increased following LPS stimulation of mouse bone marrow derived macrophages (BMDMs) (Hakimi et al. 2011). In addition, LRRK2 protein levels in these BMDMs increased following transduction of lentiviral particles (Hakimi et al. 2011). Following cranial injection of LPS, microglia also display increased expression of LRRK2 and increased kinase activity (Moehle et al. 2012). Interestingly, despite strong up-regulation of LRRK2 protein with LPS stimulation, no changes in LRRK2 mRNA levels were seen (Moehle et al. 2012). LRRK2 is also increased in the gut lamina propria macrophages, B cells, and dendritic cells of patients with Crohn's disease who experience chronic inflammation in the gut (Gardet et al. 2010). Increased LRRK2 expression after inflammatory stimulus dependent on ERK signaling implicates LRRK2 as key player in regulation of inflammatory signaling pathways.

In human umbilical vein endothelial cells (HUVECs), LRRK2 expression is increased following stimulation with IL-1 β (Hongge et al. 2014). In addition, IL-1 β also leads to increased induction of VCAM-1, an adhesion molecule important for immune cell trafficking, in HUVECs overexpressing both WT and G2019S LRRK2. This increased VCAM-1 induction appears to be kinase dependent, as the kinase dead LRRK2 mutant, K1347A, does not recapitulate this phenotype (Hongge et al. 2014). Overexpression of WT

LRRK2 and expression of the G2019S mutant led to increased phosphorylation of I κ B α allowing for increased translocation of NF κ B into the nucleus (Hongge et al. 2014). NF κ B is the transcription factor that controls expression of pro-inflammatory cytokines, chemokines, and adhesion molecules. In this way, LRRK2 levels or activity may be critical in modulating NF κ B-dependent responses in immune cells.

In support of this, the LRRK2 knockout rat was reported to be protected from LPS and α -synuclein induced neurodegeneration. There are also fewer proinflammatory myeloid cells in the brains of these rats indicating that inhibition of LRRK2 could decrease the neuroinflammation and cell loss seen in PD (Daher et al. 2014). Furthermore, there are altered immune cell frequencies detected in the spleen of these LRRK2 knockout rats (Ness et al. 2013). Under normal resting conditions, the LRRK2 knockout rat spleens have higher percentages of CD4, CD3, and CD11b positive cells, but significantly lower B cells (Ness et al. 2013). When infected with a rat-adapted influenza virus, the LRRK2 knockout rats showed a decreased percentage of CD11b+ cells, but an increase in the percentage of CD3+, CD4+, and CD8+ cells compared to their wild-type counterparts (Ness et al. 2013). Although these LRRK2 knockout animals have little-to-no PD-like pathology, they do exhibit significant pathology in the kidneys with accumulation of α -synuclein and ubiquitinated proteins, severe dysfunction in the autophagic-lysosomal pathways, and increased inflammatory and oxidative stress damage (Tong and Shen 2012; Ness et al. 2013).

In addition to kidney pathology, LRRK2 knockout rats and mice also have lung pathology with enlarged lysosome-related storage organelles called lamellar bodies (LB) in type II alveolar lung epithelial cells (Herzig et al. 2011; Baptista et al. 2013; Miklavc et al. 2014). LBs are important for storage of surfactant, a compound necessary to maintain proper surface tension and equilibrium in the lung. After ATP treatment, LRRK2 knockout rats showed increased calcium-dependent exocytosis of LBs indicating that LRRK2 plays a modulatory role in calcium signaling and exocytic pathways (Miklavc et al. 2014). With the lung being a barrier site in the immune system, the apparent homeostatic role of LRRK2 in lung function is another indication that LRRK2 is necessary for proper immune function and control.

In a study using mice overexpressing LRRK2 with the R1441G mutation, microglia stimulated with LPS had increased expression and secretion of proinflammatory cytokines compared to wild type LRRK2 microglia (Gillardon et al. 2012). Expression of LRRK2 protein was also significantly increased in both wild type and R1441G microglia following stimulation with LPS or IFN- γ (Gillardon et al. 2012). Conditioned media from the LRRK2 R1441G mutant stimulated microglia was then added to primary neuronal cultures and resulted in an increase in neuronal death compared to the conditioned media from wild type LRRK2

stimulated microglia (Gillardon et al. 2012). In an *in vitro* kinase assay, R1441C mutations lead to increased kinase activity and a decreased rate of GTP hydrolysis (Guo et al. 2007). Additionally, there is evidence supporting the idea that GTP binding stimulates LRRK2 kinase activity, indicating the potential role of LRRK2 in integrating cellular signaling pathways (Guo et al. 2007).

In a human dermal fibroblast model, a LRRK2 dependent increase in COX-2 RNA, but not protein levels, was observed in subjects with G2019S PD, R1441G PD and idiopathic PD compared to age-matched controls (Lopez de Maturana et al. 2014). Knockdown of LRRK2 also led to blunted COX-2 responses after LPS stimulation (Lopez de Maturana et al. 2014). Following stimulation with LPS, fibroblasts from subjects with idiopathic PD and subjects with LRRK2 R1441G mutations showed blunted responses as evidenced by diminished IL-6 and TNF- α RNA levels (Lopez de Maturana et al. 2014). Consistent with previous reports, in these fibroblasts LRRK2 RNA levels increased following IFN- γ stimulation, but were shown to decrease with LPS stimulation (Lopez de Maturana et al. 2014). Following LPS stimulation, in R1441G and iPD fibroblasts, NF κ B transcriptional activity was attenuated, consistent with a modulatory role of LRRK2 in NF κ B activation (Lopez de Maturana et al. 2014). Taken together, these studies implicate LRRK2 as a key regulatory protein in inflammation. These types of studies remain unreplicated in immune cells, but studies of this kind would shed light on the physiological role of LRRK2 in immune cells should the regulation turn out to be similar in nature.

9.6 LRRK2 Kinase/Small Molecule Inhibitors

Because many pathogenic LRRK2 mutations result in enhanced kinase activity, a proposed logical next step in PD therapeutics has been to develop inhibitors to dampen this increased activity. Because the sequence similarities between kinases are high, it is difficult to develop an inhibitor specific to the LRRK2 kinase domain; however, there are a few significant amino acid differences in LRRK2 that can be exploited. The DFG hinge motif in the activation loop of most kinases is DYG in LRRK2 and DYS in the G2019S mutation (West 2014; Peng et al. 2013). Despite this unique sequence, highly selective LRRK2 inhibitors remain elusive. What follows is a brief summary of important experiments using inhibitors to investigate LRRK2 function. The reader is directed to a review of LRRK2 inhibitors, their efficacy and specificity by Kramer et al. (Kramer et al. 2012) and a review of patents by Kethiri et al. (Kethiri and Bakthavatchalam 2014) for additional detail.

LRRK2-IN-1 is currently the most popular and widely used small molecule inhibitor of LRRK2; however its use has significant caveats. It does not cross the blood brain bar-

rier limiting its utility as a therapy for neurodegeneration. It also potentially inhibits ERK5, another ubiquitous kinase that regulates cell signaling, limiting its use both in research and the clinic (Deng et al. 2011). In a mouse microglial culture, treatment with LRRK2 inhibitors LRRK2-IN-1 and sunitinib decreased p38 MAPK phosphorylation and iNOS induction, two molecules important for the TNF release pathway (Moehle et al. 2012). Although, this data suggests that inhibition of LRRK2 could dampen a damaging inflammatory response in the brain, LRRK2-IN-1 was also shown to inhibit TNF and CXCL10 in LPS-stimulated astrocytes equally well in both LRRK2 wild-type and knock-out cultures, indicating the glaringly significant off-target effects of the inhibitor (Luerman et al. 2014).

LRRK2-IN-1 inhibits IFN- γ -dependent induction of LRRK2 in THP-1 differentiated macrophages and human monocyte-derived macrophages (Kuss et al. 2014). This inhibition is specific, as other LRRK2 kinase inhibitors do not prevent increased LRRK2 expression after stimulation (Kuss et al. 2014). Because of the cross-reactivity of LRRK2-IN-1 with extracellular signal-regulated kinase 5 (ERK5), IFN- γ -induced expression of LRRK2 was proposed and determined to be dependent upon ERK5 levels and activity (Kuss et al. 2014).

A promising new inhibitor, PF-06447475 was recently characterized and shown to be highly specific to LRRK2. Because it has just become available for commercial research, few experiments have been done, but *in vivo* studies from the initial characterization of the drug show high blood brain barrier (BBB) permeability and safety. Future studies will provide more accurate information on the kinase activity and specific function of LRRK2. Unfortunately, these studies indicate that the PK is too high to develop as a therapeutic in the clinic (Henderson et al. 2015).

The requirement for BBB permeability further complicates the use of LRRK2 inhibitors to treat neuronal dysfunction. As research supporting the regulatory role of LRRK2 in the peripheral immune system increases, BBB permeability may no longer be necessary for therapeutic use of inhibitors, slightly improving the ease of developing clinically relevant therapies. Currently, there are no clinical trials with LRRK2 inhibitors, however large cohorts of LRRK2 mutations carriers have been identified to expedite the process of future clinical trials (West 2014). With the current information about LRRK2 mutations, specifically G2019S, and the lack of disease-modifying therapies available, the case for LRRK2 inhibitors is strong.

9.7 Conclusion

Although the physiological roles of LRRK2 and the molecular mechanism and pathways that link it to PD pathogenesis have yet to be fully defined, it is an important kinase.

Elucidating LRRK2 function will give researchers a better understanding of the relationship between chronic inflammatory conditions and age-related neurodegenerative disease. Through the study of LRRK2 mutation carriers both with and without disease, we have gained better understanding of the multiple domains and the complex activities of this large protein in neuronal and non-neuronal cells. With widespread expression in both the immune and nervous systems, LRRK2 remains an interesting, yet challenging, pharmacological target. Through the development of more specific LRRK2 kinase inhibitors, the possibility of a disease-modifying therapy for a progressive condition that hasn't seen a new treatment in over 60 years remains bright on the horizon.

9.8 Review Questions

1. What is the role of the relationship between the GTPase and the kinase domains in LRRK2 in terms of kinase function?
2. What cell types have been shown to express LRRK2 protein?
3. What diseases have been associated with common genetic variants in LRRK2 or in mutations in LRRK2?
4. What signaling pathways has LRRK2 function been implicated in that may have a role in immune cell function?
5. How might LRRK2 kinase inhibitors be potentially beneficial even if they do not cross the blood-brain barrier?

9.9 Answers

1. The LRRK2 GTPase domain has been shown to function as a GTPase and GTP binding is essential for the regulation of LRRK2 kinase activity.
2. Full length LRRK2 has been detected via western blot in neurons, microglia, astrocytes, monocytes, dendritic cells, B cells and T cells as well as endothelial cells.
3. Common genetic variants or single nucleotide polymorphisms in LRRK2 have been associated with Crohn's disease. Mutations in LRRK2 in the GTPase and kinase domains cause dominantly inherited forms of Parkinson's disease. The R1441G, R1441C, R1441H, and Y1699C mutations are located in the ROC-COR GTPase domain, and the G2019S and I2020T mutations are in the kinase domain. Mutations in LRRK2 account for 1–2 % of all PD cases, but are particularly prevalent in the Ashkenazi Jewish population (40 %) and North African Arab population (39 %).
4. LRRK2 has been implicated in regulation of autophagy, lysosomal flux, NFAT, NFkB, and MAPK and Wnt sig-

naling, all of which play important regulatory roles in immune cell effector function.

5. LRRK2 kinase inhibitors may be beneficial in the periphery to treat the inflammatory phenotype that may be associated with LRRK2 dysfunction in immune cells in patients with PD.

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Abstract

Astrocytes being the most abundant cell types in the mammalian brain are intimately associated with a plethora of functions that are vital to central nervous system (CNS) physiology, including the development and maintenance of the vasculature scaffold and blood brain barrier, synaptogenesis, neurotransmission, and preservation of metabolic homeostasis. Astrocytes also play important roles in supporting the development and maintenance of central nervous system myelin. Accordingly, astrocytes have been directly associated with several neurological disorders including Alzheimer's disease (AD), Parkinson's disease, inflammatory demyelinating diseases, HIV-associated dementia (HAD), acute traumatic brain injury, and prion-associated spongiform encephalopathies. Other non-neuronal cell types such as, oligodendrocytes in the CNS and Schwann cells in the peripheral nervous system (PNS) are known to play an important role in the homeostasis of the nervous system. These cells wrap their plasma membranes around axons to organize myelinated nerve, thereby allowing rapid saltatory conduction. Oligodendrocytes and Schwann cells produce different type of protein, lipid and growth factors that promote neuronal survival, axonal growth and process formation.

Keywords

Astrocytes • Autoimmunity • Calcium excitability • Ceramide • CNPase • Gap junction • Glial activation • Glial fibrillary acidic protein • Glial scar • Myelination • MBP • MOG • Neurotrophins • PLP • Oligodendrocytes • Oxidative stress • Schwann cells • Vimentin

10.1 Introduction

The only difference between Albert Einstein's brain and others is the greater presence of healthy-looking and star-like astrocytes in the former. Therefore, astrocytes are not silent partners

of neurons providing only structural and metabolic support. These cells also regulate synapse formation, maturation, efficacy and plasticity. They ultimately control learning and memory and for Einstein his genius. It is now well accepted that astrocytes possess ion channels as well as G-protein coupled receptors necessary to sense and respond to neuronal activities. Similarly, myelination of neurons, oligodendrocytes, the other macroglia secrete growth factors to help neuronal growth and development and participate in cell-to-cell communication in more sophisticated way in humans than rodents, probably contributing to superior plasticity of the human brain. However, under disease conditions, astrogliosis occurs as a consequence of activation and cell proliferation. Activated astroglia also secrete proinflammatory molecules that affect neuronal loss and damage oligodendrocytes in neurodegenerative and

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neuroinflammatory demyelinating disorders. The present chapter discusses the biology and functional aspects of astrocytes, oligodendrocytes and myelinating Schwann cells ranging from their genesis to their immense role in maintaining peripheral and central nervous system (PNS and CNS) homeostasis and plasticity.

10.2 Astrocyte and Oligodendrocyte Development

The vertebrate nervous system including neurons, astrocytes, oligodendrocytes (OLs), and other cells originates from a flat sheet of neuroepithelial cells, constituent of the inner lining of neural plate along the dorsal surface of embryo (Fujita 2003). These neuroepithelial cells are the earliest precursors in the developing CNS. During embryonic development, radial glia cells derived from the neuroepithelium are the primary neural stem cells (NSCs) that produce neurons, astrocytes and OL throughout the brain (Kriegstein and Alvarez-Buylla 2009).

10.2.1 Generation of Glial Precursor Cells

During neurogenesis, neuroblasts are first derived from stem cells and then migrate peripherally to the mantle and marginal layers in the developing brain. After that, DNA synthesis in neurons is completely ceased and the progenitor cells enter into the phase of gliogenesis in the neural tube. These glioblasts eventually differentiate first into functional astrocytes and then OLs. Differentiation of cortical progenitor cells is being controlled by some transcription factors having basic-helix-loop-helix (bHLH) motifs. These are NeuroD, Neurogenin, Mash, Olig, Id, and Hes families of protein. The restricted and time-dependent binding of these transcription factors with corresponding DNA sequences present in the promoter of different developmental genes determines the outcome of final cell types.

10.2.2 Signaling Events Driving the Precursors to Astrocytes

Astrogenesis occurs toward after neurogenesis. Astrocyte development is regulated, in part, by cell-intrinsic pathways such as chromatin modifications. Extrinsic signals such as growth factors/cytokines affect the molecular mechanisms involved in astrocyte fates from neural progenitor cells (NPCs) to mature glia remain poorly understood.

One signaling pathways known operative in astrocyte growth and differentiation is Wnt signaling. It is required for critical activation of the proneural genes neurogenin (ngn) 1 and 2 in neural progenitor cells (NPCs). However, Wnt

ligands continue to be expressed in the nervous system even after the neuron to glia transition, during which time they fail to induce neurogenin expression in NPCs. Hirabayashi et al. found key intrinsic changes in histone H3 acetylation and trimethylation at the neurogenin 1 and 2 promoters in NPCs (Hirabayashi et al. 2009). Treatment with histone deacetylase (HDAC) inhibitors results in an increase in neurogenin expression in older, but not younger NPCs, which indicate that HDAC activity negatively regulates the expression of neurogenin in late NPCs. Consistent with a closed chromatin conformation at ngn loci, it was also observed that RNA polymerase II associated at relatively low rates with ngn promoters in late-stage NPCs. This epigenetic change in NPCs competence is mediated by the polycomb group complex (PcG). Knockout of key components of the complex prolongs the NPC neurogenic phase and delays the production of astrocytes. Thus despite the continued presence of Wnts, NPCs are able to make the neuron-to-glia switch in part because of intrinsic epigenetic changes that lead to PcG-dependent suppression of expression of the neurogens.

Activation of bone morphogenic protein 2/4 (BMP 2/4) signaling results in phosphorylation of SMAD transcription factors and their association with STAT3 to regulate expression of astrocyte-specific genes. It is also well established that the BMP and Notch pathways cross-talk with the JAK-STAT pathway and promote astrocyte differentiation. DNA methylation at astrocytic gene loci maintained by Dnmt1 is essential for antagonizing STAT signaling during the neurogenic phase, and the Notch-NF1A pathway has been shown to overcome this effect of Dnmt1 in inducing astrocyte differentiation. Other molecules unrelated to STAT signaling have also been implicated in the regulation of astrocyte differentiation, including Sox9, SCL, REST, SRF, KLF15, HMGN, MEK-Etv5, and NFE2L1. However, how these factors regulate astrocyte differentiation is not yet fully understood (Nagao et al. 2014).

Furthermore, β 1-integrin signaling is also becoming an important regulator of astrocytic differentiation. Conditional knockout of β 1-integrin enhances astrogliogenesis both by cultured ESCs and by subventricular zone (SVZ) progenitor cells. Integrin-linked kinase (ILK) is known to interact with the cytoplasmic domain of β 1 integrin. Recent study demonstrates an important role for β 1-ILK signaling pathway in regulating astrogliosis from ESCs and suggest ILK as a potential target for limiting glial scar formation after nervous system injury (Pan et al. 2014).

10.2.3 Signaling Events Driving the Precursors to Oligodendrocytes (OLs)

During mammalian development, oligodendrocyte precursor cells (OPCs) differentiate into OL, ultimately resulting into

myelination of axons. The OPCs in caudal as well as ventral neural tube originate under the influence of Shh protein secreted from ventral midline. At this initial stage, Shh patterns the ventral neuroepithelium by controlling the expression of a set of transcription factors PAX6, NKX 2.2, high mobility group protein SOX10, and basic helix-loop-helix proteins OLIG1 and OLIG2. These Olig genes and SOX10 are co-expressed in cells before the appearance of PDGF- α on OPCs. These PDGF-positive OPCs then proliferate and migrate away from the ventricular surface to all parts of the CNS before differentiating into functional myelin forming mature cells. Other transcription factors, including SOX9, SOX17 and Ying Ying, play a critical roles in OL specification.

In the CNS, astrocytes produce ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF). Both CNTF and LIF are capable of enhancing the generation of OL in cultures of dividing O-2A progenitors. CNTF and LIF also promoted OL maturation, as determined by expression of MBP, and could promote OL survival similar to insulin-like growth factor-1 or insulin (Mayer et al. 1994).

Activation of the Notch pathway is another important regulator of OL differentiation. It has been shown that the differentiation of OL is suppressed by the activation of the Notch signaling pathway (Yoon and Gaiano 2005). It is possible that aberrant expression of Notch ligand limits the amount of remyelination that occurs after injury.

10.3 Astrocytes: Biology and Function

In the middle of the nineteenth century, German anatomist and pathologist Rudolph Virchow was wondering about the group of cells in the brain that surround the neurons and fill the spaces between them. Dr. Virchow named these cells as “neuroglia” means “neural glue”. He used the term ‘glue’ to represent the gluing function of these cells to hold the neurons in place. Nowadays ‘neuroglia’ is collectively used for all glial cells in the CNS. Later on, due to “star-shaped” appearance, the major neuroglial cells were named as “Astrocytes” (Astra: star; cyte: cells).

10.3.1 Morphology and Markers

Morphologically, astrocytes are classified into two types: fibrous and protoplasmic astrocytes (Oberheim et al. 2012). Fibrous astrocytes are located predominantly in white matter and possess fewer but longer processes. These processes form cytoplasmic bundles of intermediate filaments (IFs). The major constituent of these are glial fibrillary acidic protein (GFAP). Under light microscope, the fibrous astrocytes look like a star-shaped cell body with finer processes.

Until recently, most studies used GFAP stains to characterize astrocyte morphology. This marker, however, reveals only the structure of primary branches, which represent a meager ~15 % of the total cell volume. Immunohistochemical staining with an aldehyde dehydrogenase 1 family member L1 (Aldh1L1)-specific polyclonal antibody divulges highly branched astrocytes morphology, including the astrocyte cell body and its extensive processes. In contrast, GFAP antibodies primarily label the thick main processes of some astrocytes. All cells positive for GFAP are also labeled with Aldh1L1, whereas Aldh1L1 strongly labels many more astrocytes (Cahoy et al. 2008).

The protoplasmic astrocytes, on the other hand, are found throughout all grey matter and generally express S100 β . They contain highly branched processes that form membranous sheets surrounding the neuronal processes, cell bodies and end-feet on capillaries. In contrast to fibrous astrocytes, these cells have fewer IFs and a greater density of organelles. Classical and modern neuroanatomical studies also indicate that both astrocytes subtypes make extensive contacts with blood vessels.

Apart from the ultrastructural study, astrocytes can also be identified on the basis of marker proteins (Table 10.1).

10.3.2 Heterogeneous Population of Astrocytes in the CNS

Astrocytes are amongst the most heterogeneous and versatile type of neuroglia. Astrocytes cover many types of cell such as, e.g., protoplasmic, fibrous, velate, perivascular and marginal astrocytes, radial glia of retina and cerebellum, Bergmann glia of the cerebellum, cribrosocytes at the optic nerve head, tanycytes and pituicytes of hypothalamus and neurohypophysis as well as ependymocytes and choroid plexus cells. Astrocytes are present throughout the CNS populating both gray and white matter (Verkhratsky et al. 2003). On the basis of morphology and antigenicity, astrocytes from optic nerve cultures were also designated as type 1 and type 2 (Raff et al. 1983). The type 1 astrocytes were originally defined as flat, polygonal cells that expressed GFAP but did not bind anti-ganglioside monoclonal antibodies A2B5 or R24 and LB1 except rat neural antigen 2 (Ran 2). These type 1 astrocytes proliferate well in the presence of epidermal growth factor and are found during gliogenesis in early developmental stage. On the other hand, type 2 astrocytes are found as GFAP⁺A2B5⁺ cells in rat optic nerve culture.

However, it is yet to be delineated whether these morphologically distinct heterogeneous populations of astrocytes are also different in their function or such morphological differences are merely intrinsic.

Table 10.1 Markers of astrocytes

Marker	Function	Cellular localization	Molecular weight
GFAP	Major constituent of intermediate filament found mostly in adult astrocytes	Cytoplasm	50 kDa (predicted)
EAAT1	Transport of amino acids	Cytoplasm	59.5 kDa
Glutamine synthase	In CNS, the enzyme found only in astrocytes; it catalyzes conversion of glutamate to glutamine	Cytoplasm	43 kDa
S-100 β	Ca-binding proteins	Cytoplasm/nucleus	21–24 kDa
Aldh1L1	Nucleotide biosynthesis and regeneration of methionine	Cytoplasm	99 kDa

GFAP glial fibrillary acidic protein, EAAT1, excitatory amino acid transporter, Aldh1L1 aldehyde dehydrogenase 1 family member L1

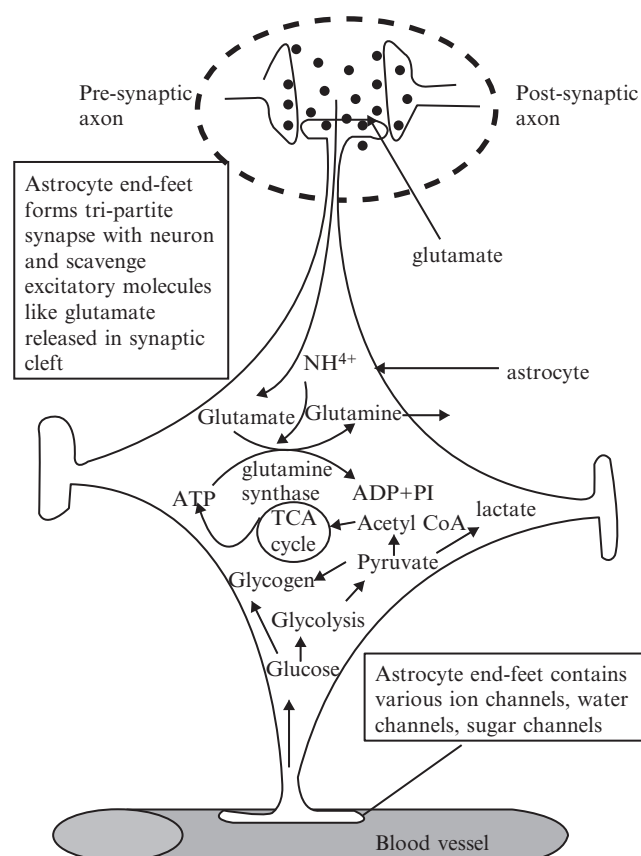


Fig. 10.1 Maintenance of CNS integrity by astrocytes. Astrocytes end-feet forms a network on blood capillary and regulate transfer of water, ions and sugars. The presynaptic position of astrocytes is critical for the uptake of excitotoxic glutamate from neuronal synapse

10.3.3 Physiological Role of Astrocytes in the CNS

10.3.3.1 Maintaining CNS Homeostasis

Providing Structural Support

Astrocytes are considered as structural support cells. The anatomy of brain microvasculature shows that astrocytic end feet constitute an envelope around blood vessels (Kacem et al. 1998). Astrocytic processes are positioned beneath the pial membrane and the ependymal surface and thereby

segregate the CNS parenchyma from external environment (Fig. 10.1). The cytoplasmic processes of astrocytes form a close network around the synaptic complex and maintain synaptic integrity (Newman 2003). Over the past 10 years, many compelling lines of evidence have shown that astrocytes powerfully control every stage of synapse formation, maturation and elimination to support the development and maintenance of neural circuits. The Barres laboratory identified the proteins involved as TSPs, a group of matricellular proteins that are expressed in astrocytes of the developing but not mature brain, and TSP1/2-deficient mice were shown to have reduced numbers of synapses in vivo (Molofsky et al. 2012)

Maintaining Water Balance

Water is essential to the CNS for formation and maintenance of cerebrospinal fluid. Water enters into the CNS either through diffusion due to difference in osmotic pressure or through some specified channels. Astrocytes, through membrane-bound transporter system, maintain water and ionic homeostasis in the brain. The co-transporter system like “sodium-glutamate co-transporter” (Na⁺-glutamate, EAAT1) and sodium-potassium-chloride ion co-transporter (Na⁺-K⁺-Cl⁻ co-transporter, NKCC) are located on astroglial membrane and regulate astrocytic water transport into the CNS (Fig. 10.1).

Apart from the co-transporters, astrocytic perivascular system in the brain involves membrane-bound water channels, called aquaporins. Among several members of the aquaporin family, aquaporin 1, 4 and 9 are expressed in astrocytes. Specifically, aquaporin 4 water channels are densely clustered along astrocyte processes that contact blood vessels and play a critical role in regulating fluid homeostasis in healthy CNS and play roles in vasogenic and cytotoxic edema (Nagelhus and Ottersen 2013). The aquaporins activities are regulated by transmembrane G-protein coupled receptor (GPCR) family of hormonal receptors and micro RNA-130a.

Maintaining Ion Homeostasis

Astrocytes are responsible in maintaining extracellular K⁺ ion concentration at a level compatible with neuronal function.

Astrocytes form a syncytium through which it efficiently redistributes K^+ from perineuronal to perivascular space. Such redistribution of K^+ is mediated by inwardly rectifying K^+ ion channels. One such K^+ ion channel Kir 4.1 is expressed in astrocytes surrounding neuronal synapses as well as blood vessels in the brain (Nagelhus et al. 2004). In addition to having K^+ channels, astrocytes bear plenty of other ion channels such as Na^+/K^+ ATPase, the K^+/Cl^- co-transporter KCC1 and $Na^+/K^+/Cl^-$ co-transporter NKCC1; function of many of these transporters is still under research. Similarly astrocytes regulate extracellular concentration of Cl^- through either Cl^- efflux via anion channels or Cl^- accumulation by NKCC1. Astrocyte cell surface bears an atypical sodium channel Nax that is assumed to be under voltage-gated sodium channel family. It has been suggested that glial cells bearing Nax channel are the first to sense a physiological increase in sodium level in body fluids (Watanabe et al. 2002).

Regulating Neurotransmitter and Amino Acid Levels

Astrocytes are active participants in the formation of tripartite synapse and modulate synaptic activity of neurons. Glutamate plays a central role in astrocytic-neuronal interactions. This excitatory amino acid released by neurons, is taken up by astrocytes from the neuronal synapses via their glutamate transporters. Astrocytes convert glutamate into glutamine and release into the synaptic cleft for being taken up by neurons (Hertz and Zielke 2004). Accumulated glutamine is converted to glutamate in glutamatergic neurons within the mitochondrial membrane, enters the mitochondrial matrix, and is returned to the cytoplasm in a pseudomaltate-aspartate shuttle". Astrocytes express several receptors linked to ion channels and second messenger pathways. Activation of receptors e.g. metabotropic glutamate receptor, in turn, elevates intracellular level of Ca^{2+} . Calcium-dependent glutamate release from astrocytes modulates the activity of both excitatory and inhibitory synapses (Fig. 10.1). Apart from glutamate, astrocytes uptake a number of neurotransmitters. This includes gamma amino butyric acid (GABA), aspartate, taurine, β -alanine, serotonin and catecholamines. The fate of such neurotransmitters is astrocyte metabolism.

Detoxifying Ammonia

The brain is especially vulnerable to ammonia as it readily crosses the blood-brain barrier in its gaseous form, NH_3 , and rapidly saturates its principal removal pathway located in astrocytes. Ammonia toxicity may result in neurological abnormalities leading to seizures, mental retardation, brain edema, convulsion, coma and death. Diverse and complex amino acid cycles have been suggested to be involved in the transport of NH_4^+ from neurons to astrocytes. NH_4^+ may be transported as such, especially since NH_4^+ is rapidly accumu-

lated in astrocytes by Na^+ , K^+ -ATPase activity. One of the most important enzymes that catalyze the formation of ammonia in the brain is glutamate dehydrogenase. The enzyme catalyzes reversible oxidative deamination of glutamate and produces ammonia particularly in astrocytes, thereby provides a mechanism for the removal of excess nitrogen from certain amino acids. Brain lacks carbamoyl phosphate synthase 1 and ornithine transcarbamylase, essential enzymes for the urea cycle, and thereby unable to remove accumulated ammonia (Cooper and Plum 1987). However, astrocytes convert excess ammonia to glutamine via glutamine synthase (Fig. 10.1). The excreted glutamine from astrocytes is taken up by neurons. In fact, either in physiological condition or even in hyperammonemic condition, rapid conversion of ammonia to glutamine is the predominant CNS detoxification event (Bak et al. 2006).

10.3.3.2 Supplying Energy

Glucose and ketone bodies are the primary source of energy in mammalian brain under normal physiological conditions. In comparison to its weight, which is only 2–3 % of total body weight, brain consumes up to one fourth of body's total glucose supply.

Glycolysis

Astrocytes are the major food depot in the CNS. The rate of glycolysis is higher in astrocytes than neurons. Astrocytes contain higher levels of a key glycolytic enzyme, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKB3) than neuron. Therefore, astrocytes defend themselves against bioenergetic crises by upregulating glycolysis, a response that is absent in neurons. Several studies suggest that glycogen phosphorylase and synthase are predominantly localized in astrocytes. Glucose is utilized in astrocytes mainly via glycolysis (Wiesinger et al. 1997). Sugars enter into the metabolic pathway through phosphorylation which is considered as rate determining step. Astrocytes express hexokinase 1, the primary isoform of hexokinase in the CNS. This enzyme is mostly localized in mitochondria, only about 30 % of it is found in cytosol. Neurons need glucose and oxygen to function, and the delivery of these essential substances from the blood is regulated by astrocytes.

Oxidative Metabolism

In order to generate energy in the form of ATP, sugars are bound to enter into oxidative metabolic pathway, the tri carboxylic acid cycle (TCA cycle) (Fig. 10.1). Glycolysis generates 2 molecules of ATP and the TCA cycle generates 30 more ATP molecules from one molecule of glucose. The formation of energy in astrocytes is either through utilization of glucose under normal physiological condition or from reserve food storage glycogen via gluconeogenesis.

10.3.3.3 Organizing the Information Network in the CNS

It is increasingly becoming clear that astrocytes form an integral and active component of the information network in the CNS and have received the ‘stardom’ reflecting their morphology (Haydon 2001; Ransom et al. 2003; Nedergaard et al. 2003). Human astrocytes are quite unique when compared to other animals; they are over 20 times larger by volume and contact up to ten-times the number of synapses as individual rodent astrocytes (Oberheim et al. 2009). Furthermore, transcriptome data of human brains demonstrate significantly increased expression of astrocyte-derived synaptogenic proteins (Caceres et al. 2007; Sloan and Barres 2014).

Astrocytes are a critical participant of “the tripartite synapse” (Araque et al. 1999; Perea and Araque 2002). Any discussion on astrocyte biology remains incomplete without a consideration of their role in information transfer and intercellular communications in the brain.

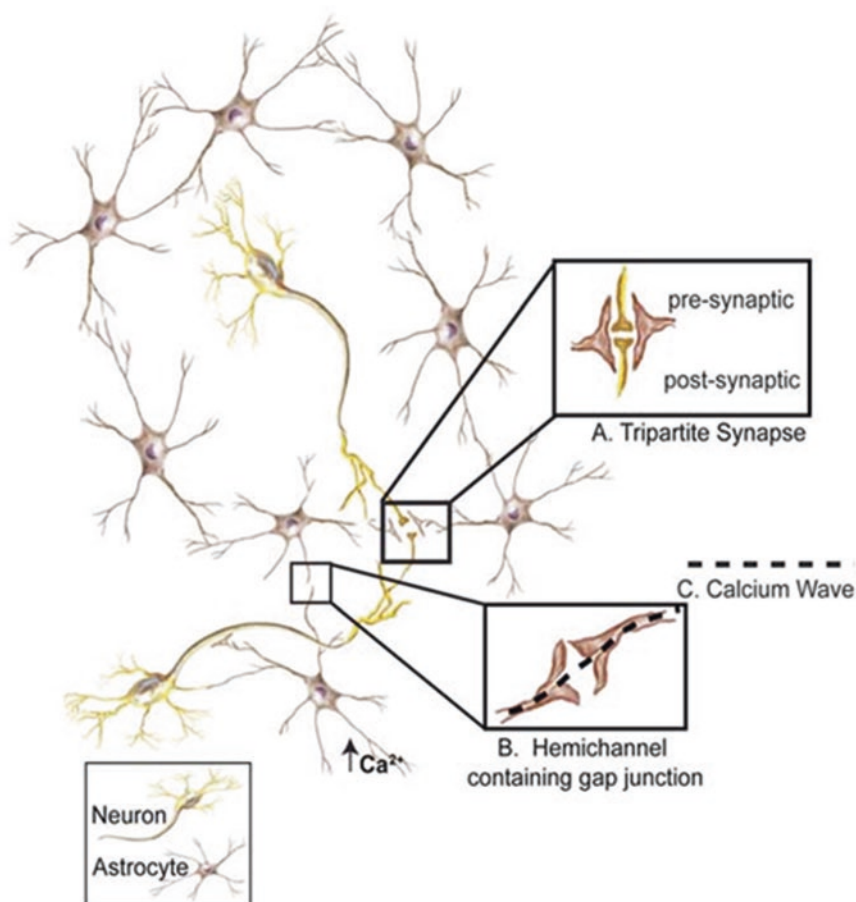
Role of GAP Junctions

In the brain, astrocytes form a syncytium, or a network of integrated cells (Scemes 2000). Such a syncytium consists of astrocytes that have cytoplasmic continuity in adjacent cells

through gap junctions (Bennett et al. 2003). There are abundant Gap junctions between astrocytes themselves, fewer between OLs and astrocytes, and few or none between OLs themselves or between neurons and glia. Gap junctions serve as a conduit between two astrocytes and consist of two hemichannels, also called connexones. These hemichannels or connexones are contributed by the juxtaposed cells and together form the gap junction (Fig. 10.2). Hemichannels assembled in the endoplasmic reticulum consist of junctional proteins belonging to the family of connexins (Contreras et al. 2003; Saez et al. 2003). Although a variety of connexins are expressed by astrocytes, connexin Cx30 and Cx43 are the predominant astrocyte connexin (Theis et al. 2005). Whereas Cx43 is expressed in early development in the nervous system and colocalizes throughout the white and gray matter in adult rodents, Cx30 is expressed later and localizes primarily to gray matter. These gap junctions form channels or pore about 1–1.5 nm in diameter and allow for the intercellular exchange of metabolites (e.g. ADP, glucose and glutathione), second messengers (e.g. cAMP and IP3) and ions (Ca²⁺, K⁺ and Na⁺) (Weber et al. 1970).

The presence of gap junctions between astrocytes and the tripartite synapse consisting of the conventional synapse ensheathed by astrocyte processes together serve as models

Fig. 10.2 The astrocyte nexus: the astrocyte processes engulf the synapse forming what is now known as the tripartite synapse, consisting of pre- and post-synaptic elements as the two components along with the astrocyte ensheathment as the third. The brain astrocytes communicate with each other through intercellular connections forming a large network on interconnected cells. These cells join through gap junctions that form a channel through which small molecules can pass from one cell to the next. Calcium, one of the molecules that can pass through these gap junctions can lead to exponential transfers within the astrocyte nexus forming a calcium wave. Such connexin 43 containing functional gap junctions are primarily observed in the astrocytes in the brain



for neuroglial interactions. Indeed, evidence from astrocyte-neuron co-cultures has demonstrated that the presence of astrocytes in neuronal cultures increases the number of synapses and their efficiency. On the other hand, gap junctional communication and function can be regulated by neurons (Nakase and Naus 2004; Orthmann-Murphy et al. 2008).

Role of Calcium

While neurons are most prominently identified with their electrical excitability and astrocytes lack such electrical impulses, calcium waves that propagate through gap junctions have emerged as the parallel mechanism to that of the transfer of electrical impulse from one neuron to another. This of course by no means suggests that calcium communication is unique to astrocytes; indeed, such signals are commonly used by a variety of cells and neurons are no exception to this. In neurons, calcium signals lead to an instant integrated electrical and chemical communication in synaptic cells. In both cultured astrocytes and astrocytes in intact brain slices, excitation of one cell can form a calcium wave transferred to several neighboring astrocytes and neurons in multiple directions. This involves elevated calcium in a single cell followed by elevated intracellular calcium in other cells. Such transfer of the calcium wave has been related to the Cx43 gap junction coupling of astrocytes both in vitro and in situ (Schipke and Kettenmann 2004). Mobilization of intracellular calcium is also widely used by astrocytes as a prominent cell signaling mechanism in response to a variety of stimuli both in physiological and pathological conditions (Verkhratsky and Kettenmann 1996).

Even more exciting is that neuronal activity can stimulate such calcium communication in astrocytes and vice versa. Thus, the role of calcium in the function of the tripartite synapse has received significant recent attention (Perea and Araque 2005; Hirrlinger et al. 2004; Araque and Perea 2004). Ca^{2+} excitability of glia is observed in response to a variety of stimuli (Verkhratsky and Kettenmann 1996). Such an elevation in intracellular calcium in a single astrocyte thus leads to the elevated calcium in the neighboring cells or the calcium wave described above. The complexity of calcium regulation in astrocytes is even greater, as has been revealed by recent studies showing that the calcium oscillations in a single cell may not even encompass the entire cell volume, but remain restricted to certain microdomains or certain processes within the astrocyte. Thus, we see the beauty of the autonomous functioning of a specific part of the cell that may have encompassed a syncytium of other cells or may ensheath certain synapses, and provide at the same time the possibility of chemical coupling of the entire cell when given the appropriate stimulus (Nett and McCarthy 2002; Kettenmann and Filippov 2002; Carmignoto and Pozzan 2002). The question as to what such calcium excitability of glia does in the neuroglial interactions is evolving.

Releasing Neuropeptides and Neurotrophins

Apart from its function as “support” cells by maintaining integrity of the CNS, astrocytes release several neuropeptides and neurotrophins. So far, five families of neuropeptides have been demonstrated to be expressed in astrocytes:

Renin-angiotensin family: The major function of renin-angiotensin system (RAS) in periphery is to maintain body-fluid homeostasis and regulate blood pressure.

Endothelins: Endothelins (ETs), a group of vasoactive peptides, acts as growth factor, exerting different functions like induction of proliferation, protein synthesis or changes in morphology. The ET1 also increases the rate of glucose 6-phosphate utilization via pentose phosphate pathway.

Enkephalins: The pentapeptide enkephalins are the ligand of orphan receptors in brain and are present mostly as precursor form in astrocytes.

Neurotrophins: Astrocytes are capable of releasing several growth factors and neurotrophic factors including epidermal growth factor (EGF), transforming growth factor (TGF), insulin like growth factor-1 (IGF-1), fibroblast growth factor-2 (FGF-2), brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), and neurotrophin-3 (NT-3) (Wu et al. 2004).

IL-6 family of neuropeptides: Astrocytes release ciliary neurotrophic factor (CNTF), interleukin 11 (IL-11) and leukemia inhibitory factor (LIF), which use the same transducer chain (signal transducer gp130) as IL-6. Interestingly, these molecules help in OL survival and differentiation (Kahn and De Vellis 1994).

10.3.3.4 Facilitating Neurogenesis

One of the key advances in the field of neurobiology is the discovery that astroglial cells can generate neurons not only during development, but also throughout adult life and potentially even after brain lesion. It has been shown that in neurogenic regions of adult brain (ventricular zone, hippocampus and olfactory subependyma), astrocytes secrete factors like FGF-2, IGF-1, Shh, BDNF, GDNF, and NT-3 that induce neurogenesis (Ruiz i Altaba et al. 2002) and support the growth of neurons and neural progenitor cells (Wu et al. 2004).

10.3.3.5 Do astrocytes Help in Myelination?

Astrocytes are themselves a major source of mitogens and differentiation factors for OPCs. For example, astrocytes release PDGF α and FGF2, which in combination promote self-renewal of the OPC population. In addition, astrocytes can secrete factors (promoting- γ -Secretase, TIMP-1, TNFR2/CXCL12, CNTF and LIF-1, Osteopontin, IGF, BDNF, NRG1, Laminin & fibronectin, NT3, GDNF & NGF) that are known to modulate myelination in a positive manner.

10.3.4 Role of Astrocytes in CNS Disorders

10.3.4.1 Activation of Astrocytes and Gliosis

Astrocytes react to various neurodegenerative insults rapidly, leading to vigorous astrogliosis. This reactive gliosis is associated with alteration in morphology and structure of activated astrocytes along with its functional characteristics (Eddleston and Mucke 1993). The astrocytic processes construct a bushy network surrounding the injury site, thus secluding the affected part from the rest of the CNS area. Subsequently, astrogliosis has been implicated in the pathogenesis of a variety of neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease, inflammatory demyelinating diseases, HIV-associated dementia (HAD), acute traumatic brain injury, and prion-associated spongiform encephalopathies (Eng and Ghirnikar 1994). Although activated astrocytes secrete different neurotrophic factors for neuronal survival, it is believed that rapid and severe activation augments/initiates inflammatory response leading to neuronal death and brain injury. This response to injury is characterized by increased expression of GFAP, vimentin, and other proteins, including CSPGs, lipocalin 2, and serpin3n (Zamanian et al. 2012). Enhanced up-regulation of GFAP is considered as a marker for astrogliosis (Eng and Ghirnikar 1994). GFAP increases at the periphery of ischemic lesion following neurodegenerative insults. Senile plaques, a pathologic hallmark of Alzheimer's disease, are associated with GFAP-positive activated astrocytes (Nagele et al. 2004). It is reported that in various neuroinflammatory diseases, the increased GFAP expression corresponds to the severity of astroglial activation (Eng and Ghirnikar 1994).

We have described that various neurotoxins increase the expression of GFAP in astrocytes via nitric oxide (NO) (Brahmachari et al. 2006), suggesting that scavenging of NO may be an important mechanism in attenuating astrogliosis. Although the activation of NF- κ B is involved in neurotoxin-induced production of NO in astrocytes, once NO is produced, it does not require the activation of NF- κ B to induce the expression of GFAP (Fig. 10.3). However, NO induces/increases the expression of GFAP in astrocytes via guanylate cyclase (GC)—cyclic GMP (cGMP)—protein kinase G (PKG) pathway (Fig. 10.3).

10.3.4.2 Release of Proinflammatory Molecules

Upon severe activation in response to various neurodegenerative and neuroinflammatory challenges, astrocytes secrete various proinflammatory molecules including proinflammatory cytokines (TNF- α , IL-1 α , IL-1 β , IL-6, and lymphotoxin), chemokines, reactive oxygen species, reactive nitrogen species, and eicosanoids (Gendelman et al. 1994;

Van Wagoner et al. 1999). These secreted proinflammatory molecules play an important role in the pathogenesis of various neurological disorders (Heales et al. 2004). In cultured murine astrocytes, bacterial lipopolysaccharides (LPS) act as a prototype inducer of various inflammatory responses. LPS is capable of inducing the expression of proinflammatory cytokines and inducible nitric oxide synthase (iNOS) in rat primary astrocytes (Pahan et al. 1997) but unable to induce the expression of iNOS in human astrocytes.

Among different proinflammatory cytokines (IL-1 β , TNF- α and IFN- γ) tested, only IL-1 β alone is capable of inducing iNOS in human primary astrocytes (Jana et al. 2005). Similarly, among different cytokine combinations, the combinations involving only IL-1 β as a partner are capable of inducing iNOS in human astrocytes (Fig. 10.4). The combination of IL-1 β and IFN- γ induces the expression of iNOS at the highest level in human astrocytes. Different proinflammatory transcription factors are involved in the transcription of iNOS in various cell types including astrocytes (Liu et al. 2002; Pahan et al. 1997). However, it has been found that the activation of CCAAT/enhancer-binding protein β (C/EBP β) plays an essential role in the expression of iNOS in human astrocytes (Jana et al. 2005). In addition to proinflammatory cytokines, viral double-stranded RNA (Auch et al. 2004) and HIV-1 Tat also induce the expression of iNOS and the production of NO in human astrocytes (Liu et al. 2002).

10.3.4.3 Do Astrocytes Present Antigen Under Autoimmune Response?

The CNS has long been known as “immunological privileged site” as it is secluded by BBB from peripheral immune system. However, this hypothesis is gradually becoming wrong. Microglia are capable of functioning as antigen-presenting cells (APC) as they express MHC I and II molecules (Dong and Benveniste 2001). In addition, microglia also expresses co-stimulatory molecules B7.1 and B7.2 molecules which play a role during antigen presentation. Another possible candidate as CNS APC is astrocyte. Expression of MHC II in astrocyte upon stimulation with IFN- γ or viruses has been demonstrated both in vivo and in vitro. However, capability of astrocytes as APC is still a controversial point. Examination of CNS tissues in MS, shows expression of B7-1 or B7-2 co-stimulatory molecules on macrophages and microglia but not on astrocytes. Human astrocytes also do not express co-stimulatory molecules B7-1 or B7-2. On the other hand, murine astrocytes express B7-1 or B7-2 either constitutively or in the presence of IFN- γ . Conflicting results are also found in case of CD40 expression. For example, CD40 expression is observed in fetal human astrocytes but not in adult human astrocytes. Therefore, functional ability of astrocytes as APC needs more research.

Fig. 10.3 Various neurotoxins induce the expression of inducible nitric oxide synthase (iNOS) via the activation of NF- κ B. Nitric oxide produced from iNOS then induces the activation of guanylate cyclase (GC) that catalyzes the production of cGMP. Inhibition of phosphodiesterase may also increase the level of cGMP. Cyclic GMP utilizes protein kinase G (PKG) to increase the expression of GFAP. *IL-1R* IL-1 receptor, *TLR4* toll-like receptor 4, *TLR2* toll-like receptor 2, *GPCR* G protein-coupled receptor, *TLR3* toll-like receptor 3

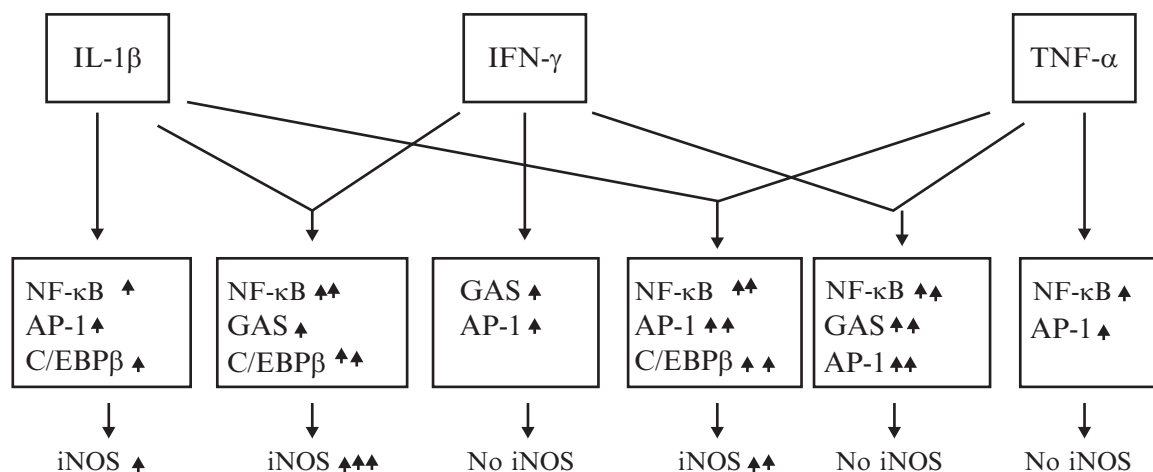
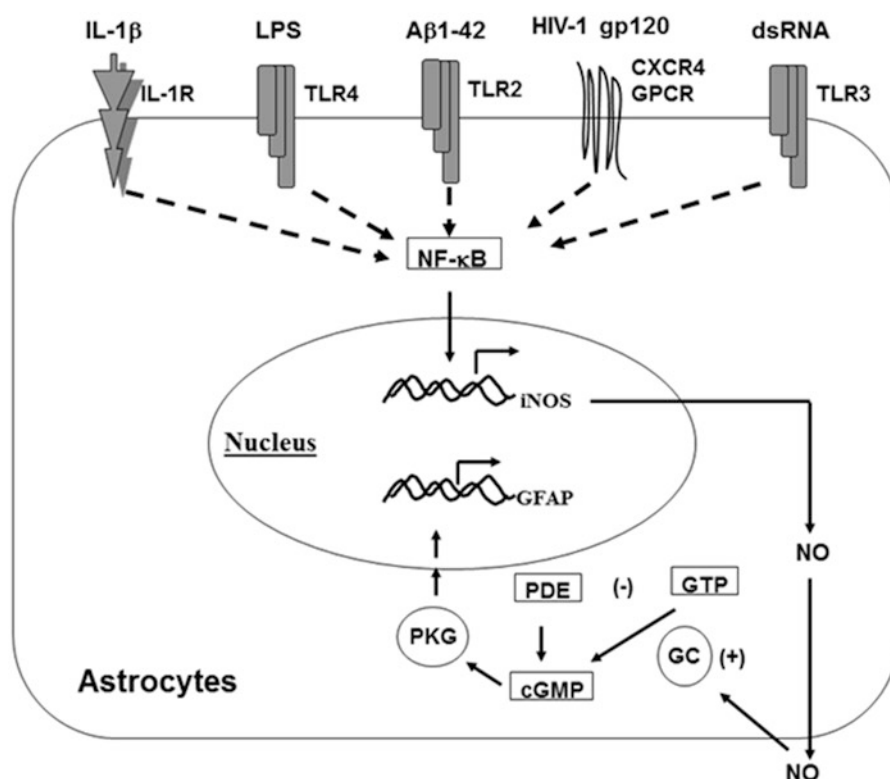


Fig. 10.4 Expression of iNOS by various proinflammatory cytokines in human primary astrocytes. TNF- α and IFN- γ alone or in combination is unable to induce the expression of iNOS. On the other hand, IL-1 β alone or in combination with other cytokines induces iNOS in human astrocytes. Activation of AP-1 and GAS together by IFN- γ is not sufficient for the expression of iNOS. Activation of AP-1 and NF- κ B together by TNF- α is also not sufficient for iNOS expression. Activation

of AP-1, NF- κ B and GAS together even at a higher level by the combination of TNF- α and IFN- γ compared to that induced by individual cytokines is also not sufficient for the expression of iNOS. However, IL-1 β capable of activating C/EBP β , AP-1 and NF- κ B induced iNOS in human astrocytes suggesting an important role of C/EBP β in the expression of iNOS in human astrocytes

10.3.4.4 Formation of Glial Scar: A Double-Edged Sword

Astrocytes play a dual role in inflammatory insults and regeneration. In one hand, activated astrocytes, characterized by cellular hypertrophy, proliferation and increased GFAP expression represent anisomorphic gliosis. This is the consequence of gross tissue damage and results in the

formation of tightly compacted limiting glial margin termed as astroglial scar or glial scar. The proinflammatory molecules released by reactive astrocytes in the scar cause tissue damage and inhibit neurite outgrowth as well as induce OL death. Chondroitin and keratin sulphate proteoglycans are among the main inhibitory extracellular matrix molecules that are produced by reactive astrocytes in the glial

scar and are believed to play a crucial part in failure to regeneration. The molecular mechanisms underlying glial scar formation have not been fully understood. STAT3, which contributes to neuroinflammation and promotes neuronal survival and regeneration after SCI, is one of the most important transducers in the process of glial scar formation. Moreover, S100A and SOX9 have also been reported to be directly involved in glial scar formation (Gris et al. 2007). More recently, it has been reported that miR-21 and miR-145 regulates astrocytic hypertrophy and glial scar progression after SCI (Yuan and He 2013). On the other hand, isomorphic gliosis, formed in response to insult, results in improved recovery and regeneration of the damaged tissue (Silver and Miller 2004). At the sites distant from injury, activated astrocytes get transformed to a more pronounced stellate shape with increased production of antioxidants and soluble growth factors that coordinate tissue remodeling in enhancing the survival of adjacent neurons and glia.

10.3.4.5 Trying to Defend Neurons Against Oxidative Stress and Excitotoxic Damage

One of the hallmarks of various neurodegenerative and neuroinflammatory disorders is oxidative stress-induced CNS damage. Such oxidative stress can damage lipids, proteins and nucleic acids of cells and power-house mitochondria causing cell death in assorted cell types including neurons and OL. However, astrocytes having high levels of antioxidant enzymes (glutathione peroxidase, catalase, glutathione reductase, and superoxide dismutase) and anti-oxidants (glutathione and ascorbic acid) try to absorb reactive oxygen species ($O_2^{\cdot-}$, O_2^- and OH^-) and reactive nitrogen species (NO , $ONOO^-$), maintain redox homeostasis and defend the insulted CNS (Dringen and Hirrlinger 2003; Chen and Swanson 2003). Accordingly, overexpression of the protective proteins, HSP72, or mitochondrial super oxide dismutase in astrocytes preserved the astrocytic glutamate transporter GLT-1 and reduced oxidative stress in the CA1 regions (Ouyang et al. 2014). In addition, astrocytes also scavenge detrimental molecules such as glutamate, produced during synaptic transmission through neurons (Hertz and Zielke 2004). Astrocytes convert glutamate to glutamine by glutamine synthetase. Moreover, activation of astrocyte specific purinergic P2Y1 receptor protects neurons from ischemic insult (Fujita et al. 2009).

10.3.4.6 Swelling of Astrocytes

Astrocytes undergo rapid swelling in certain acute pathological conditions like ischemia and traumatic brain injury. Different mechanisms are involved in such swelling process of astrocytes. Some of these are, decreasing extracellular

fluid osmolarity, intracellular acidosis, formation of ammonia, increase in Na^+ , K^+ , $2Cl^-$ co-transporter system, and due to drastically elevated levels of arachidonic acid and its metabolites. Alteration in glutamate metabolism and accumulation of glutamine and its transamination product, alanine is another possible cause of astrocytes swelling. In ischemic condition or in acute brain trauma, proton accumulation in the cytoplasm cause astroglial swelling predominantly via activation of Na^+/H^+ and Cl^-/HCO_3^- exchangers. Cytotoxic brain edema, caused due to astrocyte swelling, is a major neurological complication of the acute form of hepatic encephalopathy, a condition likely caused by elevated levels of brain ammonia. Potential mediators of ammonia-induced astrocyte swelling include oxidative/nitrosative stress (ONS), the activation of MAPKs, and the mitochondrial permeability transition (mPT) and NF- κ B. Recent studies showed that astrocyte cultures exposed to ammonia activate p53, and that such activation is mediated through ONS and MAPKs. Activation of p53 resulted in the induction of the mPT and NF- κ B activation, ultimately causing astrocyte swelling (Jayakumar et al. 2014). Blockade of p53 activation suppressed astrocyte swelling and glutamate uptake inhibition.

10.3.4.7 Apoptosis After Acute Insults

Although astrocytes usually undergo proliferation and gliosis in various neurodegenerative disorders, under acute insults, astrocytes may undergo apoptosis. Ex vivo cell culture studies demonstrate that tumor suppressor protein p53 plays a role in neuronal as well as astrocyte apoptosis (Bonini et al. 2004). HIV-1 infection of the central nervous system (CNS) frequently causes dementia and other neurological disorders in which apoptosis of astrocytes along with neuron has been found. HIV-1 infection of primary brain cultures induces the receptor tyrosine kinase c-kit and causes apoptosis of brain cells including astrocytes (He et al. 1997). The importance of c-Kit in apoptosis of astrocytes has further been confirmed by overexpressing c-Kit in an astrocyte-derived cell line in the absence of HIV-1. The mechanism of c-kit induction by HIV-1 involves transactivation of the c-kit promoter by the HIV-1 Nef protein.

10.4 Oligodendrocytes: Biology and Function

Camillo Golgi was the first to give a good description of glia. A few years later, Cajal, student of Hortega (1921) showed that there are two quite distinct cell types of neuroglia besides astrocytes using silver carbonate impregnation technique that he named oligodendrocyte (OL) and microglia. OLs are specifically the myelin-forming cells of the CNS.

10.4.1 OL Characteristics During Development

Among different brain cells, the development of OL has been well characterized. During differentiation, OL lineage cells (early OL progenitors, OL progenitors, pro-OL, immature OL, and mature OL) (Fig. 10.5) express stage-specific components that serve as markers of lineage progression (Table 10.2). Morphological characteristics of various developmental stages are shown in Fig. 10.5.

10.4.2 OL and the CNS

The major biological role of OL is myelination. However, OL may also promote neuronal survival, axonal growth and process formation. Neuronal function is also influenced by OL-derived soluble factors that induce sodium channel-clustering along axons. Trophic factors (NGF, BDNF, CNTF,

and NT3) produced from OL may provide the trophic support for both OL and local neurons.

10.4.2.1 Myelinating CNS Neurons

Myelination is a sequential multi-step process in which a myelinating cell recognizes and adheres to an axon, then ensheathes, wraps and ultimately excludes its cytoplasm from the spiraling process to form compact myelin. In humans, the period of myelination begins before birth and continues in some brain regions until 25–30 years of age. An OL is able to myelinate up to 50 axons depending on its localization. Although myelinating OLs are post mitotic, a slowly dividing population of OPCs persists in the adult brains. In addition, new OPCs are generated from subventricular zone progenitors following injury (Fancy et al. 2011).

Myelin is composed of lipids and proteins, most of which are specific for the myelin sheath. The major proteins are

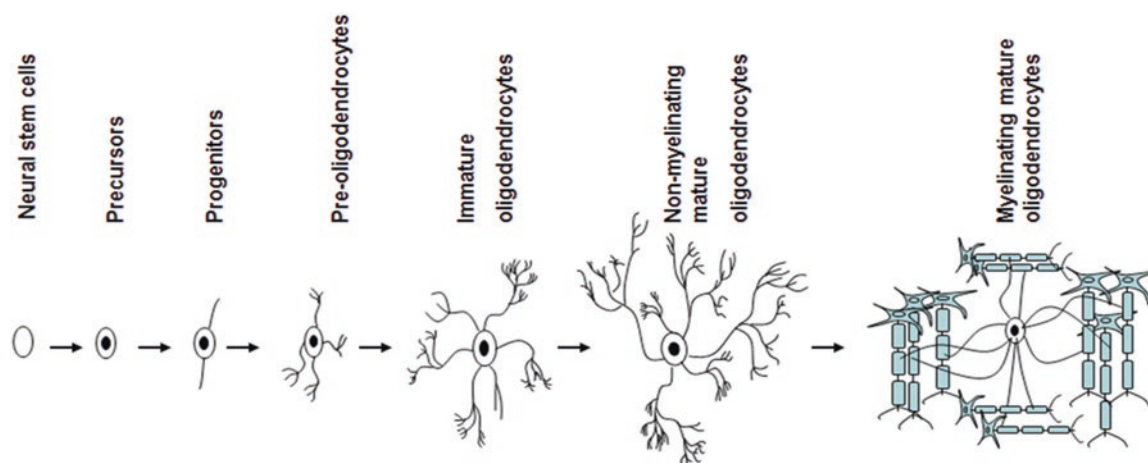


Fig. 10.5 Differential stages of oligodendroglial development

Table 10.2 Stage-specific markers of OL

Developmental stages	Markers	Detection	Characterization
Precursor	PSA-NCAM, Nestin, PDGFR- α , DM-20, PAX6, SOX10, Musashi	Anti-PDGFR- α antibody	PSA-NCAM ⁺ /Nestin ⁺ /A2B5 ⁻
Oligodendrocyte Progenitor cells (OPCs)	NG2/AN2+ proteoglycan, PDGFR- α protein or mRNA, GD3-related gangliosides, DM-20, CNPase, Nkx2.2, OLIG1 (nucleus)	Anti-NG2 antibody, Anti-PDGFR- α antibody, A2B5 antibody	A2B5 ⁺ /O4 ⁻
Pro/pre-oligodendrocyte	PDGFR α , O4, GD3, NG2/AN2+, PLP/DM20, CNPase	Anti-NG2 antibody, Anti-PDGFR- α antibody, O4 antibody	A2B5 ⁺ /O4 ⁺
Immature oligodendrocytes	GalC, O4, CNPase, PLP/DM20	O4, O1/GalC, CNPase, RIP	A2B5 ⁻ /MBP ⁻ /R-mAb ⁺
Mature oligodendrocytes	GalC, O4, CNPase, MBP, PLP, MAG, MOG, OLIG1 (cytoplasm)	MBP, MOG, PLP, MAG	A2B5 ⁻ /MBP ⁺

PSA-NCAM polysialylated form of neural cell adhesion molecule, PDGFR- α platelet-derived growth factor receptor α , MBP myelin basic proteins, PLP proteolipid protein, DM20 isoform of PLP, MOG myelin oligodendrocyte glycoprotein, MAG myelin-associated glycoprotein, CNPase 2',3'-cyclic nucleotide 3'-phosphodiesterase, OLIG1 oligodendrocyte transcription factor 1 (Baumann and Pham-Dinh 2001; Deng and Poretz 2003)

MBP, PLP, CNPase, and MAG. In the CNS, axonal factors play a critical role in the myelination process and thickness of the myelin, and that myelination in the CNS depends on a balance between positive and negative axonal signal (Sherman and Brophy 2005).

Role of Proteins

Role of CNPase: CNPase was first identified in CNS myelin and it represents about 4% of the total myelin protein in the CNS. It is localized in the inner and outer margins of myelin, paranodal loops, and OLs cytoplasm but is absent from compact myelin. It possesses enzymatic activity that catalyses the hydrolysis of 2', 3'-cyclic nucleotides into their corresponding 2'-nucleotides. However, to date any substrates of this enzyme has not been detected in the brain. Therefore, precise role of this enzyme in brain is unknown. However, it is one of the earliest myelination-specific polypeptides, synthesized by OL prior to the appearance of the myelin structural proteins (MBP and PLP) and its synthesis persists into the adulthood, suggesting a role in the synthesis and maintains of the myelin sheath. Over-expression of CNPase in transgenic mice perturbs myelin formation and creates aberrant OL membrane expansion. CNPase knockout mouse study shows that this protein is required for maintaining the integrity of para-nodes (Rasband et al. 2005). Recent studies have correlated the behavior of CNPase deficient mice with psychiatric disorders. Interestingly, CNPase null mice display progressively decreasing emotionality and lower fear expression than wild type controls. Reduced expression of CNPase in heterozygous mice increases age-induced axonal degeneration, brain inflammation, and astrogliosis (Hagemeyer et al. 2012).

Role of MBP: In the CNS, the adhesion of the cytoplasmic surfaces of the multilamellar compact myelin is maintained by MBP, one of the predominant proteins in CNS myelin. It constitute about 25–30% of the total protein. The 18.5 kDa isoform of myelin basic protein (MBP) is the exemplar of the family, being most abundant in adult myelin, and thus the most-studied. Shiverer mutation of MBP gene results in the absence of MBP proteins and morphological analysis of the CNS reveals an almost total lack of myelin in the brain, and also the existing myelin is abnormal, presenting no major dense line. Therefore, MBP is necessary for the formation of the major dense line in the CNS myelin. One of the best characterized protein ligands for MBP is calmodulin, a highly acidic calcium sensor. MBP also interacts with several other proteins, including actin, tubulin, and SH3 domain-containing proteins, and thus may be a signaling hub during myelin development and remodeling (Harauz and Libich 2009).

Role of MOG: MOG is a member of the immunoglobulin super family, preferentially localized on the outside surface of myelin sheath and on the surface of OL process.

Immunocytochemical studies demonstrate that the expression of MOG is late in OL differentiation compared with other major myelin proteins. It is used as a surface marker of OL maturation. This specific CNS protein is a minor component of myelin, constituting 0.01–0.05% of total myelin proteins. It is a 26–28 kDa integral glycoprotein and like other myelin proteins it may exist in multiple forms. Since the location of this protein in outermost surface of the myelin, it is easily accessible to a humoral immune response. MOG not only binds C1q but also may be the protein in myelin responsible for complement activation (Han et al. 2013).

Role of PLP/DM20: PLP/DM20 is the most abundant intra-membrane protein and constitutes about 17–45% of the total protein of CNS compact myelin. DM-20 and PLP arise from alternative splicing of a genomic transcript and differ by a hydrophilic peptide segment of 35 amino acids long, the presence of which generates the PLP product. PLP/DM20 is crucial for the proper assembly and physical stability of CNS myelin and thus for the transmission of nerve impulses in the CNS. PLP/DM20 participates in OL membrane adhesion, compaction of myelin, formation of the myelin intraperiod line, and wrapping the axons. In human, mutation of PLP and DM20 gene causes Pelizaeus-Merzbacher disease (PMD), an X-linked dysmyelinating neuropathy, and spastic paraplegia type II (SPG-II) (Duncan 2005). PLP has an important role in supporting normal axonal function. PLP is necessary for the transport of certain proteins, such as the NAD⁺-dependent deacetylase, sirtuin 2 and cholesterol into CNS myelin (Han et al. 2013).

Role of other myelin proteins: Besides these four proteins, myelin also contains other proteins that play a critical role in myelin compaction and neuronal function.

Myelin-associated glycoprotein (MAG): MAG is a minor constituent of both the CNS and PNS myelin, and it localizes to the noncompact regions of the myelin membrane. MAG found on the myelin membrane adjacent to the axon. MAG is a critical component of axon-glia interactions with multiple functions in the biology of both neurons and glial cells. MAG is believed to participate in axonal recognition and adhesion, inter-membrane spacing, signal transduction during glial cell differentiation, regulation of neurite out growth, and in the maintenance of myelin integrity (Jones et al. 2013).

Myelin associated/oligodendrocyte basic protein (MOBP): MOBP is abundantly expressed in the CNS myelin and shares several characteristics with MBP. MOBP is synthesized by mature OL and localized at the major dense line, suggesting a role in the myelin compaction process.

P2: P2 is a fatty acid binding protein with a molecular weight about 13.5 kDa, is located on the cytoplasmic side of the compact myelin membranes. It presents at high abundance in PNS myelin. It may serve as lipid carrier and thus could be involved in the assembly, remodeling and maintenance of myelin (Majava et al. 2010).

Oligodendrocyte-specific protein (OSP/claudin-11):

OSP/claudin-11 and PLP are both tetraspan proteins concentrated in CNS myelin. OSP represents about 7% of total myelin proteins. They possibly play an important role in myelin formation and maintenance due to their localization and concentration in membrane sheaths. Individual OSP/claudin-11- and PLP-null mice have relatively normal-appearing myelin and mild neurological deficits due to their compensatory role. However, when both OSP/claudin-11 and PLP genes are knocked out, mice show severe neurological deficits, markedly abnormal myelin compaction, and smaller axon diameters. OSP/claudin-11 in association with K(V)1 family voltage-sensitive K(+) plays a significant role in OPC proliferation, migration and myelination of axons (Tiwari-Woodruff et al. 2006).

Cx32: Cx32, an integral membrane protein, is structurally related to PMP22 with four hydrophobic transmembrane domains. Recent studies show that it is also expressed on some areas of the CNS myelin and corresponding myelinating OL. Cx32 is preferentially expressed in OL in the CNS and in Schwann cells in the PNS. In addition to forming gap junctional channels, Cx32 also forms functional hemichannels. Mutation in Cx32 causes a common peripheral demyelinating neuropathy, X-linked Charcot-Marie-Tooth disease (Schiza et al. 2014). However, mice lacking Cx32 or Cx 47 develop minimal CNS pathology and no obvious phenotype but loss of both OL connections in Cx32/Cx47 double knockout mice leads to severe and early CNS demyelination.

Oligodendrocyte-myelin glycoprotein (OMgp): It is a glycosylated protein with molecular mass 120 kDa. It is located in the para-nodal areas of myelin. During injury, it inhibits the axonal growth by interacting with Nogo-66 receptor (NgR) complex and paired Ig-like receptor-B (PiRB). Mechanistic studies reveal that Nogo-66 and OMgp suppress long term potentiation (LTP) in an NgR1-dependent manner. OMgp inhibits LTP in part through PiRB but independently of p75.

Myelin/oligodendrocyte specific protein (MOSP): MOSP is a novel surface protein which is exclusively expressed in CNS myelin. It also plays an important role in membrane/cytoskeleton interactions during the formation and maintenance of CNS myelin.

Lipids

In the CNS, lipids play an important role in myelin formation along with various protein molecules. One of the major biochemical characteristics that distinguish myelin from other biological membranes is its' high lipid-to-protein ratio. About 70–80% of the dry weight of myelin is comprised of lipid components and 20–30% protein. In every mammalian species, myelin contains cholesterol, phospholipids and glycolipids in molar ratios ranging from 4:3:2 to 4:4:2. In mature brain, cholesterol is the major lipid in myelin (about 20–25%)

but generally normal myelin does not contain any cholesterol ester. Cholesterol helps to increase membrane thickness and fluidity as well as ion leakage through membranes which may be relevant to its property of electrical insulation.

Other abundant lipids in myelin are galactosylcerebro-sides (Gal-C) and their sulfated derivatives (sulfatides). GalC represent 20% lipid dry weight in mature myelin. Immunological and chemical perturbation studies indicate that these lipids are involved in OL differentiation, myelin formation and myelin stability. These galactolipid-deficient animals exhibit severe tremor, hindlimb paralysis and display electrophysiological deficits in both CNS and PNS (Baumann and Pham-Dinh 2001).

Lysophosphatidic acid (LPA) is an intercellular signaling lipid that regulates multiple cellular functions, acting through specific G-protein coupled receptors (LPA₁₋₆). Specifically LPA1 regulates OLs differentiation and myelination in the CNS (Garcia-Diaz et al. 2014).

Molecules Involved in Positive and Negative Regulation of Myelination

The formation and maintenance of the myelin sheath require the coordination of a number of gene products. While some gene products facilitate myelination, some others try to suppress myelin formation. In the following lines, we describe such positive and negative regulatory mechanisms.

Regulation of myelination: OLIG1 and OLIG2 are closely related basic helix-loop-helix transcription factors that are expressed in myelinating OL and their progenitor cells in the developing CNS. Both OLIG1 and OLIG2 are positive regulators of myelination. Specifically OLIG1 has an essential role in OL differentiation and myelination, as it regulates the transcription of major myelin-specific genes MBP, PLP and MAG. On the other hand, OLIG2 is required for the initiation of oligodendroglialogenesis but its role in myelination is controversial (Xin et al. 2005).

Another important molecule that stimulates myelination is GPI-linked neural cell recognition molecule F3/contactin. This is a physiological ligand of Notch that signals via DTX1 to promote the development of OL (Popko 2003). Additionally, F3 also transduces signals to glial intracellular Fyn, which then interacts with Tau protein to mediate myelination. As expected, different neurotrophins also favor myelination through the maintenance of OL health and viability. For example, neurotrophin-3 (NT3) is known to induce both survival and proliferation of OL. NT3 interacts with TrkC to activate CREB that plays a critical role in proliferation and maturation of OPCs, and in the expression of myelin genes (MBP, P2, P0 MOG, PLP, and MAG) and anti-apoptotic gene Bcl-2. In addition, recent studies have identified many other molecules (e.g. PAX3, PPAR- δ , MyT1, SOX10, GTX, Sp1, YY1, Nkx2.2, Zfp488, MRF, Zfp191 and SCIP/Oct6/Tst-1) that may function as positive regulators of myelination

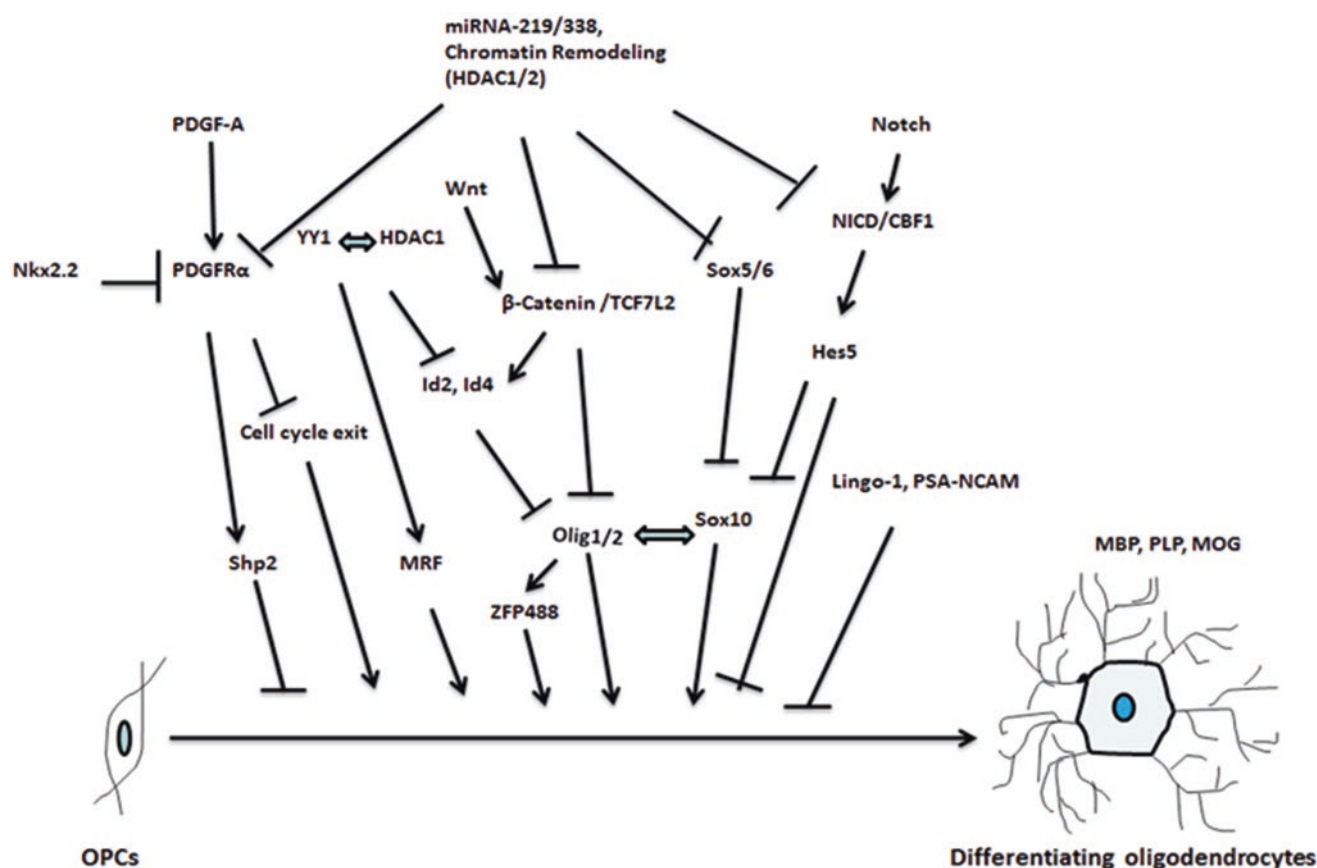


Fig. 10.6 Schematic diagram showing different molecular signalling pathways for the induction of myelin gene expression in differentiating oligodendrocytes. The crosstalk between extrinsic signal, transcription factors, miRNAs, and chromatin modifiers determine the balance

between repressive signals that inhibit OLs differentiation and de-repressive signals that OLs differentiation. Double headed arrows indicate physical interactions

(Barca-Mayo and Lu 2012) (Fig. 10.6). TACE/ADAM17 is essential for OL development and CNS myelination. Recently, it has been shown that antiaging protein klotho enhances OLs maturation and myelination through AKT and ERK signaling pathways (Chen et al. 2013).

OL differentiation is also regulated at the level of chromatin remodeling by histone deacetylases (HDACs) as pharmacological inhibition of HDAC activity in postnatal rat causes a delay in OLs differentiation and myelination. Conditional deletion of HDAC1 and HDAC2 in the OLs lineage causes a loss of OPCs and OLs, suggesting that this HDAC activity is required at multiple stages of the lineage. Histone deacetylase likely promote OL differentiation by inhibiting the expression of pathways and genes that otherwise act to block differentiation. These include HDAC-mediated inhibition of the Wnt/β-catenin pathway and HDACs acting in conjunction with the transcription factor YY1 to inhibit expression factors such as ID4 and Tcf4 (Emery 2010). Recent studies have demonstrated a critical role of miRNAs in OLs development, including cell proliferation, differentiation, and myelination. Micro-RNA -338 and miR-219 are highly expressed in OLs

and promote OLs differentiation by inhibiting the expression of differentiation inhibitors (e.g. Sox5/6 & Hes5) and OPCs proliferation factors (PDGFRα) (Fig. 10.6).

Molecules involved in negative regulation of myelination: Bone morphogenic proteins (BMP4s) should have a role in regulating bone density! Yes, they do have and in addition, these important molecules also regulate OL development. At early stage, BMPs regulate cell lineage decision and at later stage, they inhibit cell specialization in OL. For example, BMP4 signaling inhibits the generation of OL and enhances the generation of astrocytes from neural progenitor cells both in vitro and in vivo. BMP4 induces the expression of all four members of the inhibitor of differentiation (ID) family of helix-loop-helix transcriptional inhibitors and blocks OL lineage commitment through the interaction with OLIG1 and OLIG2 (Samanta and Kessler 2004). The other inhibitory signaling pathways mediate through specific downstream effectors such as Wnt/β-catenin, Notch/Hes factors, Sox5/6, and NF1a/b at different developmental stages to inhibit OL differentiation and myelination (Barca-Mayo and Lu 2012).

LINGO-1, a transmembrane protein containing a leucine-rich repeat (LRR) and immunoglobulin domain, functions as a component of the NgR1/p75 and NgR1/Taj (Troy) signaling complexes. Recent studies show that LINGO1 is also expressed in OL where it negatively regulates OL differentiation and axonal myelination by down-regulating the function of Fyn kinase and up-regulating the activity of RhoA-GTPase. Lack of LINGO-1 expression promotes more axonal myelination due to increased expression of myelin gene such as MBP, CNPase and MOG in OL. LINGO-1 knockout mice also show earlier onset of CNS myelination (Mi et al. 2005). SIRT1, a protein deacetylase inhibits the production of new OPCs in the adult mouse brain, in part by acting in NSCs and inhibits myelinating OL formation (Fig. 10.6).

10.4.3 OL and CNS Pathology

Death of OL and subsequent myelin loss has been reported in a variety of myelin disorders including, multiple sclerosis (MS), X-adrenoleukodystrophy (X-ALD), adrenomyeloneuropathy (AMN), vascular dementia, periventricular leukomalacia (PVL), hypoxia, and ischemia. Rates of OLs loss as high as 30–40% are observed following axon injury in the mature CNS after months to years. Several factors that might be associated with OL death in these pathophysiological conditions are discussed briefly in below.

10.4.3.1 Autoimmune Triggers

MS and experimental allergic encephalomyelitis (EAE), an animal model of MS, are autoimmune diseases of the CNS mediated by T cells recognizing self-myelin proteins including MBP, MOG and PLP. T cells are activated in the periphery by unknown antigens of both myelin and nonmyelin origins. After activation, T cells cross the blood-brain barrier and invade into the brain where they accumulate and proliferate in response to antigen re-stimulation. These activated T cells secrete different pro-inflammatory molecules which stimulate not only the resident glial cells (microglia and astroglia) but also other infiltrating cells. CD4⁺ and $\gamma\delta$ T cells express Fas-L which is found to be associated with OL death. Furthermore, infiltrating CD8⁺ T cells interact with MHC class 1 surface receptor of OL and in turn cause OL lysis. T cell-derived perforin may also be responsible for OL death (Scolding et al. 1990).

10.4.3.2 Cytokines

Cytokines are important mediators in the inflammatory demyelination observed in MS, EAE, X-adrenoleukodystrophy (X-ALD), and Theiler's virus infection. In these pathologies, pro-inflammatory cytokines and others factors released by endogenous glial cells and/or infiltrated macrophages and CD4⁺ Th1 cells, accumulate and exert pleiotropic effects on

OL. At lower concentrations, these cytokine may be involved in normal development of the nervous system while following brain trauma or inflammatory insults, the overproduction of these cytokines may result in a homeostatic imbalance and may contribute to the outcome of the pathological event. Various cytokines can directly kill OL or it may also affect other signaling pathways that could be involved in the susceptibility of OL. For example, IFN- γ produced by T cells may induce OL apoptosis and cell death via JAK-STAT pathway. It has been shown that CXCL10 contributes to neuropathology by promoting OPC apoptosis (Tirota et al. 2012).

Another proinflammatory cytokine TNF- α induces OL death via death signaling pathways (e.g. death initiating signaling complex (DISC), ceramide signaling pathway and stress-activated protein kinase pathways (SAPK) (Buntinx et al. 2004). IL-1 β is a strong stimulus for TNF- α release from astrocytes and microglia. Both IL-1 β and TNF- α are capable of inhibiting the expression of myelin genes via redox-sensitive mechanism (Jana and Pahan 2005). Increased level of IL-1 β is also known to induce OL excitotoxicity via P2X₇ receptor.

10.4.3.3 Nitric Acid

Nitric oxide (NO), a short-lived and highly reactive free radical, is an important physiological messenger in the CNS. However, high level of NO in the CNS has been associated with different neuroinflammatory and neurodegenerative diseases. During CNS inflammation, activated microglia, astrocytes and infiltrating cells express inducible nitric oxide synthase (iNOS) producing excessive amount of NO. OL at different stages of differentiation are differentially sensitive to NO. For example, OPCs and immature OL are more susceptible than mature OL to NO. However underlying mechanisms are poorly understood. It has been shown that NO reacts with superoxide generated by NADPH oxidase from activated glial cells and infiltrating cells to form peroxynitrite, the most reactive NO derivative. This peroxynitrite plays a critical role in the death of OL (Jana and Pahan 2013; Li et al. 2005). Combination of IFN- γ and bacterial lipopolysaccharide (LPS) or double stranded RNA in the form of polyIC induced the production of NO and decreased the expression of myelin gene in human fetal mixed glial cultures. This study illustrates a novel biological role of NO in down-regulating the expression of myelin genes preceding the death of OLs (Jana and Pahan 2013).

10.4.3.4 Oxidative Stress

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) leading to oxidative stress have been implicated as mediators of demyelination and axonal damage in both MS and EAE. Oxidative stress can damage lipids, proteins and nucleic acids of cells and mitochondria potentially causing OL cell death. During oxidative stress-induced OL apoptosis,

cytochrome c is released from damaged mitochondria, which in turn leads to the activation of the death-related caspases 3 and 10. Another study by Vollgraf et al. shows that mature OL exposed to oxidative stress undergo chromatin segmentation, condensation via mechanisms involving transcriptional activation of the immediate early stress genes (c-fos and c-jun) (Vollgraf et al. 1999). An induction of Bax protein has also been reported under oxidative stress condition in OL.

10.4.3.5 Ceramide

Ceramide, the lipid second messenger and a hydrolyzed product of sphingomyelin, is involved in apoptosis of OL. In pathological conditions, proinflammatory cytokines (TNF- α and IL-1 α) released specifically from activated microglia and astroglia leads to the activation of sphingomyelinases and production of ceramide in OL via the redox-sensitive mechanism (Singh et al. 1998). According to Jana and Pahan (2013), oxidative stress induces apoptosis in human OL via neutral sphingomyelinase (Jana and Pahan 2007). During Alzheimer's disease, amyloid- β is aggregated in the plaque region that also causes OL death by activating the NSMase-ceramide cascade via redox-sensitive pathways. In addition, ceramide can inhibit inwardly rectifying K⁺ currents and cause depolarization in OL.

10.4.4 Regeneration

Recent evidences indicate that OPCs remain intact both in normal white matter and in demyelinating CNS of patients with MS. OPCs can give rise to new OL in experimental conditions and have the ability to repopulate areas from where they are missing.

10.4.4.1 OL Regeneration

Recent identification of several genes associated with regeneration of OL has become helpful to understand the mechanism of remyelination and formulate a strategy for possible therapeutic intervention in demyelinating disorders. It has been shown that the levels of Nkx2.2, Olig1, Olig2, Ascl1 (Mash1), MYT1, and Sox9/10 at different stages of OL are similar during remyelination and myelination. Therefore, the upregulation of these factors during active remyelination likely results from the rejuvenation of a developmental genetic program enabling OPCs to actively proliferate, migrate and differentiate into new remyelinating OLs (Nakatani et al. 2013). EGFR signaling is also known to regulate oligodendrogenesis and remyelination by NG2+ MASH1+ OLIG2+ progenitors. According to Nakatani et al., GOLLI MBP is capable of stimulating OPC proliferation and differentiation in remyelinating adult mouse brain (Nakatani et al. 2013). Endothelin-1 (ET-1), which is highly expressed in reactive astrocytes of demyelinated lesions,

drastically reduces the rate of remyelination via activation of Notch signaling (Hammond et al. 2014).

10.4.4.2 Schwann Cells

During demyelination, after macrophages/microglia remove myelin debris and glial scar, SCs enter into the CNS from PNS sources such as spinal and cranial roots, meningeal fibers, or autonomic nerves where they remyelinate axons in the absence of reactive astrocytes. During this period, survival of SCs requires the axon-derived trophic factors. Schwann cell can also produce some growth factors like IGF1, FGF2 and PDGF, which promote the migration of OPCs and maturation into myelinating OL.

10.4.4.3 Thyroid Hormones

Thyroid hormone (TH) plays an important role by regulating several stages of OL development and maturation. The increasing TH levels in vivo accelerate both myelin gene expression and myelination during development, and also myelin regeneration after demyelination. OLs express TH receptors and during demyelination, TH increases the expression of NGF that protects OLs from cell death. TH induces the activation of kruppel-like factor 9 (KLF9), basic helix-loop-helix family member e22 (bHLHe22), hairless (Hr), and Albumin D box-binding protein (DBP), and thereby regulates OL differentiation and myelin regeneration (Dugas et al. 2012). TH hormone also up-regulates the expression of PDGFR- α , MBP and CNPase in CNS tissues of animals with MS (Calza et al. 2005).

10.5 Schwann Cells: Peripheral Glia

One of the major differences between the CNS and the PNS is the proportion of myelinated versus unmyelinated fibers. In the CNS, nearly all white matter tracts are myelinated, whereas in the PNS, there are approximately four times as many unmyelinated axons as myelinated ones (Griffin and Thompson 2008). The peripheral nervous system contains a number of distinct glial cells, each of which is intimately associated with different parts of the neurons or with specific neuronal cell types. Earlier, these cells were known as the supporting cells of the PNS but recent studies delineate their multifunctional role. These cells are of two types: satellite cells and Schwann cells (SCs). Satellite cells surround the neuronal cell bodies in dorsal root sensory ganglia and in sympathetic and parasympathetic ganglia. These cells help to maintain a controlled microenvironment around the nerve cell body, providing electrical insulation and a pathway for metabolic exchange. The other cells named after German physiologist Schwann are flattened cells with an elongated nucleus oriented longitudinally along the nerve fiber. Surface of all axons in peripheral nerves are ensheathed by non-myelinating or myelinating SCs.

10.5.1 Schwann Cells (SCs): Overview

In the mature nervous system, SCs can be divided into three classes based on their morphology, biochemistry and function: myelinating Schwann cells (MSCs), nonmyelinating Schwann cells (NMSCs) and perisynaptic Schwann cells (PSCs) (Fig. 10.7).

MSCs are well characterized and they wrap around axons with a diameter of 1 μm or greater, including all motor neurons and some sensory neurons. This is a mystery why they wrap a specific diameter of the axons. Smaller diameter axons including many sensory and all postganglionic sympathetic neurons are myelinated by NMSCs. The NMSCs provide the metabolic and mechanical support to the axon. The NMSCs appear later than MSCs. They express higher levels of GFAP, p75NTR and cell adhesion molecule L1 compare to MSC. The PSCs located at the neuromuscular junction incompletely wrap around the presynaptic terminal of motor axons. They help to maintain a stability of the neuromuscular junction and regulate synaptic transmission (Corfas et al. 2004).

10.5.2 Schwann Cell Development

SCs originate from the neural crest cells, a transient population of cells migrating away from the dorsal part of the neural tube. Neural Crest cells are multipotent cells that differentiate

to form neurons and glia of the PNS, and also additional cell and tissue types such as melanocytes and connective tissue of the head. Several molecules (e.g. ErbB3, transcription factor SOX10, AP2 α and Ets1, the N-Cadherin 6, the low affinity receptor for nerve growth factor p75NTR) have been shown to play important roles during the detachment of neural crest from neural tube (Jessen and Mirsky 2005).

Markers of lineage progression: Characterization of a number of specific biochemical markers has increased our knowledge on the stages of SC maturation, both in vivo and in vitro. Some of the biochemical markers have been shown to overlap partially (Table 10.3).

10.5.3 Survival, Migration and Death

10.5.3.1 Survival

The survival of immature SCs in late embryonic and prenatal nerves is probably controlled by a balance between factors that support survival and factors that cause death. Axon-derived neuregulin family (NRG-1, NRG-2 and NRG-3) have been implicated in the biological processes of SCs including fate specification, proliferation, survival, migration, regulating the extent of myelination, and triggering demyelination. It is believed that the interaction between several NRG ligands with different ERB receptors (ErbB2,

Fig. 10.7 Different stages of Schwann cell development

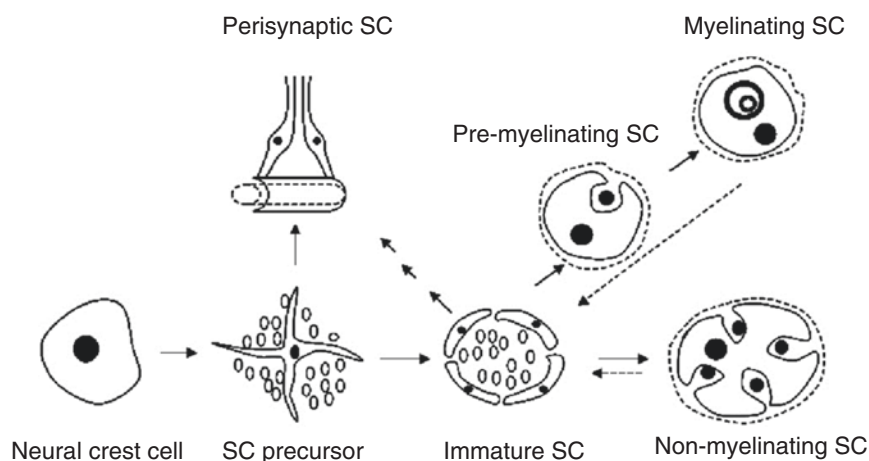


Table 10.3 Stage-specific markers of Schwann cells

Stages	Markers
Neural crest cells	SOX10, AP2 α
Schwann cell precursor (SCPs)	Cadherin19, AP2 α , low level P0, GAP43, F-spondin, SOX10, BFABP, DHH
Immature Schwann cells	S100 β , GFAP, low level of P0, SOX10, O4 antigen, BFABP, DHH
Myelinating Schwann cells	P0, PMP-22, MBP
Non myelinating Schwann cells	NCAM, GFAP

SOX10 SRY (sex determining region Y) box 10, *AP2 α* activator protein 2 α , *DHH* desert hedgehog, *GAP43* growth associated protein 43, *P0* protein zero, *O4* lipid antigen, *BFABP* brain fatty acid-binding protein, *S100* calcium-binding protein, *PMP22* peripheral 22 kDa myelin protein, *MBP* myelin basic protein, *GFAP* glial fibrillary acidic protein, *NCAM* neuronal cell-adhesion molecule

ErbB3 and ErbB4) on SCs plays a critical role in regulating these steps (Michailov et al. 2004). SCs can support their own survival by producing a number of growth factors such as IGF2, NT3, PDGF- β , LIF, and lysophosphatidic acid (LPA) in an autocrine fashion. This mechanism ensures very little SC death after axon injury, allowing SCs to play an active supportive role in PNS axon regeneration. However, following periods of chronic denervation lasting several months or more, increased SCs death is ultimately observed. The autocrine survival circuits are probably important in maintaining the survival of SCs in injured nerves.

10.5.3.2 Migration

During development of the PNS, neural crest cells migrate along the outgrowing axons and proliferate in order to produce sufficient number of cells for myelination of axons. Various factors or signaling molecules present on the neighboring cells effect Schwann cell migration in cell culture and it is possible that these signals lead to SC movements during radial sorting in vivo. Integrins, a subgroup of adhesion receptors mediate interaction between cytoplasm and the extracellular environment. This interaction influences the migration of neural crest cells, axonal out growth and SC differentiation. Integrins can interact with different growth factors, cell adhesion molecules (NCAM and F3) and intracellular cytoskeleton or adaptors proteins. This interaction is crucial for conformational changes and movement of SCs. Several growth factors that regulate the migration of SCs include NRG1, BDNF, GDNF, NT3, and IGF-1. The majority of these molecules are also expressed by SC itself. Recent

studies show that miR-221 and miR-222 promote Schwann cell proliferation and migration by targeting LASS2 after sciatic injury. The miR-182 inhibits SC proliferation and migration by targeting FGF9 and NTM, respectively at an early stage following sciatic nerve injury. On the other hand, the miR-9 inhibits SC migration by targeting Cthrc1 following sciatic nerve injury (Zhou et al. 2014).

10.5.3.3 Death

NGF acting via the p75 neurotrophin receptor promotes cell death during the SC injury or infection via activation of c-jun-N-terminal kinase (JNK). It has been found that neonatal p75 neurotrophin receptor mutant mice are less prone to cell death after nerve transection. Here it is to be noted that the same neurotrophin signaling pathway may also promote survival of SCs via activation of NF- κ B. Yeiser et al (2004) have shown that NGF signaling through the p75 receptor is deficient in TRAF-6 ($-/-$) mice and that NGF is unable to kill TRAF-6 ($-/-$) SCs (Yeiser et al. 2004). In addition, TGF β is also known to cause apoptosis of SCs via JNK in culture (Jessen and Mirsky 2005). Other inducers that also contribute to the death of SCs are P2X7 purinoreceptors and proinflammatory cytokine TNF- α .

10.5.4 OL and SC

Although both OL and SC share the common task of synthesizing myelin, there are some differences between the two cell types (see Table 10.4).

Table 10.4 Differences between oligodendrocytes (OL) and Schwann cells (SC)

OL	SC
1. OLs are present only in the CNS	1. SCs are the major glial cells in the PNS
2. The sub ventricular zone (SVZ), which is present in late gestational and early postnatal mammalian brain, is a major source of OL	2. SCs are originated from the neural crest
3. OL developmental steps are irreversible	3. Fully differentiated SCs retain an unusual plasticity throughout the life and can readily de-differentiate to form cells similar to immature SCs
4. One OL extends several processes and can myelinate up to 1–50 axons with distinct internodes	4. One SC has an intimate association with axon and each SC forms myelin around a single axon, and lines up along the axon to define a single internode
5. GM4, one of the most abundant lipids of the CNS, is present in OL	5. Some glycolipids such as sulfated glucuronyl paragloboside and its derivatives are specific to SC
6. CNS myelin contains more choline and plasmalogens than PNS myelin (Garbay et al. 2000)	6. PNS myelin contains more ethanol phosphoglycerides than CNS myelin
7. Basic proteins (MBP and PLP) are major constituents of CNS myelin (about 80 % of the total protein) (Baumann and Pham-Dinh 2001)	7. Glycoproteins (P0 and PMP22) are major constituents of PNS myelin
8. OLs have phagocytic activity	8. SCs do not have phagocytic activity
9. OLs migrate slower and divide and remyelinate at a slower rate than SCs	9. SCs migrate faster and divide and remyelinate at a faster rate than OLs
10. OLs are less resistant than SCs to injury	10. SCs are more resistant than OLs to injury
11. CNS myelin is a potent inhibitor of axon regeneration	11. PNS myelin is a less-potent inhibitor of axon regeneration
12. MiR-219, a critical miRNA implicated in OL differentiation	12. This is not present in SC

10.5.5 Biological Roles

10.5.5.1 Myelinating Peripheral Neurons

SCs cover most part of the PNS neurons by myelin sheath. Although the PNS myelin is mainly formed by the differentiation of the plasma membrane of SCs, myelination of mammalian PNS is a very complex developmental process. It requires intricate timing of several gene expression and cellular interactions between the axon and differentiated SCs (Michailov et al. 2004). In the PNS, mature SCs express *Dhh*, a family member of the Hh signaling proteins that is involved in the formation of peripheral nerve sheath and is also responsible for the formation of nerve-tissue barrier. Therefore, it has been found that *Dhh* mutant mice are defective in nerve barrier formation and unable to protect themselves against inflammatory responses. The activity of *Dhh* is regulated by several molecules such as *Notch1*, *Hes5*, *MASH-1* and others (Parmantier et al. 1999).

In addition, some well-known transcription factors and molecules such as, *KROX20*, *SOX10*, *OCT-6*, brain class III POU-domain protein (*BRN2*), *GPR126*, *SREBPs*, *NF-kB*, and *Lpin1*, also play an important role in PNS myelination. *KROX-20*, a master regulator for myelinating SCs, appears to be fundamental in controlling SC differentiation, regulating the expression of a number of genes including *periaxin*, *P0*, *MBP*, and *PMP22* by interacting with *NAB* (*NGF1-A* binding) proteins. Mutation of this transcription factor *Krox-20* is associated with lethal human neuropathy such as congenital hypomyelinating neuropathy (*CHN*), *Dejerine Sottas* syndrome (*DSS*) and the *Charcot-Marie-Tooth* (*CMT*) disease. *Oct6* and *Brn2* have been implicated in the expression of *Krox-20* and may therefore positively regulate myelination (Reiprich et al. 2010).

Histone deacetylases *HDAC1/2* are major epigenetic regulators of SC development in the PNS. It has been reported that *HDAC1/2* are required for *Pax3* expression in neural crest cells and that *Pax3* is necessary for *HDAC2*-dependent control of *Sox10* levels and expression of early determinants of SCs and satellite glia. In addition, *HDAC1/2* controls the expression of *P0* directly. By regulating the interplay between *Sox10* and *Pax3*, *HDAC1/2* is thus essential for specification of neural crest cells into peripheral glia.

After nerve injury, SCs have an important role for repair by contributing growth permissive environment that allows peripheral axons to regenerate. At the same time, Wallerian degeneration completely disrupts axon-SC contacts, which is persisted throughout normal development. After Wallerian degeneration, SC redifferentiation begins in the absence of axonal *NRG1-III* and denervated SCs require autocrine *NRG1* type 1 signaling for timely redifferentiation, whereas neuronal *NRG1* (specifically the axon-bound isoform) stimulates remyelination by itself.

10.5.5.2 Tissue Repair/Regeneration

The SCs play a pivotal role during the event of mechanical damage such as spinal or peripheral nerve injury due to their regenerative properties. Following peripheral nerve injury, distal axons degenerate while differentiated SCs and macrophages remove cell debris and inhibitory molecules via phagocytosis. SCs in the distal stumps of adult animals can survive for several months in the absence of axons due to injury/insult and these SCs provide both trophic factors and adhesive substrates that promote axonal regeneration and restore the original function. After nerve injury, SCs can transform their phenotype from differentiated myelinating state to the de-differentiating state. During this process, there is also upregulation of regeneration-associated genes such as the neurotrophin receptor *p75 NTR*, *neuregulin* and their receptors (*erbB2*, *erbB3*, *erbB4*), and *GAP-43*. They also produce different trophic factors (*BDNF*, *NGF*, *CNTF*, *FGF*, *GDNF*, *TGF-β*, *IGF-2*, *NT3*, *PDGF-β*, and *LIF*), adhesion molecules (*L1*, *NCAM*), extra-cellular matrix molecules (*laminin*, *fibronectin*, and *tenascin*), and hormones such as *progesterone* and *erythropoietin*, *NFATc4*, *proteoglycans*, and *collagen type IV* in an autocrine/paracrine manner and thereby providing a favorable environment for axonal re-growth and their own survival (Taveggia et al. 2010). Transcription factor *c-Jun* is a global regulator of a SC repair program. This program involves the regulation of molecules (surface proteins *N-cadherin*, *p75NTR* and *NCAM*, and the signaling molecules *GDNF*, *artemin*, *sonic hedgehog*, and *BDNF*) that have been directly implicated in repair.

10.6 Summary

Astrocytes, OL and SC are not silent partners of others anymore as thought a couple of decades earlier. Recent works have put these cell types in the forefront of neuroscience research. Although astrocytes being the major cell type in the CNS get more attention than the other two cell types, both OL and SC play an equally important role in human health and disease through myelination of neurons in the CNS and PNS, respectively. As a result, thousands of cutting-edge research articles are coming out each year describing biological and functional aspects of astrocytes, OL and SC. Therefore, now it is an uphill task to compile everything about these three important cell types in a single chapter. However, here we have made an honest attempt to briefly delineate major biological and functional aspects of these cell types. Although there are vast body of evidence that implicate dysfunction and dysregulation of astrocytes, OL and SC in a number of human neurological diseases, we are still more or less in the dark to draw an unifying picture from these data. An improved understanding of their genesis and

function in both healthy and diseased conditions is necessary for better preservation of brain in physiological conditions and for better repairing of this organ under pathophysiological situations.

10.7 Review Questions

- During developmental stage which cell types come last:
 - microglia
 - neuron
 - astrocyte
 - oligodendrocyte*
- If there is formation of abnormal Shh protein during embryogenesis:
 - genesis of astrocyte will be affected
 - genesis of oligodendrocytes will be affected
 - genesis of neurons will be affected
 - brain development will be impaired
 - all are true*
 - none are true
- Enzyme glutamine synthetase is not found in
 - astrocytes
 - oligodendrocytes
 - none of the above is true
 - all are true*
- Tripartite synapse is formed by
 - neurons
 - microglia
 - astrocytes
 - microglia and astrocytes
 - neuron and astrocyte*
- In the central nervous system, major role of astrocyte is to
 - scavange glutamine
 - scavange cell debris
 - produce ATP
 - all the above*
 - none the above
- In the CNS, glycogen is found only in
 - oligodendrocyte
 - microglia
 - astrocyte*
 - neuron
 - all the above
- In astrogliosis, astrocytes form
 - cluster all around the CNS
 - bushy network surrounding the injury site*
 - all are true
- Schwann cells but not oligodendrocytes have phagocytic activity.
 - True*
 - False
- Unmyelinated axons generally have a smaller diameter than myelinated axons.
 - True*
 - False
- A single Schwann cell forms myelin around one and only one axon while a single
 - oligodendrocyte forms myelin around several separate axons.
- Oligodendrocyte forms myelin around several separate axons.
 - True*
 - False
- Oligodendrocytes progenitors are identified by A2B5 antibody whereas
 - Pre-oligodendrocytes are identified by O4 antibody.
- Pre-oligodendrocytes are identified by O4 antibody.
 - True*
 - False
- Which of the sequential stage is correct for oligodendroglial development?
 - Neural stem cells, progenitors, pre-oligodendrocytes, precursors and mature oligodendrocytes.
 - Progenitors, precursors, pre-oligodendrocytes, mature oligodendrocytes and neural stem cells.
 - Neural stem cells, precursors, progenitors, pre-oligodendrocytes and mature oligodendrocytes.*
 - Pre-oligodendrocytes, precursors, mature oligodendrocytes, progenitors and neural stem cells.
 - None of the above.
- Action potentials are conducted rapidly through (choose one)
 - myelinated axons*
 - unmyelinated axons
 - large diameter axons
 - small diameter axon
 - both a and c
- Which of the following molecule is not a part of the peripheral nervous system?
 - LINGO-1*
 - BDNF
 - CNTF
 - PDGF
 - EGF
- Which of the following functions in the nervous system is not provided by the
 - oligodendrocytes?
- Oligodendrocytes?
 - Ensheath axons
 - Supply neurotrophic factors
 - Form the node of Ranvier
 - Phagocytic properties to remove debris.*
- Non-myelinating Schwann cell is characterized by the following properties EXCEPT
 - Wraps axons greater than 1 μ m.*
 - Appear later than myelinating Schwann cells.
 - Myelinate all postganglionic sympathetic neurons.
 - Produce more p75NTR and GFAP.

- (e) Provide mechanical and metabolic support to the neuron.
- 20. Gliogenesis and Neurogenesis during development
 - (a) occur simultaneously during development
 - (b) *follow this sequence i.e. gliogenesis followed by neurogenesis*
 - (c) occur one after the other after the first is completed
 - (d) occur sequentially with overlapping periods

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Abstract

The mononuclear phagocyte (MP; monocyte, macrophages, dendritic cells and microglia) is part of innate immunity that functions by nonspecific surveillance and clearing response through phagocytic and intracellular killing activities. MPs included are present within the reticular connective tissues and accumulate in lymph nodes, spleen, liver and as histiocytes, tissue macrophages, Kupffer cells and microglia. It is estimated that there are at any time six billion MP/L of blood. MPs are divided into non-professional and professional categories based on function. Phagocytosis is a key function that phagocytes possess to survey their environment, ingest and process material. This chapter expands on the mechanisms, benefits, and uses of the mononuclear phagocyte.

Keywords

Antigen presentation • Complement receptors • Experimental allergic encephalomyelitis • Lectin • Mononuclear phagocyte • Phagocytosis • Progenitor cells • Toll-like receptors

11.1 Introduction

The mononuclear phagocyte (MP) or reticuloendothelial system is part of innate immunity that functions by nonspecific surveillance and clearing response through phagocytic and intracellular killing activities. MPs included are present within the reticular connective tissues and accumulate in lymph nodes, spleen, liver and as histiocytes, tissue macrophages, Kupffer cells and microglia. These are present in connective tissue, spleen, liver and brain, respectively (Van Furth and Cohn 1968; Hickey et al. 1992). MP (monocytes, macrophages and dendritic cells) play critical first line functions in human defense after pathogens, cancerous cells and injured tissues breach the skin and mucosal surfaces. MP's abilities to ingest then destroy microorganisms such as

mycobacteria, fungi, bacteria, protozoa, and viruses define them for immune surveillance. The removal of injured tissues, senescent or dying cells that reached the end of life provide a homeostatic function for aged erythrocytes, leukocytes and megakaryocytes amongst other cell types. The mechanism operative is also their coined name that is professional phagocytes. These cells have an essential role in clearance of organs through inflammation, as well as having an engaged role in the immune tumor surveillance (Diebold 1986). Removal of damaged or destroyed biological material is through phagocytosis, digestion and intracellular degradation (Klebanoff 1999).

It is estimated that there are at any time six billion MP/L of blood. MPs are divided into non-professional and professional categories based on function. The former includes epithelial and endothelial cells, fibroblasts, mesenchymal cells, lymphocytes and natural killer cells. This category has more limited phagocytic function and unlike the macrophage and dendritic cells phagocytosis is not their principal function. While they do serve to rid the body of apoptotic cells and foreign organisms and remold scars they do not produce reactive oxygen species. In the current chapter the main focus is

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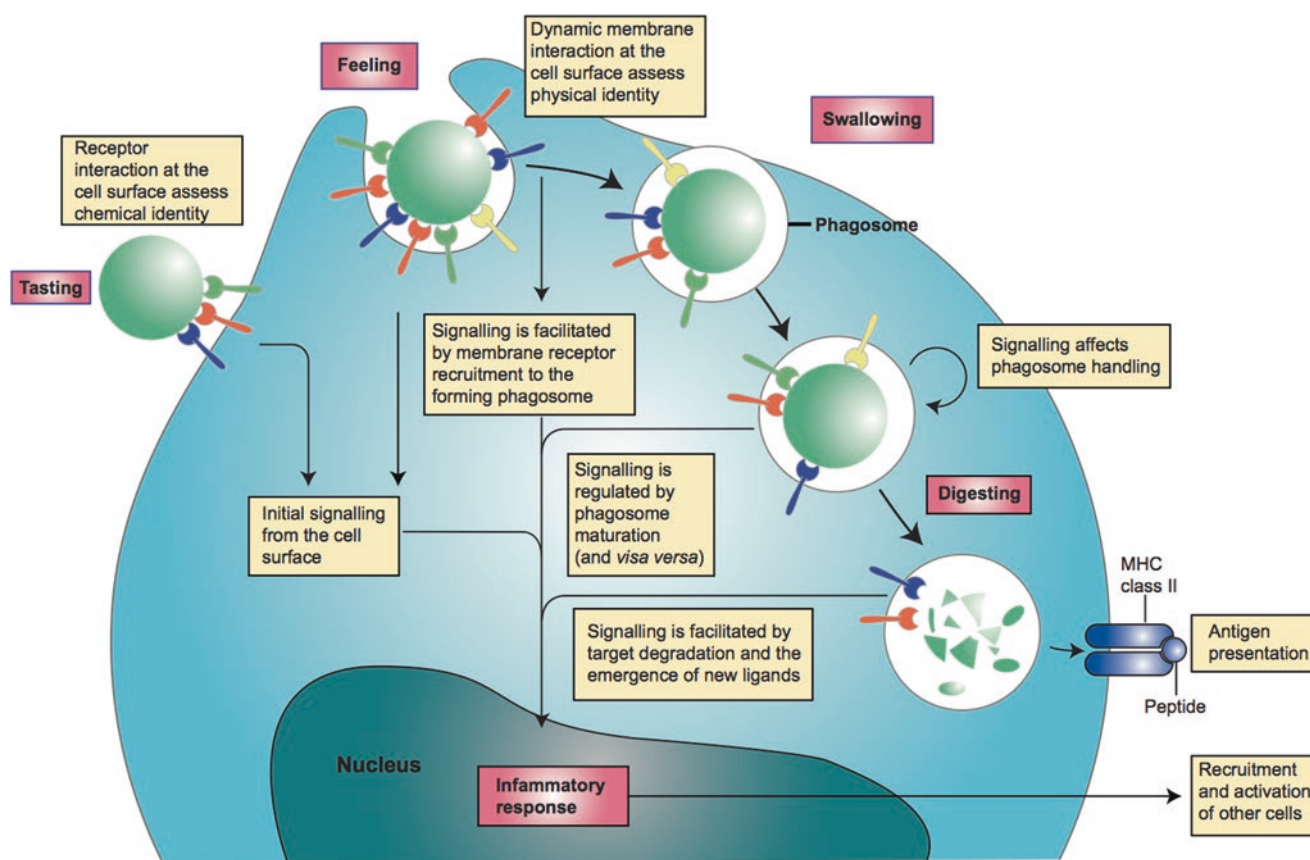


Fig. 11.1 Pathogen engulfment by phagocytosis is initiated by the pathogen-associated molecular patterns (PAMPS) on the microbe's surface. Pattern recognition receptors (PRRs) on the phagocyte sense these PAMPS and bind to them through different receptors (mannose receptors, Fc receptors, TLRs, etc.). This interaction between the receptor and ligand leads to activation of downstream pathways responsible for activation of actin filaments leading to changes in the membrane of the phagocyte to wrap around the microbe and surround it in a vesicle known as the phagosome. The phagocyte proceeds to degradation of the microbe using different intracellular killing mechanism to digest the microbe and fragment it inside the phagosome. The intracellular killings

of the phagocyte initiates appropriate inflammatory response depending on the activated downstream pathway leading to activation of other defense cells (such as T-cells and B-cells) to initiate further immune responses against the invading pathogen. Meanwhile, the phagocyte presents fragments of the digest microbe (antigens) on its MHC class II receptor. This presented antigen on the MHC II receptor would later bind to other cells to activate a full immune response against that invader. Permission was obtained to reproduce figure. Underhill DM, Goodridge HS (2012) Information processing during phagocytosis. *Nat Rev Immunol* 12(7):492–502

on professional phagocytes defined as cells expressing the major histocompatibility complex class II with specific roles in T cell immunity. The three professional phagocytes are the macrophage, the dendritic cell and B cells.

MPs evolve from blood monocytes. When they reach tissue and fully differentiate they are called by a variety of names dependent on their locale. In the liver they are called Kupffer cells, the spleen splenic macrophages, connective tissue histiocytes, brain microglia, bone osteoclasts, placenta Hofbauer cells, lymph node sinus histiocytes and perivascular macrophages through body cavities. They all together digest cellular debris and process and present antigens. In most mammalian species, monocytes make up approximately 5 % of all white blood cells. By chromosome marker and other techniques it is now well known that they are developed from pro-monocyte precursors in the bone marrow (Virolainen 1968). Following about a 3-day long devel-

opmental phase (Van Furth and Cohn 1968; Whitelaw et al. 1968) the monocytes circulate for a short period of time in the blood (less than 1 day) before they migrate to their final destinations to become fully mature macrophages where they serve as scavengers, secretory and antigen presenting cells (Volkman and Gowans 1965; Van Furth and Cohn 1968; Virolainen 1968) (Fig. 11.1).

11.2 Function

11.2.1 Phagocytosis

Phagocytosis is a key function that phagocytes possess to survey their environment, ingest and process material. The word phagocytosis is of Ancient Greek origin where phagein means “to eat/devour”, kytos means “cell”, and osis means “process”.

Hence phagocytosis means the process of cell eating or devouring. The process proceeds through endocytosis by which unicellular organisms such as protists feed while in multicellular organisms phagocytosis is a more complex process. For the latter is actin-dependent and essential for the organism's immune surveillance function and enabling the organism to face and overcome myriad of proteins, tissue metabolic products and microbial pathogens. For the immune system, phagocytosis plays an arch role in host survival. Indeed, without phagocytosis and subsequent antigen processing and presentation the ability of surveying and scanning the environment and maintaining homeostatic function would be lost rendering the organism defenseless (Volkman and Gowans 1965; Van Furth and Cohn 1968; Virolainen 1968). This is vital as a result of constant exposures to infectious agents and also to cancerous cells. MP has unique abilities to use a myriad of functions that not only include phagocytosis, by intracellular killing, secretion, migration and antigen presentation to defend itself in its environment. The vitality of the immune system rests, in large measure on the integrity of the MP system (MPS); in a study, depletion of phagocytes in the progression of a nonlethal plasmodium yoelii infection lead to severe malaria in mice accompanied with multiple organ failure and increased death. The affected mice showed critical pathological damages including coagulative necrosis in liver, fibrin deposition, and tubulonecrosis in kidney (Terkawi et al. 2016). We also posit that the broad functional capabilities of the MP can be harnessed for therapeutic gain and taking advantage of phagocytosis and antigen presentation functions to rid the body of toxic agents and microbes. Recent studies explored utilizing macrophages as depots for nanoformulated antiretroviral therapies targeting HIV reservoirs to facilitate the viral clearance (Guo et al. 2014; Martinez-Skinner et al. 2015).

During phagocytosis, the MP must recognize ligands on the microbe through specific receptors on its membrane (Kerrigan and Brown 2009). Once receptor-ligand interactions are formed, attachment takes place leading to the engulfment of that foreign body and the internalization inside the phagocyte cell; hence why phagocytosis proceeds from endocytosis (Heinrich 2015). When phagocytes come into contact with dead cells or microbes its cell surface receptors bind then engulf the microbe or cells. Destruction of these materials occurs with oxidants, nitric oxide and a range of proteases (Klebanoff 1999). Upon engulfment the foreign body persists within the phagocyte's defensive core the MP uses oxygen dependent or independent mechanisms to kill and degrade the foreign body. In most cases the process involves reactive oxygen species to degrade the engulfed substance. Innate pathogen recognition of the phagocytic cell is mediated by various germ-line PRRs found either soluble or membrane-bound on the cell such as the mannose receptor (CD206), dendritic cell-specific inter-

cellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin (L-SIGN), and macrophage galactose type C-type lectin (MGL) (Londrigan et al. 2012). PRRs are able to recognize conserved microbial structures known as PAMPs (Janeway and Medzhitov 2002). The soluble PRRs include many proteins such as ficolins, collectins, and complement that opsonize the microbe body and mark it for ingestion using specific opsonic receptors (Kerrigan and Brown 2009) In the sections following we explore the different membrane receptors that mediate the phagocytic processes.

The process of phagocytosis utilizes a variety of receptors such as mannose receptors, complements, Fc receptors, scavenger receptor, and β -glucan. Different phagocytic pathways and their signal transduction affect engulfment of pathogens (Greenberg et al. 1993; Sung et al. 1983a). Fc receptors are considered among the best-studied mediating phagocytosis. Binding of ligands and cross linking of the phagocyte's Fc receptor initiates various signaling cascades ultimately leading to formation of the activated phagosome. These receptors signal by the activation of the ITAM (immunoreceptor tyrosine-based activation motif). In 1993, experiments done on mouse macrophages revealed that the phosphorylation of tyrosines on proteins involving the Fc receptor followed signal/ligand interaction with the Fc receptor (Greenberg et al. 1993). These phosphorylated tyrosine proteins were found to be located in higher densities where filamentous actin appeared beneath phagocytic cups appearance (Greenberg et al. 1993). When they used tyrosine kinase inhibitor, genistein, they found that the appearance of tyrosine phosphoproteins were blocked along with the accumulated filamentous actin that usually formed beneath IgG opsonized particles along with their engulfment. Fc γ R-mediated phagocytosis starts by the clustering of the receptors of IgG particles. Phosphorylation of specific tyrosines with the ITAMs follows (Isakov 1997). Those ITAMs are on the cytoplasmic portion of Fc γ RIIA and also are found on γ and ζ chains joined to the Fc γ RI and Fc γ RIIIA. Src tyrosine-kinase family is the acting enzyme phosphorylating the ITAM during the initial phosphorylation (Korade-Mirnic and Corey 2000). These src kinases are inactive when a tyrosine residue that is associated with their carboxy end, SH (Src homology)-2 domain, is phosphorylated. The phosphorylated tyrosine causes a conformational change within the enzymes that leads to blocking the access to the catalytic sites of the Src enzymes rendering them inactive (Erpel and Courtneidge 1995). Upon dephosphorylation of this tyrosine residue via phosphatases, the Src kinases are active again and ready to phosphorylate the ITAMs, in leukocytes phosphatase CD45 is responsible for this dephosphorylation process (Adamczewski et al. 1995). In other instances, it was found that protein-protein interaction leading to receptor

cross-linking could also activate Src kinases. This process is thought to involve autophosphorylation and protein-protein interaction (Korade-Mirmics and Corey 2000). Few active Src kinases are thought to be joined to the cytoplasmic tails of those ITAM-containing Fc γ R chains, as a result of these receptors being close, cross-linking induce the local kinases activation (Isakov 1997; Strzelecka et al. 1997). Hence, Fc receptors can be activated by binding to IgG-coated particles where phagocytosis is initiated. In 1980, it was demonstrated that the phagocytosis of IgG-tagged lipid vesicles was dependent on the Fc surface receptors on RAW264 macrophages (Petty et al. 1980). This was measured using rosette assays for receptors measuring and monitoring the loss Fc receptors on the surface of cells when incubated with these antibody-coated lipid vesicles, in comparison with C3b receptor activity (Petty et al. 1980). The experimental results suggested that Fc receptors were depleted through IgG-mediated phagocytosis independent of the IgG density, highlighting the specific receptor-mediated phagocytic activity of macrophages. IgG-coated and uncoated erythrocytes were used to treat mouse peritoneal macrophages to determine the fate of the internalized Fc receptors. Receptor-mediated phagocytosis resulted in more than 50 % removal of the surface-expressed Fc receptors from the macrophages, compared to other surface receptors such as complements (Mellman et al. 1983). These phagocytized-internalized receptors were rapidly degraded; this was measured by immunoprecipitation experiments from lysates of 125 I-labeled macrophages. A mathematical model was developed to measure the effects of antibodies concentration on the phagocytic index of the Fc and complement receptors. The model consisted of macrophages and *Cryptococcus neoformans* fungus as the foreign pathogen. In this model, phagocytosis studies of the fungus occurred in the presence of opsonins which are the antibodies in this experiment. The system also showed antibody-mediated phagocytosis involving both the Fc and unusually complement receptors in the absence of complement (Macura et al. 2007). Experimental yields revealed reduction in phagocytic index when treating the cells with excess Fc-antibodies that blocked the Fc-mediated phagocytosis while phagocytosis via the complement receptors made up for this negative effect, pointing out the complement receptors' phagocytic function. When further increasing the antibody concentrations, the phagocytic index was also further reduced due to blockage and interference with the complement receptors (Macura et al. 2007). The engulfing mechanisms of particles through the Fc receptor differs from the complement receptor; particles coated with IgG are engulfed by lamellipodia projecting from the surface of the phagocyte while particles opsonized with complement sink in the cell (Allen and Aderem 1996). Immunofluorescence and confocal microscopy were applied to examine the different association of cytoskeletal proteins that form with phagosomes

containing IgG beads and complement-coated particles in peritoneal macrophages. During the Fc-mediated phagocytosis, proteins involved in the formation of phagosomes like F-actin, vinculin, alpha-actinin, paxillin, and phosphotyrosine-containing proteins were found to be uniformly spreading near the phagosome forming surface. While with the phagocytosis of particles coated with complement, these proteins were distributed over the phagosomal area forming a foci underneath the bound particle in less than a minute of cellular incubation in the presence of active protein kinase C (PKC) (Allen and Aderem 1996).

Complement receptors on phagocytes naturally are involved in phagocytic function and as a result are implicated in autoimmune diseases resulting from the demyelination of axons in the central nervous system such as multiple sclerosis where invading macrophages phagocytose myelin. This was demonstrated by experiments on the multiple sclerosis animal model; experimental allergic encephalomyelitis (EAE) in rats. Huitinga et al. demonstrated the critical role macrophages play in the progress of EAE. When the macrophages were eliminated, in vivo, by injecting mannosylated dichloromethylene diphosphate-containing liposomes that are capable of crossing the blood brain barrier, the liposomes were able to deplete almost all macrophages in spleen and liver due to the toxic effect manifested once these liposomes are phagocytosed. Treatment of the animals with these liposomes, after the onset of EAE, significantly slowed down the expression of the diseases (Huitinga et al. 1990). In 1991, Brück and Friede demonstrated myelin phagocytosis mediation by the complement receptor type 3 on macrophages. Macrophages attack degenerating nerves, however incubating macrophages with degenerating nerves in a C3-deficient media showed no invasion of the macrophages on the nerves. C3-deficient media meant that it lacked the complement opsonins that would have coated the myelin that shed from the degenerated nerves and hence the receptors could not detect them. Furthermore, exchanging the normal media with C3-deficient media after macrophage invasion on these nerves resulted in loss of the phagocytic activity of these cells for the myelin produced from the degenerating nerves. This highlights the crucial role of macrophages' CR3 in myelin phagocytosis. To confirm their results, an in vivo experiment was done using monoclonal antibody to the CR3 showing reduced myelin phagocytosis due to blockage of these receptors (Brück and Friede 1990). In a similar experiment, Lewis EAE model rats were treated with cobra venom factor (CVF) to abolish the serum's complement resulting in noticeably reduced clinical expression of the disease, further establishing the involvement of complements and complement receptors in the progression of EAE and similar demyelination-associated diseases (Linnington et al. 1989).

Phagocytosis of apoptotic cells is an essential physiological cell death process. Apoptotic cells are phagocytosed in an

early stage of the apoptotic onset; with intact plasma membrane before any toxic leakage occurs from the cells, avoiding activation of inflammation pathways. Unlike lipopolysaccharide (LPS), apoptotic cells do not induce pro-inflammatory cytokines' production from macrophages. These dying cells are marked by phagocytes for phagocytosis by the cluster of differentiation receptor CD14 (Devitt et al. 1998). CD14 recognizes LPS on bacterial cells as well as markers on the apoptotic cells and the same receptor mediates different macrophage-induced responses. Reacting with LPS activates pro-inflammatory function of the macrophages while interaction with apoptotic cellular markers engages the macrophages in apoptotic clearance. This highlights the effects of self and nonself stimulants on the phagocyte's role and function. Apoptotic cells interact with CD14 inducing the phagocytic function of the phagocytes to engulf the apoptotic cells (Devitt et al. 1998).

The C-type lectin superfamily are characterized by the presence of C-type lectin-like domains. The C-type lectin superfamily consists of 17 groups and categorized based on their phylogeny and domain organization (Drickamer and Fadden 2002; Zelensky and Gready 2005). Mannose receptor is an endocytic carbohydrate-binding receptor found on populations of macrophages, dendritic cells, and nonvascular endothelium. The receptor is characterized as a type-I transmembrane protein belonging to the Group VI C-type lectin and is involved in many functions among them are clearance of endogenous molecules, promotion of antigen presentation, and mediation of cellular activation and trafficking. Mannose receptor Cluster of Differentiation 206 (CD206) is considered the prototype member of the mannose receptor family of proteins. Members of this family are known to be endocytic receptors sharing common structures like the N-terminal, Cysteine rich domains, fibronectin type II domains, and many CTLDs (C-type lectin-like domains). CD206 stands out in that it is the only member of the family with a functional cysteine rich domain. It consists of a cysteine rich amino terminal in the extracellular region, fibronectin type II repeat domain, eight CTLDs, a transmembrane hydrophobic region and a short cytoplasmic tail with a single tyrosine residue occurring within a diaromatic amino acid sequence (Kruskal et al. 1992). Mutation of that single cytoplasmic tyrosine can lead to reduction of phagocytosis but would not compromise it completely (Kruskal et al. 1992). Boskovic and his colleagues had proposed a structural model of the receptor where two conformations of the receptor exist; extended form and bent compacted form (Boskovic et al. 2006). CD206 expression is up-regulated by cytokines; IL-4, IL-13, and IL-10, while interleukin IFN γ shows a down-regulatory effect on the receptor's expression (Harris et al. 1992; Stein et al. 1992; Doyle et al. 1994; Martinez-Pomares 2012). The mannose receptor binds to numerous harmful microorganisms, including *Leishmania*

donovani and *Mycobacterium tuberculosis* (Schlesinger 1993; Chakraborty et al. 2001). The receptor recognizes sugar moieties on the microbe; mannose, fucose or N-acetylglucosamine sugar residues (Largent et al. 1984). Recognition of the carbohydrates resides in the C-type lectin-like domains (CTLDs) 4-8 (Taylor et al. 1992). Taylor, Bezouska, and Drickamer found that at least three carbohydrate-recognition domains (CRDs) are needed for high affinity binding to the receptor. Several CRDs with weak affinity for single sugars are grouped and cluster to achieve that high affinity binding needed for endocytosis induction. In the case of mannose receptor, these clustered weak interactions are obtained via many active CRDs from a single polypeptide chain (Taylor et al. 1992). While there are only few examples showing the important role mannose receptor plays in the MR-dependent phagocytosis, there are enough experiments done to remove the doubt that mannose receptors are not implicated in phagocytosis. The first experiment done in 1983 showed the mannose receptor as a phagocytic receptor by demonstrating inhibition of zymosan uptake by mice peritoneal macrophages (Sung et al. 1983b), using yeast mannan which is also recognized by other receptors rendering the experiment unreliable since uptake inhibition could have been induced by those other receptors inhibited by mannan as well. However, other studies followed that proved the role of mannose receptor in phagocytosis; experiments done in 1990 showed that transfecting non-phagocytic COS-1 cell line with the receptor's cDNA resulted in the receptor-mediated phagocytosis of mannose-rich glycoconjugate and yeasts (Ezekowitz et al. 1990). Ezekowitz and colleagues also showed the importance of the cytoplasmic tail of the mannose receptor for phagocytosis to take place, they transfected the same non-phagocytic COS-1 cells with mutant mannose receptor cDNA that expressed the mannose receptor minus the cytoplasmic tail. This mutation compromised phagocytosis and showed a great reduction in the uptake of radiolabeled mannose-BSA (Ezekowitz et al. 1990). In another study using cells of the murine macrophage cell line J774A.1 to measure the phagocytosis of *Francisella tularensis*; a gram-negative bacteria and tularemia's causative agent. Expressing the mannose receptors in the cell line J774A (MR-positive J774 cells) was found to be sufficient to enhance *Francisella tularensis* phagocytosis; the cells ingested nearly threefold more bacteria than the negative control (MR-negative J774) cells (Schulert and Allen 2006). There remain contradictions regarding the importance of mannose receptors in mediating phagocytosis and the research is ongoing to clear those doubts. Various players are recruited to the phagosome; F-actin, talin, PKC α , MARCKS and myosin I. However, in contrast to phagocytosis mediated by Fc γ Rs and complement, vinculin and paxillin are not recruited during phagocytosis via the mannose receptors (Allen and Aderem 1996; Kerrigan and Brown 2009).

Toll-Like receptors are members of the pattern recognition receptors family that also recognize the PAMPs on foreign microbes. TLRs were recently found to be involved in phagocytosis. Stimulation of microglial TLR1/2, TLR3, TLR4 and TLR9 with their corresponding agonists leads to an increased phagocytic function of the cells and increased the phagocytosis of *Cryptococcus neoformans* (Redlich et al. 2013). These investigators showed that stimulating these receptors lead to increased production and release of TNF- α , CXCL1 (KC), IL-6, IL-10 and MIP-2 and reflecting microglial activation. Compared to TLR-stimulated cells, they found that unstimulated cells had a noticeable reduced phagocytosis of the *Cryptococcus neoformans* as well as decreased intracellular killing ability. That experiment was successful to show that stimulation of TLR receptors of brain macrophages (microglia) increased the immunity function of these cells against CNS pathogen/microbes. In another experiment, phagocytosis of the gram-negative bacterium *E-coli* was increased in the RAW264.7 cell line, which normally has a low basal phagocytic activity, upon stimulation of the TLR9 (Doyle et al. 2004). Doyle et al. also found that phagocytosis stimulation by TLR9 induction relied on the myeloid differentiation factor 88-dependent signaling mediated by the interleukin 1 receptor-associated kinase-4 and p38 which eventually lead to up-regulation of scavenger receptors that are heavily involved in phagocytosis (Doyle et al. 2004), hence there is a positive feedback. Moreover, stimulation of TLR3 on mouse peritoneal macrophages was found to improve bacterial uptake by inducing the activation of interferon-regulating factor 3, which plays an essential role in the innate immune system response against viral pathogens (Deng et al. 2013).

11.2.2 Intracellular Killing

After the formation of a phagolysosome, the phagocyte begins to kill/degrade the engulfed foreign body it phagocytosed. The phagolysosomal pH could be as low as 4.0 as a result of lactic acid accumulating inside; the acidity is sufficient to halt majority of pathogens growth and as a result, survival. Upon engulfment of bacteria, there is almost an immediate loss of viability so as to stop it from reproducing. The phagocyte then proceeds to lyse and digest the pathogenic body by lysosomal enzymes. This microbicidal lysis/degradation activity can be divided in two categories depending on the type of the phagocytic cell; oxygen-dependent killing and oxygen-independent killing. In the oxygen-independent intracellular killing utilizes the lysosomal granules to render the germ inactive. The lysosomal granules contain different cationic basic proteins that are able to damage the germ cell's permeability barriers, for example the lysosomal granules in the neutrophils contain

lactoferrin, which is known to be a strong iron-chelating agent that stops bacterial growth by sequestering the iron needed for the bacterial growth and survival. This is in addition to the already acidic phagolysosomal environment which optimizes the degradative lysosomal enzymes that could include nucleases, phospholipases, lysozymes, and glycosylases (Cooper 2000).

As noted crosslinking of Fc receptors on neutrophils, monocytes, or macrophages, and mannose receptors on macrophages and dendritic cells eventually leads to the formation of the activated phagosome (Greenberg et al. 1993). The signaling of the Fc and mannose receptors leads to increases of cell oxygen uptake causing a case known as respiratory burst (Root et al. 1975). The burst is initiated by the phagocytic cell receptors; Fc and mannose receptors, that are capable of activating a membrane-bound NADPH oxidase that acts to produce reactive oxygen species by reducing the oxygen to a superoxide that can be further reduced to hydroxyl radical or dismutated to hydrogen peroxides via the action of the enzyme superoxide dismutase. NADPH oxidase gets assembled into activity upon the translocation of its cytosolic proteins components; gp40^{phox}, gp47^{phox}, gp67^{phox} and Rho-family GTPase Rac2. In addition to being activated by the phagocytic receptors, NADPH oxidase can be induced by microbial products, i.e. endotoxins, also by IFN- γ , and IL-8. Those reactive oxygen species are powerful oxidizing agents capable of inflicting irreversible damage to cellular structures; membranes and nucleic acids. In addition to the reactive oxygen species; myeloperoxidase (MPO) plays an important role in intracellular killing. MPO is a heme protein that is found in the granules of monocytes and neutrophils. Once the neutrophil is activated, it released the myeloperoxidase into the phagolysosome formed with the engulfed microbe. Myeloperoxidase can also be released in the extracellular space following the binding of specific agonists. During oxygen-dependent activity, the NADPH-dependent oxidase is activated leading to the generation of hydrogen peroxides. In an activated neutrophile, both MPO and hydrogen peroxides form MPO-hydrogen-peroxide-halide system, toxic hypohalites (Klebanoff 1999); this has a powerful and potent microbicidal activity. Mice lacking MPO production in their phagocytes showed increased susceptibility to *Candida albicans* (Aratani et al. 1999). In addition to the presence of ROS, reactive nitrogen species (RNS) that are synthesized via the iNOS, macNOS, or NOS2 enzyme (inducible isoforms of nitric oxide synthase), play important and potent microbicidal role. NOS is activated by cytokines and various immunological triggers as well as being regulated on transcriptional and post-transcriptional levels including various signal transduction pathways and molecules (Bogdan et al. 2000) Both ROS and RNS exist in most phagocytic cells however each species can be more/less present depending on the phagocytic cell in topic; greater

amounts of ROS is produced in neutrophils than in macrophages, in the meantime more ROS is generally produced in macrophages (Nathan and Shiloh 2000; Fang 2004). Reactive nitrogen species can also be referred to as reactive oxygen intermediates and they range from nitric oxides to nitrates, including S-nitrosothiols, peroxynitrite, and dinitrosyl-iron complexes. While reactive oxygen and nitrogen species might seem to be redundant functionally they both play essential roles in the host immunity against harmful attacks and often work synergistically (Rutkowski et al. 2007). “A phenotype indicating that a given gene product is important for resistance to a pathogen does not imply that other gene products are unimportant in defense against the same pathogen.” (Nathan and Shiloh 2000). The oxygen and nitrogen species intermediates can and do in fact damage the host DNA and kill the cells eventually in many cases but in the immediate immunity, the phagocytic cells are quick to sacrifice themselves to prevent microbial spread (Nathan and Shiloh 2000). Mice that showed deficiency in the phagocyte oxidase (phox), the major source of microbe-induced reactive oxygen intermediate production, were found to be at higher risk of various inoculated microbes such as chronic granulomatous disease (Sharon et al. 1995; Pollock et al. 1995), CGM is characterized by repeated life-compromising bacteria and fungal infections and granuloma formation in the patient’s tissue. Intracellular digestion results from the phagocytic killing of the microbes that are now dead and degraded in the phagolysosomes. Microbes are broken down to lower molecular weight fragments using hydrolytic enzymes that include proteases, lipases, lysozymes, nucleases, and glycosylases. Neutrophils lyse following a prolonged phagocytosis and macrophages digest the microbe as well but also undergo antigen presentation into the plasma membrane for presentation to the lymphocytes for a full immune response.

11.2.3 Secretory Activities

In addition to their phagocytic activity of foreign invading microbes, phagocytes have a critical secretory role by which mediate and are involved in inflammation especially in the case of the tissue macrophages that exist throughout the human body. The table shown below lists some of the different secretory products of the MPs such as lysosomal enzymes, complement proteins, prostaglandins and interferon. In this section, we’ll focus on the importance of some of these secretory activity products secreted by the monocytes-macrophages and how they contribute to the immunity homeostasis and inflammation; the different secretions work in concert with each other and at times against each other. Activated macrophages secrete various neutral proteinases; collagenases, elastases, plasminogen, and a

cytolytic type proteinase (Adams et al. 1980; Mainardi et al. 1980; Werb and Gordon 1975a, b; Unkeless et al. 1974).

MP secrete soluble products that enhance the immune system by activating more T-cells rendering an active immune attack against invading pathogens. Endogenous leukocytic pyrogen monokine produced by phagocytic cells was shown to augment the antigen-induced proliferation of murine T cells in a similar manner as macrophage cultures containing LAF (lymphocyte activating factor—IL-1) activity (Rosenwasser and Dinarello 1981). Moreover, Takemura and Werb were able to show that the macrophage secretions of proteinases’ concentrations (elastase and plasminogen activator) can be different depending on what receptor on the macrophage gets activated by the foreign pathogen (Takemura and Werb 1984). They were able to observe different rates of proteinases secretions when IgG erythrocytes and complement-coated erythrocytes interacted with the Fc receptors and complement receptors on the macrophages, respectively. They noticed that activation of the Fc receptors resulted in accelerated synthesis of the enzyme while activation of the complement receptors resulted in a transient secretion that dropped back to control levels sooner. Different macrophage secretion products have different and multiple activities. Human recombinant IL-1 is responsible for T-lymphocytes potentiation and activation (Gery et al. 1972). In addition, IL-1 produced by the human synovial cells was found to have a great role in collagenase and prostaglandin E2 production (Dayer et al. 1986).

Some macrophage secretions can enhance each others’ release and effects in a positive or negative manner. Production of TNF (tumor necrosis factor) enhances the macrophage ability to produce its intracellular reactive oxygen intermediates which enhances TNF performance since both have antitumor activity (Badwey and Karnovsky 1980; Hori et al. 1987). Macrophage-derived interferon- α , on the other hand, can have a negative effect on the production of interferon- γ or the macrophages’ superoxides (Yoshida et al. 1988).

11.2.4 Migration

Monocytes have essential roles in both innate and adaptive immune responses. Derived from bone marrow progenitor cells (CD34⁺), monocytes circulate the blood and differentiate into myeloid dendritic cells or tissue macrophages in response to the cytokine gradient or by chemokines released in response to a microbe or toxic proteins (Volkman and Gowans 1965; Van Furth and Cohn 1968; Virolainen 1968; Wang et al. 1988; Sozzani et al. 1991; Randolph and Furie 1995). Within hours of infection, neutrophils are mobilized to the site of attack; which is now characterized with inflammatory cytokines gradient production. Those mature

neutrophils are considered the chief population of circulating granulocytes (Imhof and Aurrand-Lions 2004). On the other hand, the monocytes are not mature (undifferentiated) when released from the bone marrow. These monocytes circulate in the blood for a time span of 1–3 days (Van Furth and Cohn 1968).

Monocytes are recruited to non-inflamed tissues as well as inflamed tissues; different cytokines recruit the monocytes to different conditions of tissues. In the absence of inflammatory stimuli, cytokines as well as specific adhesion molecules are the main players in the differentiation of monocytes and their accumulation into macrophages and dendritic cells in the peripheral tissues. Those cytokines and adhesion molecules are constitutively active to allow the accumulation of macrophages and dendritic cells at all time to set up a ready immune system soldiers at all time. One of the chemoattractant proposed to play a role in the constitutive trafficking of monocytes is BRAK (breast and kidney-expressed chemokine); also known as CXCL14 (Kurth et al. 2001). There have been numerous studies done to investigate monocytes' migration and recruitment to inflamed tissue. With the use of flow cytometry, classification of the monocytes was revealed based on the expression of surface markers that included chemokine receptors like CCR2, CCR5, or CXCR3 (Grage-Griebenow et al. 2001). The inflammatory monocytes known as CD14⁺ express increased levels of CCR2 which is the receptor for CCL2; monocyte chemoattractant protein 1, MCP1 (Boring et al. 1997). Boring and his colleagues showed that CCR2 minus mice's recruitment of peritoneal macrophages was critically decreased; leukocytes failed to migrate in response to the receptor's ligand MCP1. They, hence, concluded that CCR2 played a critical role in the immune response; being involved in the recruitment of monocytes/macrophages to the inflammatory sites (Boring et al. 1997). Additional experiments on mice deficient for the expression of the CCR2 yielded the same results, reduction of the monocyte recruitments (Kuziel et al. 1997; Kurihara et al. 1997). Mice deficient in the CCR2 ligand, MCP-1, also yielded the same result of abnormally decreased monocyte recruitment (Lu et al. 1998). These experiments confirmed the critical role CCR2 and its ligand play in modulating the immune system and recruitment of the inflammatory monocytes to sites of attacks.

Many others proteins play important roles in the migration of phagocytes; of these proteins MRP8 and MRP14. The two proteins were found to be involved in the transendothelial migration of phagocytes on the level of microtubule reorganization (Vogl et al. 2004). Major calcium-binding proteins of monocytes and neutrophils, gene disruption of MRP14 (S100A9) revealed its role in transendothelial migration. MRP14 binds to MRP8 (S100A8) in a complex to induce polymerization of microtubules. The phosphorylation of MRP14 by p38 mitogen-activated protein kinase (MAPK)

results in the disruption of the microtubules polymerization induced by the MRP14/MRP8 complex. When phosphorylated, higher levels of MRP8 are needed to overcome the antagonistic effect of phosphorylated MRP14 on the microtubule polymerization (Vogl et al. 2004). The microtubule polymerization takes place in a calcium-dependent manner. Polymerization decreases with increased activity of the MAPK phosphorylation activity on the MRP14 and increases with higher levels of calcium to overcome the conformational change resulting from the phosphorylation. Another factor that induces monocyte migration is the recombinant macrophage colony-stimulating factor (M-CSF) (Wang et al. 1988). Macrophage colony stimulating factor was found to be a potent chemoattractant with a preferential effect on the monocyte-macrophage cell lineage.

11.2.5 Antigen Presentation

Most phagocytes are antigen presenting cells (APC); to mention the main phagocytic cells macrophages, dendritic cells, and B cells. There are two main classes of MHC, also known as the human leukocyte antigen (HLA); MHC I and MHC II. MHC I is recognized by CD8 T cytotoxic cells while MHC II is strictly recognized by CD4⁺ T helper cells. MHCs were first discovered during mice studies involving tissue transplantation for its critical role in histocompatibility. Antigen presenting cells express MHC II (Class II histocompatibility molecules) in order to present fragments of the antigen that the APC had encountered, to the T helper cells. Intracellular killing and processing of the antigen is a necessary step before proceeding to present the antigen to the T helper cells. The degraded processed antigen fragments are known as determinant peptides or antigen epitopes. Processing the antigen into smaller peptides/molecules is followed by the presentation of these fragments on the MHC II molecules where they can be seen by CD4⁺ (T helper cells), which then establishes and maximizes the adaptive immune response against that antigen. Upon binding of the T lymphocytes to the MHC molecule, the antigen epitope held in the binding groove of the MHC molecule then interacts with the variable Ig-like domain of the T-cell receptor (TCR) to trigger its activation.

MHC I has three main regions; telomere class I, centromeric class II, and the central class III each with different functional roles (Dukkipati et al. 2006). General structure of MHC is conserved throughout the different mammalian species. MHC genes are polymorphic; there are various alleles in a population where MHC genes are codominantly expressed markers (Amills et al. 1998). MHC I molecules are glycoproteins molecules that are, as previously mentioned, expressed on every nucleated somatic cell. MHC I is a heterodimer. Its subunits consist of a heavy chain,

composed of three extracellular subunits $\alpha 1$, $\alpha 2$, $\alpha 3$, that is bound non-covalently to a light chain ($\beta 2$ -microglobulin chain) (Bjorkman et al. 1987). Only the heavy chain of the MHC-I glycoprotein is a transmembrane segment that transverse the APC's membrane.

MHC II molecule is also a heterodimer consisting of two transmembrane chains; α II and β II chains; α II chain consists of $\alpha 1$ and $\alpha 2$ domains and β II chain consists of $\beta 1$ and $\beta 2$ domains. The chains have N-linked glycosylation sites, connecting peptides, transmembrane regions to transverse the cell's membrane, and the cytoplasmic domains (Ottaviani et al. 2008). The α and β domains form the peptide-binding groove where they present the antigenic peptide to the T cell. And as with MHC I, α and β domains are also polymorphic showing high variability, which indicates the limitless diversity of the antigens to be presented (Brown et al. 1993).

11.2.6 Drug Carriage and Depots

Targeted drug nanoparticles are the recent drug evolution that is now taking over drug research and industry. Targeted-nanoparticles delivery enables the specific and localized targeting of the drug to the cells that the drug need to be in. In some cases those receptors being targeted could be redundant between cells however the targeted system still is much more efficient and focused on fewer cells in that case rather than the whole population of innocent cells in the drug's path/vicinity. This can improve the drug pharmacodynamics and pharmacokinetics as well as bioavailability; since only specific cells are being targeted drugs could be synthesized so that they would not biodegrade/unravel from their formula unless they are in the specified cell's environment and that would not take place until they are endocytosed/phagocytosed in their targeted cells. This is one important feature about using the nanoparticles for drug delivery because it can provide controlled release hence enabling a longer half life of the drug which is an issue with almost any drug that would indicate the potency of the drug and hence the dosage and frequency of drug administration. Complex biological barriers can block treatments to prevent and treat different diseases such as the blood brain barrier. Nanocarriers can be used to reach and penetrate these biological barriers that while are vital to maintain the system viability, could prevent drug delivery from reaching the infected and diseased cells (Table 11.1).

These targeting systems for nanocarriers can utilize lipids, biodegradable polymers, or even nonbiodegradable materials. The main prototypic example of lipid-based nanocarriers are the liposomes that were first described by Bangham in 1965. Following Bangham's discovery, 30 years passed before the first nontargeted liposomal formulated drug, Doxil, was made and approved by the FDA. Using

liposomes for drug delivery proved to be efficient for many ways; since liposomes are basically consisted of lipids; hence they are poor immunogenic and as a result tolerated by the body. The other advantage of liposomes is the possibility to synthesize them in different size which gives the option of packing the liposomes with a specified amount of drug and control the payload hence minimizing the side effects. The size of the nanoparticle is essential because it would determine whether it is eliminated easily from the body or accumulated in certain organs representing a danger to the whole system. In a study with quantum dots, it was found that particles smaller than 5.5 nm resulted in a rapid and efficient urinary excretion, otherwise excretion was slow or halted with bigger particles causing particles to be accumulated in certain organs such as the liver causing detrimental pharmacotoxicity (Choi et al. 2007). These liposomes could be modified with ligands that would target certain receptors on certain cells. The more specific and localized that receptor is the more of a localized, focused targeting these liposomes would have when launched in the system/body. Another important advantage of using liposomes is their ability to be loaded with hydrophobic or hydrophilic agents to deliver into the cells, which could prove to be challenging using any other techniques. In a study targeting monocyte-derived myeloid dendritic cells, mainly targeting the DC-SIGN (CD-209), a MyDC-associated C-type lectin involved in the transmission of HIV-1 to the T helper cell, liposomes encapsulated with fluorochrome targeted the receptor on the MyDCs showing a high liposomal binding and uptake visualized by the fluorochrome (Gieseler et al. 2004).

11.3 History and Cell Discovery

The discovery of phagocytosis by the Ukrainian Zoologist, Elie Metchnikoff, in 1882 marked the official beginning of the modern immunology field as we know it. Prior to Metchnikoff's discovery for phagocytosis by 30 years, there were evidences of endocytosis (phagocytosis is a type of endocytosis) by leukocytes, the main players of the immune system, however it was Metchnikoff who officially brought phagocytosis to the light by explaining its function and crucial role in immunity. To achieve recognition of the phagocytosis theory, Metchnikoff diligently worked on it for 25 years and as a result the theory was viewed to be "the first experimentally based theory in immunology" (Heifets 1982).

In Messina, while continuing a work on comparative embryology that he had started, Mechnikov stumbled on the phenomenon of phagocytosis. This finding was a result of observations he made with starfish larvae where he saw mobile cells which he hypothesized to have connection with the organism's defense/immune system. To prove his hypothesis, Mechnikov introduced thorns from a tangerine

Table 11.1 Secretory products of mononuclear phagocytes^a

Polypeptide hormones	Inhibitors of enzymes and cytokines
Interleukin 1- α and 1- β (collectively, IL-1)	Protease inhibitors: α -2-macroglobulin, α -1-antiprotease, plasminogen activator inhibitors, plasmin inhibitors, collagenase inhibitor
Tumor necrosis factor- α (cachectin) (TNF)	Phospholipase inhibitor: lipomodulin (macroscortin)
Interferons- α	IL-1 inhibitors
Interferon- γ (confirmation needed)	
Platelet-derived growth factor(s)	Proteins of extracellular matrix or cell adhesion
Fibroblast growth factors	Fibronectin
Fibroblast activating factors	Gelatin-binding protein of 95 kD
Transforming growth factor- β	Thrombospondin
Insulinlike activity	Chondroitin sulfate proteoglycans
Thymosin B4	
Erythropoietin	Other binding proteins
Colony-stimulating factor for granulocytes and macrophages (CSF-G/M)	For metals: transferrin, acidic isoferitins, transcobalamin II
Colony-stimulating factor for granulocytes (CSF-G)	For lipids apolipoprotein E, lipid transfer protein
Erythroid colony-potentiating factor	For biotin: avidin
Factor-inducing monocytopenia	
β -Endorphin	Bioactive oligopeptides
Adrenocorticotrophic hormone	Glutathione
Plasmacytoma growth factor	
Neutrophil-activating factor	Bioactive lipids
Complement (C) components	Cyclooxygenase products: prostaglandin E ₂ (PGE ₂), prostaglandin F _{2a} , prostacyclin, thromboxane
Classical path: C1, C4, C2, C3, C5	Lipoxygenase products: monohydroxyeicosatetraenoic acids, dihydroxyeicosatetraenoic acids, leukotrienes B ₄ , C, D, E
Alternative path: factor B, factor D, properdin	Platelet-activating factors (1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine)
Inhibitors: C3b inactivator, β -1H	
Active fragments generated by macrophage proteases: C3a, C3b, C5a, Bb	Sterol hormones
Coagulation factors	1 α , 25-Dihydroxyvitamin D ₃
Intrinsic path: IX, X, V, prothrombin	
Extrinsic path: VII	Purine and pyrimidine products
Surface activities: tissue factor, prothrombinase	Thymidine, uracil, uric acid, deoxycytidine
Prothrombolytic activity: plasminogen activator	Neopterin (2-amino-4-oxo-6-trihydroxypropylpteridine)
Antithrombolytic activities: plasminogen activator inhibitors, plasmin inhibitors	
Other enzymes	Reactive oxygen intermediates
Neutral proteases: plasminogen activator, elastase, collagenases, angiotensin convertase, others	Superoxide, hydrogen peroxide, hydroxyl radical, hypohalous acids
Lipases: lipoprotein lipase, phospholipase A ₂	
Glucosaminidase: lysozyme	Reactive nitrogen intermediates
Lysosomal acid hydrolases: proteases, lipases (deoxy) ribonucleases, phosphatases, glycosidases, sulfatases	Nitrites, nitrates
Deaminase: arginase	

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tree that he had prepared as a Christmas tree for his family. Next morning, Mechnikov noticed that the thorns he had introduced were surrounded by those mobile cells he had observed in the starfish larvae. Making the connection between those mobile cells and the leukocytes that migrate

in the blood stream of organisms with a blood vascular system, play similar role in the host's defenses where they could be engulfing and eating up foreign and harmful bacteria the same way the mobile cells of the starfish larvae had eaten up the tangerine thorns. In Vienna, Mechnikov shared

his ideas with Claus, professor of Zoology, who suggested the name *Phagocytes* for those mobile cells that engulfed the tangerine thorns. In 1883, in Odessa, he gave his first paper on phagocytosis marking a fundamental breakthrough in immunology. In Paris, at the Pasteur Institute, he kept busy on establishing his theory of cellular immunity. During that time, he wrote several papers and two volumes on the comparative pathology of inflammation and in 1901 he published his famous treatise known entitled *Immunity in Infectious Disease*. In 1908 he was honored, along with Paul Ehrlich, with the Nobel Prize for Physiology or Medicine (Nobel Lectures 1967). Metchnikoff's discovery of the phagocytic function of leukocytes to rid the body of harmful bacteria lead to a huge advancement and progress in immunology and earned him the title "Father of natural immunity". The first person known to have observed phagocytosis in action under the microscope is a German clergyman and an amateur naturalist, Johann August Ephraim Goeze. In 1777 Goeze published his observations of protists ingesting other protists. In his publication, Goeze writes his observations of predators and preys as some protists ingest other "weaker" or smaller protists.

11.4 Review Questions

1. Tissue macrophages evolve from:
 - (a) Neutrophils
 - (b) Basophils
 - (c) *Monocytes*
 - (d) Dendritic cells
2. Two types of phagocytes are the _____ and _____ phagocytes.
3. Some of the critical phagocytes' receptors that play a significant role in recognizing PAMPS on foreign antigens are _____, _____, and _____.
4. This receptor was found to be implicated in demyelination-associated diseases:
 - (a) Mannose receptor
 - (b) TLR4
 - (c) CD209
 - (d) *Complement receptor 3*
5. Intracellular killing of the engulfed pathogens takes place through _____ or _____.
6. Unlike all the other cells, antigen presenting cells also express this receptor on their surface
 - (a) MHC I
 - (b) *MHC II*
 - (c) CD206
 - (d) FcR
7. Macrophage secretions can enhance each others' release and effect in a _____ or _____ manner.

8. Monocytes circulate the blood and differentiate into myeloid dendritic cells or tissue macrophages due to the _____ released in response to a microbe or toxic proteins.
9. All of these steps are involved in phagocytosis except:
 - (a) Intracellular killing
 - (b) Phagolysosome formation
 - (c) Receptor recognition
 - (d) Secretory activity
 - (e) *None of the above*
10. This protein is found to be involved in the phagosome formation mediated by Fc-mediated phagocytosis
 - (a) F-actin
 - (b) Vinculin
 - (c) Alpha-actinin
 - (d) Paxillin
 - (e) *All of the above*

11.5 Answers

2. Professional; nonprofessional
3. Fc receptors; C-type lectin receptors; Toll-like receptors
5. Oxygen-dependent killing; oxygen-independent killing.
7. Positive; negative
8. Cytokine gradient

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James Hilaire and Howard E. Gendelman

Abstract

Mononuclear phagocytes (MP; monocytes, macrophages, microglia and dendritic cells) are joined together based on common function and phenotypic properties. In regards to phagocytic, interacellular killing, secretory, antigen presentation and mobility properties differentiating the cell types can be difficult. Ontogeny, locale and modest phenotype differences underlie differences. Similarities abound in innate immunity and protection of a steady-state homeostatic environment and engaging inflammatory responses that direct surveillance and clearance responses. A clear benefit of spanning tissue origins and species differences underlies the importance of these cells that serve as the first line of immune defense.

Keywords

Dendritic cells • Macrophages • Major histocompatibility • Microglia • Mononuclear phagocytes • Multiple sclerosis • Neurodegeneration • Neuroprotection • Pathogen-associated patterns • Progenitor cells • Reactive oxygen species • Toll-like receptors

12.1 Mononuclear Phagocytes (MP)

Mononuclear phagocytes (MP) encompass a diverse collection of immune cells including monocytes, tissue-resident macrophages, microglia, and dendritic cells (DC) (Chow et al. 2011). Seemingly distinct, MP all possess the ability to facilitate phagocytosis, secrete cytokines, and initiate lymphocyte interactions (Wiktor-Jedrzejczak and Gordon 1996). Early description of MP ontogeny reported bone marrow (BM) derived promonocytes giving rise to circulating monocytes, which subsequently can populate peripheral tissue, ultimately generating tissue-resident macrophages (van Furth and Cohn 1968). Further investigation yielded the discovery of a novel cell type of discrete lineage and morphologically distinct from macrophages, termed dendritic cells (DC) (Steinman and Cohn 1973). Current research has expanded and redefined the

origins of all MP. Specifically, common myeloid progenitors (CMP) have the potential to give rise to all cells of myeloid lineage, and in more immediate terms, granulocyte/macrophage (GMP) and monocyte-macrophage-DC precursors (MDP) (Akashi et al. 2000; Geissmann et al. 2010). Further downstream, monocytes, macrophages, and dendritic cells each arise from a defined set of precursors specific to each cell type and will be expanded upon in this chapter. Each MP has a defined role in the immune system, protecting the body from and/or responding to potential pathogens. Interestingly, during infection or an inflammatory response, increased expression of macrophage colony-stimulating factor (MCSF), can directly effect the differentiation of hematopoietic stem cells (HSC) by interacting with myeloid master regulator PU.1 to transiently increase myeloid progeny (Mossadegh-Keller et al. 2013). MCSF modulation of HSC differentiation is a deviation from previous theories suggesting the influence of MCSF was on lineage-committed progenitors such as GMP, not the HSC themselves (Rieger et al. 2009; Mossadegh-Keller et al. 2013). This chapter will focus on each subtype of MP, with a specific emphasis on their interactions with the central nervous system (CNS).

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12.1.1 Monocytes

12.1.1.1 Origin and Development

Monocytes represent a diverse leukocyte subset that rapidly responds to injury or infection, aiding in the scavenging of toxic compounds or apoptotic cells (Geissmann et al. 2008; Auffray et al. 2009; Ginhoux and Jung 2014). Originating from HSCs in the bone marrow, the two main mouse monocyte subsets (LY6C^{Hi} and LY6C^{Low}) emerge from differentiation of the monocyte-macrophage DC progenitor (MDP) and subsequently the common monocyte progenitor (cMoP) (Fogg et al. 2006; Hettinger et al. 2013; Ginhoux and Jung 2014). Upon entry into the bloodstream, LY6C^{Hi} monocytes have the potential to differentiate into the LY6C^{Low} subset (Sunderkotter et al. 2004). In addition, LY6C^{Hi} monocytes actively migrate to sites of inflammation where they can differentiate into monocyte-derived macrophages (Geissmann et al. 2003). Interestingly, in the absence of inflammation, LY6C^{Hi} monocytes retain the potential to circulate back to the BM and undergo further differentiation (Varol et al. 2007). In contrast, LY6C^{Low} monocytes constitutively provide immune surveillance by patrolling endothelial cells, thereby allowing prompt responses to infection leads to inflammation (Auffray et al. 2007). Although monocytes foster the ability to differentiate into macrophages, they are not the primary source of tissue-resident macrophages (Ginhoux and Jung 2014). Certainly, this provides a stark shift from the traditional ideology of monocyte function and behavior. Tissue macrophage populations, such as those of the brain, liver, spleen, and lung are embryonically derived, thus undergo self-renewal in adult tissue (Ginhoux et al. 2010; Hashimoto et al. 2013; Jakubzick et al. 2013; Yona et al. 2013). Exceptions, such as intestinal macrophages persist, where circulating monocytes are principally responsible for the tissue macrophage population (Zigmond and Jung 2013). Monocytes are a fundamental component of the innate immune system and their biological function in the presence of neuropathology is discussed below.

12.1.1.2 Monocyte Neuroimmunology

Monocytes have a substantial role in HIV-1 pathogenesis. Infected monocytes cross the blood brain barrier (BBB) via surface adhesion molecules expressed on brain microvascular endothelial cells (BMVEC) such as E-selectin and VCAM-1 (Nottet and Gendelman 1995). Upon CNS entry, monocytes differentiate into macrophages, and develop a viral reservoir in the brain (Koenig et al. 1986). Specifically, HIV-1 infected CD14⁺/CD16⁺ monocytes cross the BBB in a CCL2-mediated mechanism and do so at a rate higher than uninfected counterparts (Eugenin et al. 2006; Williams et al. 2013). Populations of circulating CD14⁺/CD16⁺ monocytes increase during late stages of HIV-1 infection and not only accumulate in perivascular regions of the CNS, but also pre-

ferentially harbor HIV-1 compared with CD16⁻ subsets (Thieblemont et al. 1995; Fischer-Smith et al. 2001; Ellery et al. 2007). Furthermore, HIV-1 infected monocytes are resistant to apoptosis, thereby maintaining a cell population that is susceptible to infection (Giri et al. 2009). HIV-1 seeding within the CNS by monocytes significantly impacts the development of HIV-1 associated cognitive disorders (HAND). Continued research into the effect of monocytes on the progression of HIV-1 neuropathogenesis is imperative for the advancement of new therapeutic designs.

In mouse models, LY6C^{Hi} monocytes migrate to sites of infection and further differentiate within inflamed tissue. For example, in experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis (MS), significant portions of LY6C^{Hi} monocytes infiltrate the CNS, where they differentiate into monocyte-derived macrophages (King et al. 2009). CNS infiltrating monocytes during EAE may have a detrimental effect as evidence suggests monocytes recruited to the CNS via a CCL2/CCR2 mediated mechanism actually increase disease progression (Ajami et al. 2011). In terms of relapsing EAE, the balance between pro-inflammatory M1 and immunoregulatory M2 phenotypes is important. Specifically, it was observed that when the monocyte population in the blood is shifted toward M1, relapse is favored, thus subsequent administration of ex vivo M2 monocytes can suppress EAE, thereby balancing the M1/M2 equilibrium (Mikita et al. 2011).

12.1.2 Dendritic Cells

12.1.2.1 Background

Dendritic cells (DC) were discovered in the 1970s when Steinman and Cohn observed a novel cell type with a stellate and elongated morphology, constantly using its cellular processes to probe its environment (Steinman and Cohn 1973; Steinman 2007). Observed in both lymphatic and non-lymphatic tissue, dendritic cells have the capacity to ingest antigens and provide surface presentation in conjunction with major histocompatibility complex (MHC) molecules (Steinman 2007; Ransohoff and Cardona 2010). Therefore, immature DCs probe their environment for potential antigens; possess a vast array of pathogen associated molecular pattern receptors (e.g. TLRs), and react to numerous cytokines (Iwasaki and Medzhitov 2004; Villadangos and Schnorrer 2007). Receptor engagement triggers migration to lymphoid organs, where DCs can specifically engage and activate T-cells (Banchereau and Steinman 1998). Therefore DCs are essential for presenting antigen to T-cells in lymph nodes, thereby facilitating an appropriate immune response (Villadangos and Schnorrer 2007). Further detail into the ontology and biological role of DCs during neuropathology will be presented in the proceeding section.

12.1.2.2 Ontology

Dendritic cells (DC), generally categorized as either classical (cDC) or plasmacytoid (pDC), arise from progenitors within the bone marrow (Merad et al. 2013). Specifically, MDPs possess the capacity to differentiate into a common DC progenitor (CDP) (Onai et al. 2007; Liu et al. 2009). Flt3 ligand (Flt3L) is imperative for further downstream development to either cDC or pDC, as Flt3L-deficient mice have severe deficiencies in these cell types (McKenna et al. 2000). In terms of differentiation, CDPs can produce pDCs or Pre-cDCs, with only Pre-cDCs having the capacity to further differentiate into cDCs (Naik et al. 2006). The specific functions and characteristics of each cell type (cDC or pDC) will be expanded upon below.

12.1.2.3 Classical Dendritic Cells (cDC)

Pre-cDCs, generated in the bone marrow, enter the bloodstream and distribute to lymphatic tissue ultimately generating both subsets (CD11b⁺/CD8⁺) of mature cDCs (Liu et al. 2009; Merad et al. 2013). Specifically, pre-DCs gain entrance into lymph nodes through high endothelial venules (HEV), where they mature and distribute (Liu et al. 2009). Additionally, populations of CD103⁺ cDCs (resemble CD8⁺ cDCs) found in nonlymphoid tissue are also derived from pre-cDCs (Ginhoux et al. 2009). In terms of the CNS, DCs have been observed in meninges and/or choroid plexus, suggesting their potential importance in autoimmune diseases of the brain (Matyszak and Perry 1996; McMenamin 1999; Zozulya et al. 2010). Specifically, DCs populate this area in an Flt3L specific manner, in which replenishment from the bone marrow occurs every 5–7 days (Anandasabapathy et al. 2011). In mouse models of multiple sclerosis (MS), such as experimental autoimmune encephalomyelitis (EAE), disease progression can be introduced through adoptive transfer of encephalitogenic CD4⁺ T helper (T_H) cells (Greter et al. 2005). Interestingly, Greter et al. observed CD11c⁺ DCs associated with the meninges and CNS blood vessels can prime encephalitogenic CD4⁺ T_H cells, resulting in CNS inflammation and disease progression. In fact during the onset of EAE, infiltrating cDCs are found in perivascular regions, ultimately migrating to the spinal cord during disease progression (Clarkson et al. 2014). Further investigation into the biological role of DCs during EAE is necessary and may yield substantial insights in regards to MS therapeutic development.

12.1.2.4 Plasmacytoid Dendritic Cells (pDC)

pDCs, which are characterized morphologically by a rounded shape, circulate in the bloodstream and enter the lymph nodes in a CCR7 dependent manner upon activation (Sozzani et al. 1998; Cella et al. 1999; Reizis et al. 2011). Additionally, the defining characteristic of pDCs remains its

capacity to secrete large quantities of Type 1 Interferon (IFN- α/β) upon detection of foreign nucleic acids by intracellular Toll-like receptors (TLRs) (Liu 2005; Reizis et al. 2011). Specialized for viral nucleic acid detection, pDCs are able to ingest foreign antigens and engage in cross presentation to T-cells, when activated by a TLR-7/9 dependent mechanism (Kadowaki et al. 2001; Mouries et al. 2008). pDCs are active participants during neuroinflammation, exemplified by their significant infiltration into the CNS during MS and its subsequent animal model, experimental autoimmune encephalomyelitis (EAE). In EAE, pDCs accumulate around white matter lesions, but have also been observed in the cerebrospinal fluid (CSF) of MS patients (Pashenkov et al. 2001; Bailey-Bucktrout et al. 2008; Lande et al. 2008). Characterization of pDCs isolated from MS patients' revealed drastic reductions in co-stimulatory molecules CD86 and 4-1BBL expression leading to inefficient maturation, and a potential source of the immune dysregulation observed in MS (Stasiolek et al. 2006). Furthermore, Derkow et al. investigated the mechanism behind interferon- β treatment in relapse-remitting MS (RRMS) patients. Interestingly, it was observed interferon- β upregulates TLR7 in pDCs, thereby increasing its immunoregulatory activity, a possible source of protection from virus-triggered relapse in MS patients (Derkow et al. 2013). Certainly, the role of pDCs in CNS autoimmune diseases such as MS is complex and research is beginning to elucidate their pathological influence.

A hallmark of progressive immune system suppression in HIV-1 infection is reduction of CD4⁺ T-cells. In addition, infected individuals exhibit decreased levels of circulating pDCs facilitating further suppression of the immune system and development of opportunistic infections (Soumelis et al. 2001). Although numerically reduced, pDCs of HIV-1 infected individuals exhibit a hyperactivated state whereby TLR mediated chemokine/cytokine production is significantly higher than uninfected controls (Sabado et al. 2010). HIV-1 activates pDCs by interacting with CD4 on the cell surface, as well as intracellularly via TLR7 viral RNA recognition (Beignon et al. 2005). This activation produces IFN- α/β thereby facilitating activation of neighboring cDCs (Beignon et al. 2005). In addition, HIV-1 infected patients termed long-term non-progressors (LTNP) do not show deficiencies in pDC levels suggesting an important role in disease progression (Almeida et al. 2005). On the contrary, continual activation of pDCs may be detrimental as persistent IFN- α expression leads to IDO production, as well as specialized DCs with the ability to kill uninfected CD4⁺ T-cells (Fitzgerald-Bocarsly and Jacobs 2010). Certainly, pDCs play a complex role in HIV-1 infection and continued research is necessary to elucidate its role in disease progression and potential therapeutic target.

12.1.2.5 DC Vaccines

Dendritic cells constitute an integral arm of the immune system by facilitating antigen ingestion, presentation, and subsequent T-cell activation. Therefore, the design of immune therapies harnessing the specificity and potency of DCs has tremendous potential. One such therapeutic option involves the injection of ex vivo generated DCs loaded with tumor antigens in an effort to activate a tumor-specific T-cell response in patients (Steinman and Banchereau 2007). To this end, investigators have explored DC vaccines as an alternative strategy for the treatment of primary malignant gliomas, where current treatment focuses on radiation therapy and surgery (Liau et al. 1999). In early research conducted in animal models, Liau et al. pulsed dendritic cells with glioma antigens, thereby generating a DC population, which could facilitate a cytotoxic T-lymphocyte (CTL) response against the tumor and significantly increased the survival of treated mice compared to controls. Development of DC vaccines has progressed from animals into Phase I/II clinical trials in glioma patients and is thoroughly reviewed by Kim and Liau (2010) and Sampson and Mitchell (2015). Currently, DC vaccines have been predominantly clinically ineffective, although clinical trials have deemed the approach feasible and well tolerated (Sampson and Mitchell 2015). Lack of clinical efficacy may lie in the inability of a significant portion of injected DCs to migrate from injection site to the lymph nodes (Steinman and Banchereau 2007; Sabado and Bhardwaj 2015). To this end, Mitchell et al., primed DC injection sites with tetanus/diphtheria (Td) toxin and significantly increased DCs in draining lymph nodes compared to controls (Mitchell et al. 2015). Focus of current research centers around the development of adjuvants to improve the efficacy of DC vaccines and its progress bear monitoring (Sampson and Mitchell 2015).

12.2 Mononuclear Phagocytes in the CNS

The CNS contains a heterogeneous population of myeloid derived mononuclear phagocytes. Specific anatomical regions such as the choroid plexus, meninges, and perivascular space are occupied by hematopoietically-derived macrophages under pathological conditions (Prinz et al. 2011; Prinz and Priller 2014). In fact, CNS inflammation is a consistent hallmark of neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease (AD), MS, Amyotrophic Lateral Sclerosis (ALS), despite their discordant etiologies (Schwartz and Baruch 2014). Alternatively, microglia reside within the CNS parenchyma as a self-renewing resident macrophage population during both physiological and pathological states. Microglia have a distinct ontology compared to the BM-derived macrophages, which transiently infiltrate areas of the CNS during brain pathology. This section will describe in

detail the mononuclear phagocytes in the CNS and their role in the contexts of neuroimmunology.

12.2.1 Choroid Plexus (CP)

The blood-CSF barrier (BCSFB) surrounding the choroid plexus is structurally and functionally distinct from the blood-brain barrier (BBB) (Schwartz and Baruch 2014). In fact it is more permissive, exemplified by observations of CD4⁺ T-memory cells in the CSF of healthy individuals performing immune surveillance (Kivisakk et al. 2003). During incidence of neuropathological conditions such as spinal cord injury (SCI), an IFN- γ /IFN- γ R pathway is used to recruit leukocytes to the choroid plexus (Kunis et al. 2013). In models of SCI, Ly6c^{Low} monocytes infiltrate the CNS via the choroid plexus and subsequently differentiate into M2 monocyte-derived macrophages (Shechter et al. 2013). Monocyte exit from the bloodstream and subsequent passage across the BCSFB, involves adhesion molecules (VCAM-1/VLA-4, ICAM-1) expressed on the BCSFB surface, as well as CD73, an epithelial enzyme that can assist movement across epithelial cell barriers (Steffen et al. 1996; Szmydynger-Chodobska et al. 2012; Shechter et al. 2013; Schwartz and Baruch 2014).

12.2.2 Perivascular Space

Perivascular macrophages (PVM) reside within spaces surrounding cerebral vasculature and present a convergence between the immune system and the CNS (Williams et al. 2001; Kim et al. 2006). Continuously replaced by bone marrow derived monocytes, perivascular macrophages maintain functional phagocytic and antigen presentation capabilities (Hickey and Kimura 1988; Williams et al. 2001). Identification of PVM from invading monocytes and resident microglia is often difficult. Originally, CD163 was used to specify PVM, but further evidence revealed their expression in microglia as well as subsets of monocytes (Kim et al. 2006; Holder et al. 2014). Therefore, CD206 or the macrophage mannose receptor is used to differentiate PVM from CNS resident microglia and other infiltrating monocytes (Galea et al. 2005; Holder et al. 2014). In terms of neuropathological conditions such as HIV/SIV, PVM are the major CNS cell type infected in SIV and significantly increase in numbers post infection (Lane et al. 1996; Williams et al. 2001). PVM provide a means of HIV entry (other than cell free mechanisms) and facilitate the progression of disease within the CNS. Furthermore, patients exhibiting HIV-1 related neurocognitive disorders (HAND) present with increased levels of infiltrating perivascular macrophages in the brain parenchyma and represent the main population of infected cells in the CNS (Fischer-Smith et al. 2001).

12.2.3 Microglia

12.2.3.1 Background

Microglia are highly dynamic resident immune cells of the brain. Originally considered “resting” during normal physiological conditions, recently it was determined microglia constantly probe their microenvironment in an effort to thwart potential pathogens or localized cellular injury (Nimmerjahn et al. 2005). Subtle changes in the CNS microenvironment in terms of foreign DNA, antibodies, cytokines, chemokines, or abnormal endogenous protein levels, are sensed by receptors (TLRs, Scavenger receptors, MAC1, etc.), which facilitate a transition to an “activated” microglial state more apt to provide an appropriate cellular response (Block et al. 2007; Hanisch and Kettenmann 2007). Microglial activation causes polarization a kin to what occurs in macrophages, thus developing an M1 or M2-like phenotype, each presenting a markedly different cellular response (Prinz and Priller 2014). The rest of this section will focus on the mechanisms by which microglia survey its microenvironment, its reaction to environmental changes, and its role during neurological pathology.

12.2.3.2 Origin and Development

Microglia, the tissue-resident macrophage of the brain, regulates tissue homeostasis during normal physiology, as well as in CNS pathogenesis (Prinz and Priller 2014). Originally conceived as BM-derived, current evidence suggests microglia arise from a yolk-sac lineage during the early stages of embryogenesis (Ginhoux et al. 2010). Ginhoux et al., determined yolk sac macrophages arising before E8.0 significantly contribute to the homeostatic pool of microglia in the healthy adult brain. Therefore, under the current model, macrophages of the yolk sac enter the brain via the circulatory system, subsequently enter the neuroepithelium around E9.5 resembling macrophages in terms of morphology and F4/80 and CD11b surface expression (Ginhoux et al. 2010; Perry and Teeling 2013; Nayak et al. 2014). Once in the CNS, the development and differentiation of microglia is not completely defined. New molecules are continually being identified as effectors/mediators of microglia development. One such molecule, Colony stimulating factor 1 receptor (Csf1-r), is indispensable in microglial development, in that Csf1-r knockout mice exhibit extensive reductions (~99%) in microglia postnatally (Erblich et al. 2011). In addition, microgliogenesis, the process by which microglia emerge from yolk sac-derived erthromyeloid precursors is dependent on an interferon regulatory factor 8 (Irf8)/PU.1 specific pathway (Kierdorf et al. 2013). Runt related transcription factor 1 (Runx1), a regulator of proliferation and differentiation in myeloid progenitors promotes the transition of phagocytic prenatal amoeboid microglia into the ramified cells found in the healthy developed brain (Kurokawa 2006; Zusso

et al. 2012; Prinz and Priller 2014). Certainly continued research is necessary to elucidate the complex molecular and transcriptional regulation required for successful microglial development and differentiation.

12.2.3.3 Microglia Function in the Healthy Brain

Research in microglial biology often focuses on cellular behaviors during disease or tissue injury/inflammation. Interestingly, microglia exhibit dynamic behavior in the CNS under normal physiological conditions. Morphologically, “resting” microglia exhibit a ramified architecture and express low levels of CD45, MHC II, and Fc receptors on their cell surface (Perry and Teeling 2013). In terms of gene expression, neighboring neurons provide substantial input, exemplified by nerve growth factors (NGF) inhibitory effect on MHC II expression in “resting” microglia (Neumann et al. 1998). In fact, evidence continues to emerge for the prominent role of microglia during brain development and synaptic maintenance (Graeber 2010). Therefore, firm understanding of microglia cell biology under steady state conditions may provide insight into neurocognitive or degenerative conditions associated with synaptic loss or aberration.

During development, microglia actively participate in synaptic pruning (Paolicelli et al. 2011). In fact, studies utilizing two-photon microscopy confirm “resting” microglia frequently contact neuronal synapses, thereby efficiently monitoring synaptic health (Wake et al. 2009). Furthermore, interactions were prolonged in response to transient ischemia, ultimately resulting in presynaptic terminal removal (Wake et al. 2009). Moreover, microglia phagocytize retinal ganglion cell (RGC) presynaptic inputs in an activity dependent manner during development (Schafer et al. 2012). This interaction was facilitated by complement component 3 (C3) and its receptor (CR3), in what is deemed to be a microglia-specific phagocytic pathway (Schafer et al. 2012). This evidence corroborates previous work in which mice deficient of C3 exhibit developmental deficiencies in terms of synaptic elimination (Stevens et al. 2007). Furthermore, Cx3cr1 KO mice, which lack Cxcr1 expression on the microglial surface, exhibit decreased levels of microglia, as well as deficient synaptic pruning, therefore leading to increased persistence of immature synapses (Harrison et al. 1998; Jung et al. 2000; Paolicelli et al. 2011). Moreover, the neurodevelopmental deficiencies observed in Cx3cr1-KO mice manifested in decreased social interaction, coupled with increased repetitive behaviors; phenotypes often associated with models of autism spectrum disorders (Zhan et al. 2014). Research into the role of microglia during development, as well as, normal physiological conditions continues reshape our conceptions. Continued research investigating the role of microglia in maintenance and/or remodeling of synaptic networks is currently being investigated.

12.2.3.4 Microglia and CNS Homeostasis

Microglia constantly probe their microenvironment garnering information necessary to maintain CNS tissue homeostasis. Detection of environmental aberrations, such as foreign molecules or those familiar but at atypical concentrations, cause microglia to shift toward one of two activated states (Hanisch and Kettenmann 2007). This shift in phenotype, which is coupled with a transition to a more amoeboid morphology and altered surface receptor expression (CD14, MHC), facilitates an effective response to the perceived environmental changes (Block et al. 2007; Kettenmann et al. 2011). Traditional macrophages become polarized to either M1 or M2 macrophages. Phenotypically, M1 macrophages exhibit a rounded morphology and produce pro-inflammatory cytokines (IL-1, IL-6, IL-23), while conversely M2 macrophages possess a bipolar nature, and have the potential to induce T_H2 cells responses and secrete pro-inflammatory cytokines (e.g. IL-10) (Mosser and Edwards 2008; Durafour et al. 2012; Prinz and Priller 2014). Microglia in the CNS adopt similar but not identical phenotypes, thus their polarization is termed M1 and M2-like (Durafour et al. 2012). Certainly the balance between these two phenotypes is imperative. Prolonged release of pro-inflammatory cytokines by M1-like activated microglia activates NADPH oxidase and production of reactive oxygen species (ROS), potentially a source of cytotoxicity (Block et al. 2007). In the next few sections, the role of microglia (M1/M2) will be explained in terms of different disease states, yielding a better understanding into the functional role of each distinct phenotype in various disease models.

12.2.3.5 Alzheimer's Disease (AD)

Post-mortem studies of AD patients reveal accumulation of β -amyloid (A β) protein in conjunction with activated microglia, rendering a prominent pathological hallmark (Dickson et al. 1988; Hickman et al. 2008). Microglia become activated in the presence of aggregated β -amyloid and subsequently release proinflammatory cytokines, such as TNF- α (Meda et al. 1995). Additionally, microglia have been observed to secrete proteolytic enzymes in attempts to degrade these protein accumulations (Qiu et al. 1998). Receptor complexes on the microglia surface mainly comprised of CD36, $\alpha_6\beta_1$ -integrin, and CD47, bind fibrillar A β , thereby activating intracellular signaling cascades resulting in proinflammatory cytokine release (Bamberger et al. 2003). Additionally, further evidence revealed the TLR4, TLR2, and CD14, function on the surface of microglia to aid in fibrillar A β phagocytosis and microglial activation (Reed-Geaghan et al. 2009). Early phase microglial β -amyloid scavenging has been hypothesized to slow the onset of disease progression (Solito and Sastre 2012). Studies in PS1-APP mice models of AD, reveal that over time microglia lose

their ability to effectively clear A β , as evidenced by reduced expression of surface receptors responsible for A β clearance (Hickman et al. 2008). Additionally, not only do they become inefficient in A β clearance, but they continue to secrete pro-inflammatory cytokines, potentially causing cytotoxicity (Hickman et al. 2008). Continued research into the behavior of microglia during AD is critically important. Understanding the molecular biology of microglia during its different activation states, will allow the design of new targeted therapeutics slow the progression of AD.

12.2.3.6 Parkinson's Disease (PD)

PD is notoriously hallmarked by the presence of aggregated α -synuclein (Lewy bodies) and massive neuronal loss within dopaminergic neurons of the substantia nigra pars compacta (SNpc) (Braak et al. 2003). Furthermore, it has been observed that activated microglia accumulate in the SNpc of PD patients (McGeer et al. 1988). In fact, PD patients exhibit enhanced reactive microgliosis, a process of recruitment, proliferation, and activation of microglia in sites of neuronal injury (Hu et al. 2008). Upon aggregation, microglia phagocytize α -synuclein and become subsequently activated, thereby facilitating neuronal toxicity (Zhang et al. 2005). Activated microglia, which present with an upregulation of MHC-II and release TNF- α and IL-6, are observed to not only interact with lewy bodies, but also damaged neurons themselves (Imamura et al. 2003). Furthermore, activated microglia in early PD present an M1-like phenotype, which is coupled with an upregulation of NADPH-oxidase and subsequent ROS production are observed in the substantia nigra of PD patients and MPTP mouse models (Wu et al. 2003). Prolonged microglial activation may have a negative impact on disease progression. In fact, microglia is hypothesized to be the prominent facilitator of neuroinflammation during states of neurodegeneration such as PD, exemplified by blockade of microglial activation has a neuroprotective effect (Wu et al. 2002; Gao and Hong 2008). Efforts to control the inflammatory response provoked by activated microglia has been studied with the use of non-steroidal anti-inflammatory drugs (NSAIDs), anti-inflammatory cytokines, as well as treatment strategies centered on regulatory T-cells (T_{Reg}) (Qian et al. 2010). Specifically, adoptive transfer of Tregs in MPTP mice was able to produce a neuroprotective effects by modulating the response of microglia to neural injury (Reynolds et al. 2007). Subsequent studies using GM-CSF to induce a Treg cell response in MPTP mice provided reductions in microgliosis, as well as neuroprotection (Kosloski et al. 2013). Restoring the balance between T_H1/T_{Reg} cells in response to neuroinflammation in diseases such as PD may have significant therapeutic importance and are currently under development (Mosley et al. 2012).

12.2.3.7 HIV-1 Associated Neurocognitive Disorders (HAND)

Early studies with HIV-1 infected patients observed evidence of progressive cognitive impairments, often coupled with motor and behavioral aberrations, and was originally termed AIDS dementia complex (Navia et al. 1986). Research involving HIV-1 infection of the CNS and its clinical effects has significantly progressed and currently are described as HIV-associated neurocognitive disorders (HAND), which can be further broken down into asymptomatic neurocognitive impairment (ANI), mild neurocognitive disorder (MND), or HIV-1 associated dementia (HAD) (Antinori et al. 2007). The advent of combination antiretroviral therapy (cART) greatly improved patient's quality of life, whereby patients exhibit viral suppression and immune function restoration (Katlama et al. 2013). The effect of cART on HAND is still being investigated but reports suggest pre-cART patients have a prevalence for motor skill aberration, while cART treated patients effected by HAND tend to be deficient in learning/memory tasks. Interestingly, cART adherent patients that are cognitively asymptomatic still harbor activated microglia (Garvey et al. 2014). In fact, HIV-1 DNA has been isolated from parenchymal microglial in presymptomatic HIV-1 infected individuals (Thompson et al. 2011). HIV-1 infection of microglia is mediated not only by CD4, but co-receptors CCR5 and CCR3 (He et al. 1997). Microglia have low surface expression of CD4 and evidence suggests that viral variants, which can infect cells with very low CD4 expression, are present in the CSF of HAD patients (Schnell et al. 2011). In fact, this Microglia/macrophage tropic viral variant is differentially distributed as its found in very low levels in the blood compared with the CSF (Schnell et al. 2011). HIV-1 infection of microglia and macrophages promote the secretion of neurotoxins that facilitate the progression of HAND (Nottet and Gendelman 1995). Specifically, recent research suggests HIV-1 infected microglia can induce neurotoxicity by increased release of glutamate by means of upregulation of glutaminase C (Huang et al. 2011). Further research on microglial HIV-1 infection and its subsequent effects on HAND progression is currently being delineated and imperative towards the design of future therapeutics.

12.3 Future Perspectives

Mononuclear phagocytes (MP), consisting of monocytes, macrophages, microglia, and dendritic cells are prominent cells types of the innate immune system (Chow et al. 2011). A dynamic interplay between MP and the CNS is critical during several neuropathologies. In fact, neuroinflammation is conspicuous in neuropathological conditions of diverse etiologies ranging from PD, AD, and HAND, thus illuminating the importance of MP and the immune system during

CNS disorders. Harnessing and altering the response of MP and the rest of the innate and adaptive immune system during CNS diseases has vast therapeutic potential. For example, immunomodulation of PD, in which agents promoting Treg production can attenuate the neurotoxic effects of activated microglia by stimulating an anti-inflammatory and neuroprotective environment have been proposed and are being investigated (Olson and Gendelman 2016). Moreover, dendritic cells continue to be harnessed as potential treatments for cancers affecting the CNS, such as gliomas. (Sampson and Mitchell 2015) Furthermore, in numerous neurological conditions, infiltrating monocytes tend to have a negative effect on disease progression, often exacerbating pathological conditions. Therefore, harnessing the phagocytic and migratory capabilities of monocyte and monocyte-derived macrophages (MDM) to design new therapeutic treatments has immense potential. Specifically, nanoparticles surface functionalized with ligands (e.g. Folic Acid) which specifically bind and enter MDM create a cell-based delivery system to the brain to treat CNS disorders (Gendelman et al. 2015). Thus, advancements in MP biology will not only improve our understanding of the innate immune system, but also elucidate the intricate mechanisms of neurodegeneration. Understanding the dynamic interplay between CNS resident cells and infiltrating immune cells may be essential in halting the progression of numerous neuropathologies, thus moving neuroimmunological research to the forefront of disease modification efforts.

12.4 Review Questions

- The functions of mononuclear phagocytes include all except:
 - Phagocytic
 - Interacellular killing
 - Secretory
 - Antigen presentation
 - Transformation to lymphoblasts*
- Mononuclear phagocytes include each of the following except:
 - Monocytes
 - Macrophages
 - Dendritic Cells
 - B Cells*
 - Kupffer Cells
- Neuroinflammation is conspicuous in neuropathological conditions of diverse disorders ranging from PD, AD, and HAND. The following are produced by activated microglia and macrophages known to negatively affect neuronal integrity except:
 - Tumor necrosis factor alpha
 - Interleukin-1 beta

- (c) Interleukin-6
 - (d) *Brain derived neurotrophic factor*
 - (e) Quinolinic acid.
4. Harnessing MP function can be used in the following ways for treatment of CNS disease.
- (a) Anti-inflammatory
 - (b) De-activation
 - (c) Phenotypic transformation from an M1 to an M2
 - (d) Secretion of trophic factors
 - (e) *All of the above*
5. Which of the following cells do not arise from progenitor cells in the bone marrow.
- (a) Dendritic Cells
 - (b) *Microglia*
 - (c) Perivascular Macrophages
 - (d) Monocytes
 - (e) b and c

True/False

- 6. Microglia express both CD4 and CCR5, two receptors essential for R5-tropic HIV cellular entry.
- 7. LY6C^{Hi} and LY6C^{Low} monocytes both emerge from cMoP in the bone marrow.
- 8. The number of circulating pDCs increases during the course of HIV-1 infection.
- 9. During normal brain physiology, microglia express high levels of MHC II on the cell surface.
- 10. VCAM-1 and I-CAM-1 are essential for the passage of circulating monocytes across the BCSFB and entering the choroid plexus.

12.5 Answers

- 6. T
- 7. T
- 8. F
- 9. F
- 10. T

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Abstract

Microglia are resident cells of the brain involved in regulatory processes critical for development, maintenance of the neural environment, injury and repair. They originate from erythromyeloid progenitors (EMPs) in the yolk sac and develop in the forming CNS. Microglia serve as brain immune cells to orchestrate innate immune responses, however, they are distinct from other tissue macrophages due to their unique homeostatic phenotype and tight regulation by the CNS microenvironment. They exhibit multiple morphological phenotypes and functional profiles depending on their environment. Microglia actively survey the surrounding parenchyma and respond rapidly to changes such that any disruption to neural architecture or function can contribute to the loss in regulation of the microglia phenotype. Alterations in microglia functionality are involved in brain aging, as well as in neurodegenerative diseases. In many models of neurodegeneration and neurotoxicity, early events of synaptic degeneration and neuronal loss are accompanied by an inflammatory response including activation of microglia, perivascular monocytes, and recruitment of leukocytes. As the primary source for pro-inflammatory cytokines, microglia are implicated as pivotal mediators of neuroinflammation and can induce or modulate a broad spectrum of cellular responses. One key question in determining the consequence of neuroinflammation is whether the response is an initiating event or the consequence of tissue damage. Microglia execute varied tasks, not typical of those carried out by peripheral macrophages, which support optimal function of neurons and neuronal networks. Recent observations about microglia ontogeny combined with extensive gene expression profiling and emerged novel tools to study microglia biology allow us to characterize the variety of microglial phenotypes during development, homeostasis and disease.

Keywords

Microglia • Origin • Phenotypes • Functions • Regulation • Homeostasis • Neurotoxicity • Neurodegeneration

13.1 Introduction: History of Microglia

Microglia—from micro (small) and glia (glue)—are the resident innate immune myeloid cells in the central nervous system (CNS) parenchyma. These cells constitute about 5–12 % of the total glial population (Ling and Leblond 1973). They are present throughout the CNS, including the spinal cord, although some regions are more populated than others. The white matter generally contains fewer microglia than the

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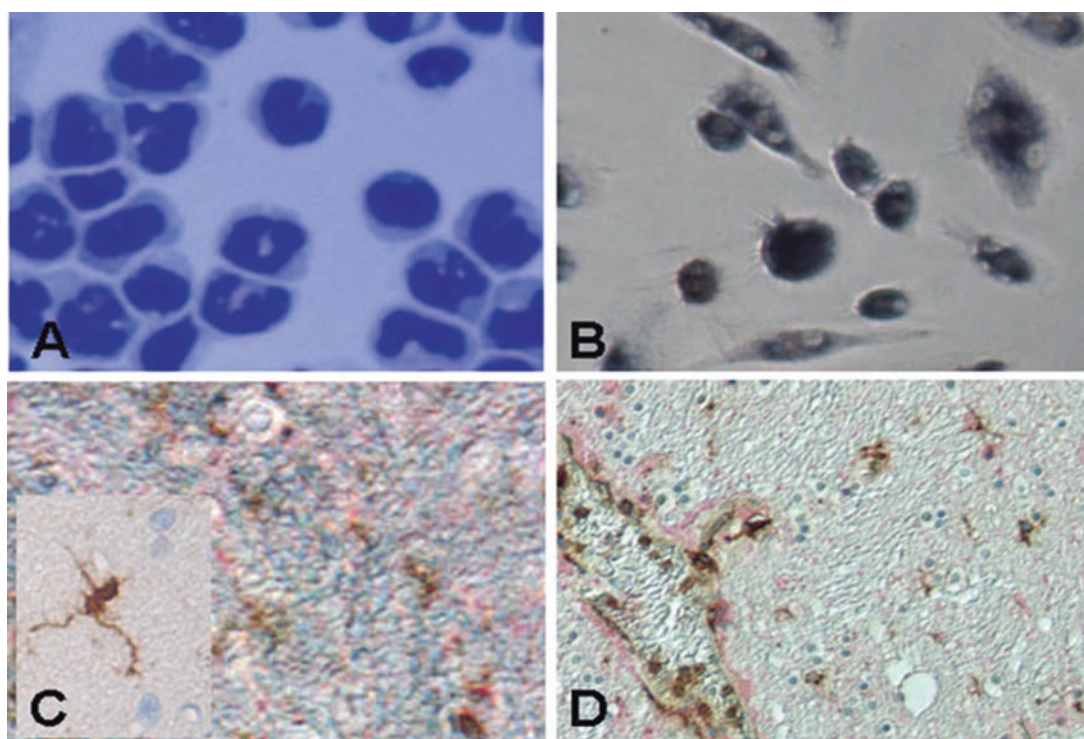


Fig. 131 Morphological and immunohistochemical characteristics of monocytes and macrophages. *Panel A* demonstrates Wright's stain demonstrating the classical monocyte kidney-shaped nucleus. *Panel B* demonstrates the fine processes on microglial cells called the fimbriae. In the

grey matter. Robertson and Nissl were the first to describe these cells at the end of the nineteenth-century. Nissl first recognized microglia in the brain (1899). However, microglia were first identified as a defined cellular component or “mesoglia” by Pio del Rio Hortega in 1919 (Rio-Hortega and Sociedad española de historia natural Madrid. [from old catalog] 1932). He first characterized a small cell in the neuroectodermal tissue, which apparently was of mesodermal origin and seemed to be related to other tissue macrophages in the body (Rio-Hortega and Sociedad española de historia natural Madrid. [from old catalog] 1932). Rio Hortega postulated that microglia enter the brain during early development and take on a distinct amoeboid morphology. Moreover, he described functional features, such as the ramified phenotype in a mature brain (Fig. 131), or amoeboid phenotype in case of lesion. Based on these features he hypothesized that microglia have a defined territory to survey but they are able to migrate, proliferate, and phagocytize debris in the brain. These conclusions remain valid but they can be refined with the increased amount of research generated on microglia over the last few decades. In particular, new exciting results showing that microglia are highly dynamic and frequently interact with neuronal elements, raised the possibility that microglia play a fundamental role in monitoring of neuronal activity, the surveillance of the neuronal milieu, structural remodeling of neurons and modulation of neuronal and synaptic plasticity

brain, microglial cells demonstrate a typical ramified phenotype as shown in *Panel C*. In neuroinflammatory diseases, such as HAD, cells from the periphery infiltrate the brain as shown in *Panel D*. A blood vessel in brain parenchyma is surrounded by perivascular macrophages

(Blais and Rivest 2003; Hanisch 2013; Kettenmann et al. 2011, 2013; Prinz et al. 2014).

Microglia are one of the first cells to respond to an environmental change in the brain. Perivascular cells such as CD163⁺ macrophages, at the blood brain barrier (BBB) located in the circumventricular organs, will sense any stimulus coming from the periphery and relay this information to microglia. When a tissue is damaged or a bacterial or viral infection is detected in the CNS, microglia is playing a critical role to clean and restore brain homeostasis. Microglia are not only essential under pathological conditions but they also maintain homeostasis in non-inflammatory conditions. They constantly sense their environment by extending and retracting their processes to probe the brain parenchyma for any tissue/cell damages or intruders. Microglia contribute also actively to shape the brain during neurodevelopment. They act on neuronal proliferation, neurogenesis and formation/suppression of synaptic connections by phagocytizing.

13.2 Origin of Microglia

Defining the origin of microglia has been an intensive question and a longstanding issue of debate. Traditionally, microglia were considered as the brain macrophages defending the CNS following acute lesions and neurodegenerative

diseases. An increasing number of studies pointed to a very early colonization of the CNS by mesodermal progenitors (Kaur et al. 2001) and indicated that microglial progenitors arise from the yolk sac (Alliot et al. 1999). Since the work of Ginhoux et al. (2010), microglia are now known to originate from the yolk sac during embryogenesis (Alliot et al. 1999; Ginhoux et al. 2010; Herbolme et al. 2001). Although there is a consensus about the myeloid origin of microglia, much controversy remained concerning the origin of microglia progenitors.

Microglia were shown to derive from primitive myeloid progenitors of the yolk sac that invade the CNS during early embryonic development around E8.5 (Ginhoux et al. 2013) after the establishment of the blood circulation. Once in the parenchyma, they start to proliferate and differentiate in mature microglia during postnatal brain development. Microglia were first thought to be similar to macrophages but it is now apparent that they differ in ontogeny and functional capacity (Nayak et al. 2014). Microglia are derived from an uncommitted F4/80-negative erythromyeloid precursor in the yolk sac that develops via immature F4/80-CX3CR1⁻ myeloid progenitor subsets into F4/80⁺CX3CR1⁺ mature macrophages, which finally colonize the CNS to give rise to microglia (Kierdorf et al. 2013). Fate-mapping studies have established that transcription factor Myb, required for hematopoiesis is not necessary for the generation of microglia (Schulz et al. 2012) and that they specifically originate from CD45-c-kit⁺ erythromyeloid progenitor cells (Kierdorf et al. 2013).

Circulating monocytes and tissue macrophages thought to participate to microglia population are no longer considered as microglia progenitors in a healthy brain (Gomez Perdiguer et al. 2013; Kierdorf et al. 2013; Neumann and Wekerle 2013). Indeed, knocking-down of Pu.1 gene leads to the absence of yolk sac-derived microglia in the postnatal brain (Beers et al. 2006), and infiltration of bone marrow-derived myeloid cells in the brain. Peripheral monocytes and macrophages derive from hematopoietic stem cells initially located in the fetal liver and later in the bone marrow (Gomez Perdiguer et al. 2013). Monocytes can infiltrate the CNS and play the role of microglia under specific conditions (Fig. 131) (Ajami et al. 2007; Ginhoux et al. 2013; Varvel et al. 2012).

In the adult brain, microglia population is exclusively maintained by self-renewal in physiological conditions (Ginhoux et al. 2013). It was originally hypothesized that continual replenishment of the microglia population occurred into adulthood via peripheral recruitment of circulating monocytes, followed by subsequent differentiation steps. However, although murine monocytes have been shown to invade the CNS amidst insult (Andersson et al. 1992) and microglia demonstrate the potential to differentiate into either CNS-macrophage or dendritic cell profiles in vitro

(Santambrogio et al. 2001), evidence for monocyte to microglial differentiation in the CNS is lacking (Ginhoux et al. 2010; Ajami et al. 2007). Microglia are then the only immune cells, and permanently reside in the CNS parenchyma, alongside neurons, astrocytes, and oligodendrocytes. Specifically, colony-stimulating factor 1 receptor (CSF1-R) but not colony-stimulating factor 1, is necessary for regulating the microglia lineage, because inhibition of CSF1-R in mice leads to depletion of 99 % of the microglial population (Elmore et al. 2014a). Microglia, upon successful CNS-seeding of their progenitors in early development, act as an independent, self-renewing population into adulthood (Ginhoux et al. 2010; Bruttger et al. 2015).

13.3 Phenotypic Diversity in Development and Aging

Microglia cells are often sub-divided in either resting or activated states depending on the inflammatory milieu (Ransohoff and Cardona 2010; Kettenmann et al. 2011; Hanisch and Kettenmann 2007; Hanisch 2013). Contrary to this simplistic view, microglia can also be considered “active” in normal conditions. Microglia show high level of phenotypic and functional plasticity in healthy and inflamed brains. Indeed, multiples microglial phenotypes exist and they will adapt and switch phenotype based on alterations in response to cues from the microenvironment (Hanisch 2013, 2014). The spectrum of activated microglia phenotypes is based on macrophages plasticity at the periphery. Peripheral macrophages are known to show phenotype plasticity represented by varied expression of cell surface receptors (Mantovani et al. 2005). Microglia phenotypes following polarization have been adapted from macrophage polarization studies which include M1-like (proinflammatory signaling and neurotoxicity) vs. M2-like phenotype (resolution of inflammation) (Martinez and Gordon 2014). M1/M2 phenotypes represent different states of macrophage activation, based on Th1/Th2 phenotypes for lymphocytes. This last decade, scientists have been focusing on microglia plasticity trying to demonstrate that microglia can adopt different phenotypes such as M1/M2 phenotypes in disease. Microglia were then thought to present different states of activation including classical activation state M1 (in response to interferon gamma and LPS) and alternative activation state M2 (after exposure to IL-4 and IL-13) (Liao et al. 2012; Gordon and Martinez 2010; Colton 2009). Classical activated microglia were considered neurotoxic whereas alternatively-activated microglia would be neuroprotective (Liao et al. 2012; Kigerl et al. 2009). However, this is a simplistic view of microglia activation. Indeed, microglia can adopt a pro-inflammatory M1 phenotype and become neurotoxic in advanced stages of neurodegenerative diseases, such as

Alzheimer's disease, multiple sclerosis and amyotrophic lateral sclerosis (Liao et al. 2012; Muzio et al. 2007; Butovsky et al. 2012), but microglia phenotype and function can be difficult to classify based on these characteristics. During disease, different microglia phenotypes are apparent, however, these phenotypes differ with the brain structure and pathology. Moreover, in the past, disease-associated microglial phenotypes have been studied using approaches that could not distinguish between resident microglia and recruited myeloid cells. However, these phenotypes do not represent microglia states in health or disease and increasing evidence, including data from this report, suggest that the M1/M2 paradigm does not apply to microglia in disease (Butovsky et al. 2014; Holtman et al. 2015a, b; Hickman et al. 2013; Chiu et al. 2013). Thus, characterization of microglia diversity in pathology and physiology is complex and may be left to interpretation but with the help of new developed technologies, research on microglia phenotypic diversity will be more efficient in next few years.

13.3.1 Development

During prenatal development, fetal microglia is characterized by an amoeboid morphology which is not conserved after maturation of the CNS when they become ramified. However, this morphology can be found in the adult under certain pathological conditions during a lesion or an infection of the CNS. Fetal microglia are characterized by a large cell body, a large amount of cytoplasm and the expression of specific markers. Microglia during development express low-density protein receptors (LDL) (Giulian and Baker 1986) and scavenger-type receptors (Husemann et al. 2002). Fetal microglia have been shown to express Iba1, CX3CR1 and CD45 molecules around E12–E15 days of development (Harry 2013). Recently, fetal microglia have been shown to present a specific molecular signature during development and before maturation during postnatal period (Butovsky et al. 2014). Indeed, they express molecules that are usually present after an immune activation and during phagocytosis.

13.3.2 Adulthood

The idea of microglia phenotypic plasticity is not new. Microglia plasticity corresponds to the diversity of phenotype and functions the cells can adopt and do to face a challenge. This is already known in the context of neuroinflammation and during microglia activation. Indeed, it is known that during neuroinflammation, microglia will become amoeboid and secrete inflammatory factors. However, microglia plasticity is a more complex phenomenon that some have observed in inflammatory conditions based on the concept of macrophage morphoplasticity already detailed at the periphery (Mantovani

et al. 2005). In physiology, this is more complex to describe and is biased by the techniques employed to observe microglia. Recently, several groups identified a unique homeostatic signature for microglia at different ages and in different conditions. Microglia homeostatic signature has been shown to be dependent on TGF β signaling (Butovsky et al. 2014) with the expression of specific genes such as *P2ry12*, *Tgfb1*, *Tmem119*, *Fcrls*, *Hexb*, *Cx3cr1* (Butovsky et al. 2014). Microglia present a unique signature at the cell surface but also a specific sensome which is dysregulated during aging (Hickman et al. 2013).

13.3.3 Aging

Aging is a complex process of cumulative changes. A key feature is the progressive decline in physiological functions and behavioral capacity, which is observed at various levels of the organism and in particular in the CNS. Some responses of the immune system also decline with age increasing the vulnerability to infections and cancer. On the opposite, other immune responses are exacerbated such as generation of chronic neuroinflammation mediated by the dysregulation of the innate immune system. Microglia is undergoing several age-related changes that contribute to a chronic inflammatory environment including production of inflammatory cytokines and reactive oxygen species. Microglia are activated in nearly all CNS diseases (Kreutzberg 1996a; Hanisch and Kettenmann 2007; Neumann et al. 2009). Genes that encode microglia surface receptors for neuron-microglia crosstalk are affected. Microglia with aging are controlled by transcription factors such as Runx1, Irf8, Pu.1 and Tal1 (Kierdorf and Prinz 2013). Aged microglia display morphological changes with fewer and shorter processes, an increased cell body and formation of spheroid swellings, which refers to a dystrophic microglia (Streit et al. 2004; Streit 2006; Flanary et al. 2007). Microglia colocalize with neurodegenerating neurons and present an accumulation of phagocytic inclusions (Hefendehl et al. 2014; Tremblay et al. 2012). The unique signature present during adulthood is dysregulated during aging with the downregulation of most of their homeostatic genes and upregulation of genes associated to inflammation (Holtman et al. 2015b).

13.4 Morphological Characterization

13.4.1 Homeostasis

Microglial cells have been classified into three types: ramified, amoeboid and reactive. In the prenatal brain, the amoeboid phagocytic microglia are the predominant form, with a large spherical cell body and short processes (Hess et al. 2004). During postnatal maturation, amoeboid microglia

Table 131 Surface markers for macrophages/microglial

Chemokine receptors
CCR1, CCR2, CCR3, CCR5, CCR8, CCR9, CXCR1, CXCR2, CXCR4, CX3CR1
Complement receptors
C3b, C3d, C3bi, C1q
Cytokine receptors
Interleukins 1, 2, 3, 4, 6, 7, 10, 13, 16, 17
Interferon α , β , γ
Colony stimulating factors
Fc receptors
IgG2a, IgG2b, IgG1, IgG3, IgA, IgE
Fibronectin and Laminin receptors
Mannose, fucose, galactose
Integrins (CFA-1, CR3, CR4, VLA-4)
Toll-like receptors (pattern recognition)
CD4, CD14, CD16, CD45, CD68, CD163, HLA-DR

transform into ramified resting microglia, and these cells remain a semi-permanent population with relatively slow turnover rates when compared to peripheral macrophages (Dick et al. 1995; Becher and Antel 1996). Ramified microglia act as surveying cells by actively sensing the surrounding environment via dynamic processes (Schlegelmilch et al. 2011; Stence et al. 2001; Hanisch and Kettenmann 2007; Nimmerjahn et al. 2005). As homeostatic (“resting” previous term) ramified microglia, they monitor their microenvironment and adapt their morphology and express cell surface markers accordingly (Table 131) (Lawson et al. 1990, 1992). The resident microglial cells in the healthy brain can be seen in all regions of the CNS including optic nerve and retina (Kaur et al. 2001). They remain homeostatic (“quiescent”-previous term) until stimuli from injury, infection or neurodegenerative process activate their transformation into amoeboid phagocytic cells (Schroeter et al. 1997; Zhang et al. 2001). Microglia exhibit multiple morphological phenotypes and, presumably, multiple functional profiles depending on their environment (Streit 2006; Streit et al. 2004). Structurally, microglia display a dynamic and active phenotype with ongoing retraction and extension of processes into the brain parenchyma (Raivich 2005). The morphology of a “resting” microglial cell is characterized by a very small cell soma with elongated ramified processes (Cuadros and Navascues 1998). Under healthy conditions, microglial cell processes do not overlap with processes of neighboring cells and each cell seems to have a scavenger function for its own immediate area. The position of the cell soma remains stable, whereas the processes of the resting microglia are continuously elongating and retracting to explore the tissue environment. In vivo imaging of microglia in intact brain tissue demonstrated highly dynamic processes which continuously scan the surrounding microenvironment (Nimmerjahn et al. 2005).

13.4.2 Disease

Amoeboid microglia are highly motile and participate in phagocytosis (Suzumura et al. 1991; Szabo and Gulya 2013). In addition, bipolar/rod-shaped microglia transiently form trains of cells aligned end-to-end at the damaged site after brain injury (Taylor et al. 2014). Bipolar/rod-shaped microglia are also found in the cerebral cortex of Alzheimer’s subjects (Wierzb-Bobrowicz et al. 2002). Upon recognition of a pathogen or other inflammatory stimuli, microglia can rapidly retract their processes and become efficient mobile effector cells (Davalos et al. 2005). These observations highlighted the important immune surveillance function of microglia in the healthy CNS parenchyma. In general, microglia activation is triggered by a plethora of well-described subsets of immune receptors such as Toll-like receptors (TLRs), scavenger receptors, and numerous cytokine and chemokine receptors. This supports the idea of a surveillance function for microglia in the healthy brain and indicates that these cells are poised to rapidly respond to environmental changes. This concept has been supported by the observation that microglial activation is likely an early event in all forms of pathology. In the human brain, microglial activation and neuroinflammation have been associated with viral or bacterial infection, autoimmune disease such as multiple sclerosis, head trauma, vascular system damage, neuropsychiatric disorders, and neurodegenerative diseases. The presence of activated microglia was initially considered as a sensitive marker to identify sites predestined for imminent tissue destruction. Based upon this, a role for microglial activation and neuroinflammation has more recently been considered as an underlying and, possibly, unifying factor of neurotoxicity from environmental exposures.

13.5 Neuroinflammation

Upon infection or insults within the adult CNS parenchyma, microglia are rapidly activated and efficiently phagocytose pathogens and dying cells (Hanisch and Kettenmann 2007; Ransohoff and Perry 2009) and release effector molecules for the recruitment of other immune cells from the blood to limit infections in the CNS (Saijo and Glass 2011). In addition, they help in the regeneration of damaged tissue by secretion of growth factors and anti-inflammatory molecules (Saijo and Glass 2011). Therefore, microglia are indispensable in the adult CNS as stabilizers and modulators of tissue homeostasis under physiological conditions.

Inflammation is a host defense response to injury, tissue ischemia, autoimmune responses or infectious agents. Classical signs of swelling, redness, heat and pain are witnessed in all tissues except that of brain as manifestations of inflammation. These symptoms are caused as a result of increased amount of blood flow to the site of injury to get more nutrients and immune cells to an area in need (Benacerraf and McCluskey 1963). Invasion of circulating immune cells (lymphocytes and macrophages), induction or activation of inflammatory mediators such as cytokines, kinins, reducing and oxidizing species aid the repair and removal of injured or infected cells (Eming et al. 2007).

The central nervous system differs from the other systems and its response to pathogenic challenges is significantly different. In contrast to the historic view that the CNS is an immune-privileged organ, lacking a lymphatic system and shielded from the circulatory system by the blood-brain barrier recent studies and evidences have revised the idea significantly. The CNS has been found to have its own system of combat through inflammatory response and an adapted system of immunosurveillance with coordination with the systemic immune system is also evident (Badie and Schartner 2000). Nevertheless the inflammatory response of the other tissues and the brain are varied. This is most evident in leukocyte recruitment, which is rapid in many systemic organs, but modest and delayed in the brain. While leukocyte invasion may be delayed in response to acute insults, activation of brain's own immune cells and release of inflammatory mediators are rapid, occurring within minutes or hours (Lucas et al. 2006).

Inflammation in the brain is characterized by activation of glial cells (mainly microglia and astrocytes) and expression of key inflammatory mediators as well as neurotoxic free radicals. In the central nervous system, microglia is the resident phagocytes of the innate immune system and astrocytes provide nutrients to the nervous tissue and help maintain the extracellular ion balance (Stoll and Jander 1999).

A role for immune responses, involving antigen presentation and immune-response-generating cytokines, in neurodegenerative diseases such as Alzheimer's disease was recognized for a decade before the term neuroinflammation

came into widespread use. While some chronic/remitting neurological diseases, such as multiple sclerosis, have long been recognized as inflammatory, the term neuroinflammation has come to denote chronic, CNS-specific, inflammation-like glial responses that do not reproduce the classic characteristics of inflammation in the periphery but that may engender neurodegenerative events; including plaque formation, dystrophic neurite growth, and excessive tau phosphorylation. In this way, neuroinflammation has been implicated in chronic unremitting neurodegenerative diseases such as Alzheimer's disease – diseases that historically have not been thought of as inflammatory diseases.

This new understanding has come from rapid advances in the field of microglial and astrocytic neurobiology over the past 15–20 years. These advances have led to the recognition that glia, particularly microglia, respond to tissue insult with a complex array of inflammatory cytokines and actions, and that these actions transcend the historical vision of phagocytosis and structural support that has long been enshrined in the term “reactive gliosis.” Microglia are now recognized as the prime components of an intrinsic brain immune system, and as such they have become a main focus in cellular neuroimmunology and therefore in neuroinflammation. This is not the inflammation of the adaptive mammalian immune response, with its array of specialized T-cells and the made-to-order antibodies produced through complex gene rearrangements. This is, instead, the innate immune system, upon which adaptive immunity is built.

Many researchers now consider this innate immune response in the brain to be a potentially pathogenic factor in a number of CNS diseases that lack the prominent leukocytic infiltrates of adaptive immune responses, but that do have activated microglia and astrocytes, i.e., neuroinflammation.

The idea that neuroinflammation is detrimental implies that glial cell activation precedes and causes neuronal degeneration, a sequence of events that appears to be at odds with experimental models of neurodegeneration in which glial cell activation occurs secondary to neuronal damage. What is missing from this simple linear model is the understanding that chronic neurological diseases are just that—chronic, and that this chronicity introduces complex interactions and feedback loops between neurons and glia that render attempts to construct simple, linear cascades of cause and effect inelegant. Neuroinflammation can be further explained as two distinct responses- acute and chronic neuroinflammation.

13.5.1 Neuroinflammation as Physiological Response

Neuroinflammation is a process in which the brain responds to infections, diseases and injuries through release of proinflammatory molecules (Nencini et al. 2003; Schmidt 2005).

These responses are mediated by two types of immune cells: lymphocytes, monocytes and macrophages of the hematopoietic system and glial cells of the CNS (astrocytes and microglia—the supporting cells of the CNS) (Stoll and Jander 1999; Streit and Stern 1999).

In response to a brain insult, glial cells are the first to be activated. The astrocytes upon activation, increases expression of glial fibrillary acidic protein, and produces cytokines; also contributing to the formation of the glial scar, which isolates the damaged area. These reactive astrocytes produce neurotrophic factors such as nerve growth factor and brain-derived growth factor which help in blood brain barrier repair and remyelination (Faulkner et al. 2004). On the other hand, within any scenario of immune-mediated brain injury, microglia qualifies as the main intrinsic immune effector cells of the brain. They are potentially phagocytic cells, have a pronounced cytotoxic potential (reviewed by (Banati et al. 1993)) may express several immunomolecules on their surface, may effectively present antigen to T-lymphocytes (Matsumoto 1992) and are capable of releasing a plethora of mediator substances such as inflammatory cytokines.

Most inflammatory mediators have relatively few actions in healthy CNS tissue and are expressed at very low or undetectable levels. Nevertheless some cytokines also modulate neuronal activities in the mature CNS and participate in neuro-immune–endocrine communication. However, they are induced rapidly in response to tissue injury or infection; certain cytokines appear in the affected brain region and the cerebrospinal fluid (CSF) when the CNS homeostasis is disturbed as a result of trauma, stroke, ischemia, infection, or degenerative processes. This increased cytokine levels in the CNS may result from blood–brain barrier (BBB) disruption or synthesis by invading immune cells, both of which originate from extraneuronal sources. These disruption of the blood-brain barrier (BBB), allows cells from the hematopoietic immune cells to leave the blood stream and come in contact to the injury site (Lossinsky and Shivers 2004). The immune cells respond to injuries by eliminating debris and, synthesizing and releasing a host of powerful regulatory substances, like complements, cytokines, chemokines, glutamate, interleukins, nitric oxide, reactive oxygen species and transforming growth factors (Bonifati 2002) which in turn start the cycle all over.

13.5.1.1 Acute Neuroinflammation

Acute neuroinflammation is more of a physiological response either to injury or insult to the CNS. Before “neuroinflammation” became a commonly used term, endogenous CNS tissue responses to injury were referred to as ‘reactive gliosis’. Reactive gliosis entails accumulation of enlarged glial cells, notably microglia and astrocytes, appearing immediately after CNS injury has occurred (Wolfgang 1999). Glial reactivity is majorly a passive response to injury whereas glial

activation implies a more aggressive role in responding to activating stimuli. Activated glial cells release factors that act on and engender responses in target cells equivalent to the responses of activated immune cells in the periphery; however, peripheral immune cells activation leads to leukocyte infiltration of tissues, which is notably absent in the brain unless there has been destruction or compromise of the blood brain barrier (Stoll and Jander 1999). In the presence of such destruction or compromise, peripheral leukocytes do enter the brain producing a scenario similar to that seen in inflammatory responses in the periphery.

In limited, acute reactions to injury, in the absence of blood-brain barrier breakdown, there is the subtler response of the brain’s own immune system, composed largely of rapid activation of glial cells. These responses represent the other end of the spectrum of CNS injury, where limited neuronal insults trigger glial cell activation without breakdown of the blood brain barrier and without concomitant leukocytic infiltration. This form of “pure” glial response occurs in neuronal injury caused by either loss of afferents (Kreutzberg 1996a) or loss of efferents (Ito et al. 1997).

The term “neuroinflammation” as generally used and understood applies to more chronic, sustained cycles of injury and response, in which the cumulative ill effects of immunological microglial and astrocytic activation contribute to and expand the initial neurodestructive effects, thus maintaining and worsening the disease process through their actions.

Before “neuroinflammation” became a commonly used term, neuroscientists spoke of “reactive gliosis” in describing endogenous CNS tissue responses to injury. Reactive gliosis specifically referred to the accumulation of enlarged glial cells, notably microglia and astrocytes, appearing immediately after CNS injury has occurred. In contrast to glial reactivity, which suggests a largely passive response to injury; glial activation implies a more aggressive role in responding to activating stimuli: activated glial cells release factors that act on and engender responses in target cells analogous to the responses of activated immune cells in the periphery. Activation of immune cells in the periphery leads to leukocyte infiltration of tissues, but this is notably absent in the brain unless there has been destruction or compromise of the blood brain barrier (Streit et al. 1998; Sroga et al. 2003). In the presence of such destruction or compromise, peripheral leukocytes do enter the brain producing a scenario similar to that seen in inflammatory responses in the periphery.

In limited, acute reactions to injury, in the absence of blood–brain barrier breakdown, there is the subtler response of the brain’s own immune system, composed largely of rapid activation of glial cells. These responses represent the other end of the spectrum of CNS injury, where limited neuronal insults trigger glial cell activation without breakdown

of the blood brain barrier and without concomitant leukocytic infiltration. This form of “pure” glial response occurs in neuronal injury caused by either loss of afferents (Kreutzberg 1996b) or loss of efferents (Ito et al. 1997). Axotomy, for instance, results in neuronal chromatolysis, the classic example of potentially reversible neuronal injury (Kreutzberg 1996b). It is in these situations that microglial and astrocytic responses (like their peripheral counterparts) fulfill their evolutionarily programmed functions of a reparative response to the benefit of the organism as a whole.

13.5.1.2 Chronic Neuroinflammation

Chronic inflammation is often associated in the understanding of CNS disease as opposed to acute inflammation which is linked with CNS injury. It is proposed that chronic inflammation is a causative factor to the pathogenesis of neurological diseases and disorders (Eikelenboom et al. 2006; Whitton 2007). The immune cells and pro-inflammatory chemicals involved in neuroinflammation would underlie the mechanisms of diseases and neurodegeneration. The activation, or over activation, of immune cells involved in neuroinflammation and release of pro-inflammatory substances would result in reduced neuroprotection and neuronal repair, and increased neurodegeneration, leading to neurodegenerative diseases (Zilkova et al. 2006). To elaborate, during disease states (for example, Parkinson’s (PD), Multiple sclerosis (MS), Alzheimer’s disease (AD) the inflammatory responses damage the BBB, increase oxidative stress and release pro-inflammatory and pro-apoptotic cytokines and other neurotoxic factors that affect neuronal damage or dropout. The damage and stress signals enhance microglial activation, resulting in positive feedback in the release of chemokines and cytotoxic cytokines that cause further ingress of immune cells into the brain and expand inflammatory responses.

The concept of chronic inflammation (as opposed to acute inflammation) is more relevant in the context of understanding CNS disease (as opposed to CNS injury), as the very term “disease” implies chronicity. Multiple sclerosis is, of course, an unequivocal and long-recognized example of an inflammatory brain disease. Although the underlying cause(s) of multiple sclerosis have not been elucidated, it is probably safe to say that the persistent injurious stimulus that accounts for neuroinflammation in multiple sclerosis is a myelin-related protein that has escaped self-tolerance and become an autoimmunogen. Consistent with the chronic persistence of this autoimmunogen is a persistent accumulation of blood-derived mononuclear leukocytes in the CNS parenchyma, a phenomenon that is similar to what is found in other autoimmune diseases such as rheumatoid arthritis or polymyositis.

Infections are another group of diseases that are classically recognized as inflammatory in nature, with meningeal, perivascular, or even parenchymal infiltrates of peripheral

leukocytes. There are, however, exceptions. Rabies is a disease in which the peripheral immune response is slow and inadequate, and in which classic inflammatory changes are less striking than those found in other viral encephalidites. Babes, in 1897, described microglial activation in rabies infection, although he did not recognize the nodules he found as clusters of activated microglia. Similar small collections of activated microglia were subsequently found to occur in a wide variety of viral brain infections.

Today, the most important example of a chronic brain infection is human immunodeficiency virus (HIV). Chronic HIV encephalitis is characterized by the same nodules of activated microglia that Babes described in rabies. HIV enters and persists in the CNS via myelomonocytic cells: monocytes, perivascular cells, and microglia (Garden 2013). HIV infection is uniquely different from most other infectious diseases affecting the CNS in that the virus targets and disables precisely those cells that are key players in neuroinflammation; microglia in the brain and T lymphocytes in the periphery. It therefore comes as no surprise that prominent T cell infiltrates do not occur in HIV encephalopathy.

Prion diseases represent another chronic infectious CNS disease that is not accompanied by leukocytic infiltrates. Microglial activation, again, appears to be the most prominent inflammatory component of prion diseases (Eikelenboom et al. 2002; Perry et al. 2002), although there are a few reports describing T cell infiltration as well (Betmouni et al. 1996). Prion diseases share interesting parallels to rabies infection in that infected cells are unrecognized by peripheral immune responses. This may explain in part the unusual patterns of neuroinflammation in prion diseases—manifest not only in atypical cellular infiltrates but also in unusual cytokine profiles (Baker et al. 2002). Both HIV and prion infections probably produce an altered microglial physiology that is likely to translate into cycles of neurodegeneration, which could be a contributing factor in the development of dementia that occurs in these conditions.

13.5.2 Neuroinflammation in Neurodegenerative Diseases

Chronic inflammation could influence the pathogenesis of neurodegenerative diseases. It is clearly evident that physiological or pathological responses like neuroinflammation, if goes awry, is tightly linked to the neurodegenerative disorders and diseases. A few examples neuroinflammatory response being the key drivers in disease and infection progression would bring out a better understanding of this concept.

Neurodegenerative diseases particularly Alzheimer’s disease, amyotrophic lateral sclerosis, Parkinson’s disease, and Huntington’s disease (HT) lack the prominent infiltrates of

blood-derived mononuclear cells that characterize autoimmune diseases. On the other hand, there is abundant evidence that many substances involved in the promotion of neuroinflammatory processes are present in the CNS of patients with such neurodegenerative diseases.

Alzheimer's disease (AD) is characterized by the progressive inability to form new memories and access existing ones, mainly because of neuronal cell death in the hippocampus and frontal cortex of the brain (Selkoe 2002). Besides such atrophy in the brain, microscopically there are a number of changes in the AD patient's brain too. The two major findings in the Alzheimer's brain are amyloid plaques and neurofibrillary tangles. β amyloid ($A\beta$) is a peptide that forms insoluble and pathological extracellular aggregates forming plaques, which seem to attract microglial cells, as suggested by the clustering of microglia at sites of $A\beta$ deposition (reviewed by (Streit 2004)). This proinflammatory response of microglial recruitment initially poses as a neuroprotective effect, but when prolonged or continuous turns into a neurotoxic effect. This suggests that CNS inflammation at least participates in amplification of the disease state.

Parkinson's disease (PD) whose clinical symptoms include tremor, postural instability and slowness of movement (Gao et al. 2003) is attributed to the loss of dopaminergic neurons in the substantia nigra pars compacta and the subsequent loss of projecting nerve fibres in the striatum (Block et al. 2004). PD is sporadic, and various environmental agents including pesticides and infections, may contribute to the disease. A role of inflammation has been strongly implicated (reviewed by (Gao et al. 2003)) and activated microglia are found close to degenerating substantia nigral neurones of patients with PD.

Multiple sclerosis (MS) is a chronic disorder in which inflammation plays a clear role. Invasion of the CNS by T cells and macrophages leads to damage to the myelin sheaths surrounding axons, loss of neuronal function and death. Since the injured areas of the CNS vary widely, the clinical symptoms are heterogeneous, and can include fatigue, muscle weakness, areas of numbness and paralysis. Microarray analysis has revealed that many genes related to inflammatory processes are upregulated in the marginal zones of active demyelinating lesions (Mycko et al. 2003).

Autism is a neurodevelopmental disorder characterized by impaired communication and social interaction and may be accompanied by mental retardation and epilepsy. Its cause remains unknown, despite evidence that genetic, environmental, and immunological factors may play a role in its pathogenesis. Recent studies show that in autistic cases, microglial and astroglial activation was present in the absence of lymphocyte infiltration or immunoglobulin deposition in the CNS (Vargas et al. 2005).

Inflammation undoubtedly contributes to other chronic CNS disorders, such as amyotrophic lateral sclerosis (ALS)

and Creutzfeldt–Jakob disease (CJD). ALS, a rapidly progressing motor neuron disease, is associated with mutation of superoxide dismutase (SOD1) gene, and mice that overexpress mutant SOD1 show upregulation of proinflammatory cytokines (Yoshihara et al. 2002), suggesting activation of microglia. Similarly, cytokine levels are elevated in CSF of CJD patients, and activated microglia are detected in mice infected with CJD (Van Everbroeck et al. 2002).

Infections are another group of diseases that are classically recognized as inflammatory in nature, with meningeal, perivascular, or even parenchymal infiltrates of peripheral leukocytes. However there are exceptions, microglial cells have found to be the potential mediator of neurodegeneration in some infections. Rabies is a disease in which the peripheral immune response is slow and inadequate, and in which classic inflammatory changes are less striking than those found in other viral encephalidites. Babes (1892), described microglial activation in rabies infection, although he did not recognize the nodules he found as clusters of activated microglia. Similar small collections of activated microglia are subsequently found to occur in a wide variety of viral brain infections. Presently, the most important example of a chronic brain infection is human immunodeficiency virus (HIV). The virus targets and disables precisely those cells that are key players in neuroinflammation; microglia in the brain and T lymphocytes in the periphery. HIV enters and persists in the CNS *via* myelomonocytic cells: monocytes, perivascular cells, and microglia (Garden 2002). Chronic HIV encephalitis is characterized by the same nodules of activated microglia that was described in rabies. Overall the activated microglial cells contribute significantly to the HIV associated neuropathogenic processes.

Prion diseases represent another chronic infectious CNS disease that is not accompanied by leukocytic infiltrates. Microglial activation, again, appears to be the most prominent inflammatory component of prion diseases (Eikelenboom et al. 2002; Perry et al. 2002), although there are a few reports describing T cell infiltration as well (Betmouni et al. 1996).

Thus it clearly evident that chronic microglial activation is an important component of neurodegenerative diseases, and this chronic neuroinflammatory component likely contributes to neuronal dysfunction, injury, and loss (and hence to disease progression) in these diseases. The recognition of microglia as the brain's intrinsic immune system, and the understanding that chronic activation of this system leads to pathologic sequel, has widened the modern concept of neuroinflammation. The idea of microglia-driven neuroinflammatory responses, with neuropathological consequences, has comprehensively replaced the older vision of passive glial responses inherent in the reactive gliosis hypothesis.

13.5.3 Microglia as Mediators of Inflammation

Once the microenvironment of the CNS becomes activated, local cells also produce proinflammatory cytokines, chemokines and upregulate immunomodulatory surface markers. These changes in turn decrease the stringency of the blood–brain barrier, allowing entry of soluble factors and peripheral immune cells, including macrophages, natural killer cells and lymphocytes. The specific sequence of events demonstrating that microglia activation precedes peripheral cell infiltration has been demonstrated in bone marrow chimeric mice (Schilling et al. 2003). For self-protection against oxidative stress, microglial cells are equipped with efficient antioxidative defense mechanisms. Microglial cells contain glutathione, substantial activities of the antioxidative enzymes, superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase as well as nicotinamide adenine dinucleotide phosphate (NADPH)-regenerating enzymes. Their antioxidative potential protects microglial cells against oxidative damage (Dringen 2005).

Activated microglia cells trigger and maintain an inflammatory response, deluging neurons with a whole host of inflammatory mediators that may ultimately lead to neuronal cell death. Thus microglial activation and chronic inflammation thereafter is the starting point for elevated levels of a wide array of potentially neurotoxic molecules which are believed to contribute to neurodegenerative processes. Several methods have become available for identifying activated microglia, and their presence has been demonstrated in a variety of neuroinflammatory/neurodegenerative diseases such as AD, PD, ALS and MS (Chen et al. 2004); (Turner et al. 2004). This participation of activated microglia and the release of neurotoxic products in the demise of neurons have now been postulated in most, if not all, neurodegenerative diseases.

13.5.4 Signaling Pathways Mediating Microglial Homeostasis and Activation

The transition from the homeostatic microglial phenotype to an activated stage is tightly regulated by several intrinsic (e.g., Runx-1, Irf8, and Pu.1) and extrinsic factors (e.g., CD200, CX3CR1, and TREM2).

The surface molecule CD200 is expressed by neural cells including neurons, astrocytes and oligodendrocytes (Barclay et al. 2002). Its receptor CD200R is expressed on microglia. The interaction of neuronal CD200 with CD200R leads to inactivation of microglia and keeps them in a resting state (Hoek et al. 2000). Following facial nerve axotomy, a model for local neuronal degeneration, CD200-deficient neurons elicited an accelerated microglial response in the lesioned nucleus. In the animal model for multiple sclerosis (MS), experimental autoimmune encephalomyelitis (EAE), defi-

ciency of CD200 resulted in a more rapid onset of disease (Hoek et al. 2000). These findings indicate that without the CD200–CD200R signaling, microglia develop an activated phenotype in the CNS.

The G-protein-coupled seven-transmembrane chemokine receptor, CX3CR1, is expressed on myeloid and natural killer (NK) cells and microglia (Jung et al. 2000). Its ligand, fractalkine or neurotactin, CX3CL1, is expressed on different neuronal subsets (Kim et al. 2011). Binding between neuronal CX3CL1 to the microglial CX3CR1 plays in the interaction of neurons and microglia (Harrison et al. 1998). Furthermore, loss of CX3CR1 in microglia led to an enhanced neuronal cell death in animal models for Parkinson's disease and other motor neuron disorders (Cardona et al. 2006b). However, several studies on the role of CX3CR1-deficiency in animal models for Alzheimer's disease (AD) showed that CX3CR1 deficiency appeared to be beneficial by resulting in reduced neuronal loss and improved behavioral deficits (Fuhrmann et al. 2010; Lee et al. 2010; Liu et al. 2010). These results indicated that CX3CR1-deficiency could play different roles under inflammatory and neurodegenerative CNS conditions. In addition, CX3CR1 is an important regulator of microglial surveillance. Thus, Paolicelli et al. reported CX3CR1-deficient animals showed a reduced number of microglia during the first weeks after birth (Paolicelli et al. 2011). Reduced microglial cell number in CX3CR1-deficient animals resulted in the development of immature neuronal circuits. In contrast, CX3CR1-deficient animals revealed a high density of spines and functional excitatory synapses, which are more known to be related to a mature phenotype than delayed brain development. In addition, another study reported that CX3CR1-deficient animals exhibited an inhibition in microglial recruitment to forming synapses in the barrel field of the somatosensory cortex, leading to an abnormal synapse formation in this area (Hoshiko et al. 2012). Therefore, interaction of neurons and microglia appears to be essential for proper synapse formation in the postnatal cortex. Recent investigations in healthy CX3CR1-deficient animals showed a harmful effect of this mutation for adult neurogenesis and hippocampal circuit integrity. The number of neuronal precursors in the hippocampus was decreased and led to diminished adult neurogenesis (Bachstetter et al. 2011). Moreover, CX3CR1-deficient animals display cognitive impairment (Rogers et al. 2011). These studies support the role of CX3CR1 signaling in keeping microglia in a homeostatic state.

Another glycoprotein identified on microglia is known as the triggering receptor expressed on myeloid cells 2 (TREM2), which was linked to an anti-inflammatory phenotype (Colonna 2003). TREM2 is associated with the adaptor protein DNAX-activating protein of 12-kDa (DAP12), which transmits the signal from the receptor to the intracellular signaling cascade. TREM2 is essential for phagocytosis of apoptotic cell by microglia (Neumann and Takahashi 2007). Furthermore,

TREM2 was shown to have beneficial effects in autoimmune CNS demyelination (Takahashi et al. 2007). Mutations in TREM2 or DAP12 are associated with a severe neurodegenerative disease known as Nasu-Hakola disease, which is further characterized by a form of early onset dementia and formation of bone cysts (Paloneva et al. 2001). Although TREM2 polymorphisms are known to be associated with a higher risk for the development of late onset AD (Guerreiro and Hardy 2013), the role of TREM2 in neurodegenerative diseases remains controversial. Murine studies suggest a beneficial role for TREM2 in suppressing inflammation (Piccio et al. 2007; Takahashi et al. 2005) and promoting phagocytosis of amyloid- β peptide (Wang et al. 2015). However, a recent study reported that *Trem2* deficiency in APP-PS1 mice reduces brain inflammation and plaque burden (Jay et al. 2015). Consistent with this, increased *TREM2* expression is associated with the CD33 AD risk allele (Chan et al. 2015) and recent data show that soluble TREM2 levels in the CSF are increased in the early symptomatic phase of AD in association with neuronal injury markers (Heslegrave et al. 2016; Suarez-Calvet et al. 2016). This may reflect a dysregulation of homeostatic microglia in response to neuronal injury.

Kinase and phosphate cascades mediate microglial response to extracellular stimuli. p38 mitogen-activated protein kinases are a class of mitogen-activated protein kinases (MAPK) that are responsive to stress stimuli. Several reports have demonstrated that p38 and p44/42 families of mitogen activated protein kinase pathways play a significant role in activation of microglial cells which in turn leads to release of neurotoxic molecules and neuroinflammation (Lee et al. 2000; Li et al. 2001; Waetzig et al. 2005). A MAPK pathway generally consists of four sub- pathways: (1) extracellular-signal-regulated kinases (ERK1/2, also known as p44/42 MAPK); (2) c-jun N-terminal kinases/stress-activated protein kinases (JNKs./SAPKs); (3) ERK5/big MAPK 1 (BMK1), and (4) p38 MAPK. Microglia activation could effect through any these if not all of the MAPK pathways. In vivo evidence also implicates that p38 and p44/42 MAPKs play an important role in microglial activation in acute brain injury such as stroke and in chronic neurodegenerative diseases such as Alzheimer's disease (Koistinaho and Koistinaho 2002). The other proinflammatory pathway that could respond to microglia activation is the NF κ B pathway. Any of the microglia mediated pro-inflammatory stimuli can activate NF κ B expression (Sparacio et al. 1992), which can further induce specific genes that regulate inflammation.

13.6 Physiological Functions

The main function of immune cells and in particular microglia is to secrete cytokines to fight first against an invasion and promote an inflammatory reaction. Microglia are also known

to produce cytokines in the context of synaptic modulation. However, microglia also have diverse functions.

13.6.1 Motility/Surveillance

In 2005, discovery of high degree of motility of microglia in the healthy brain brought a new view on their active role in surveying the microenvironment of neurons. These studies revealed that microglia were not static cells as microglia processes protract and retract, scanning the molecular and cellular microenvironment in proximity to each individual microglia. Thus, under physiological conditions microglia present a constant motility of their ramifications in the cerebral cortex (Davalos et al. 2005; Nimmerjahn et al. 2005; Stence et al. 2001). These surveying movements are dependent on ATP and gap junction proteins or connexins in steady-state (Davalos et al. 2005). More recently, the CX3 chemokine receptor 1 (CX₃CR1) also known as the fractalkine receptor, which is mainly expressed by microglia in the CNS, has been shown to regulate microglia surveillance dynamics in the retina. For instance, basal microglia motility is decreased in retina explant of CX₃CR1-knockout mice (Liang et al. 2009). The constant surveillance of specific microenvironments allows microglia to rapidly extend processes toward a lesion site in CNS ($\sim 1.25 \mu\text{m}/\text{min}$) (Davalos et al. 2005; Nimmerjahn et al. 2005). Several extrinsic and intrinsic factors are implicated in the process of microglia extension toward a lesion. Rapid extension of these processes is also dependent on extracellular ATP detected by microglia through the purinergic receptor, P2Y₁₂ (Davalos et al. 2005; Haynes et al. 2006). Activation of P2Y₁₂ receptors is associated with outward potassium current in microglia (Wu et al. 2007). Outward potassium current could contribute to regulation of microglia volume during chemoattraction (Eder 2005). Glutamate also attracts microglia through an ATP independent mechanism as shown in mouse spinal cord (Liu and Kielian 2009). In a model of retina lesion, CX₃CR1 has been shown to be implicated in the fast response of microglia toward the lesion (Liang et al. 2009). Interestingly, in vivo activated microglia by bacterial endotoxin such as lipopolysaccharide (LPS) exhibit retracted ramifications (Madore et al. 2013), this in response to ATP detected by the adenosine receptor 2A (Orr et al. 2009). Activation of this receptor could explain morphological changes from ramified to amoeboid microglia. Microglia are considered as a protective agent in healthy tissues able to very rapidly extend their migrating and converging processes toward a lesion site. Indeed microglia processes convergence is quick after a laser beam lesion on brain slices. This rapid reaction limits lesion propagation to neighboring neuronal tissues (Hines et al. 2009). Moreover, microglia motility is also regulated by neuronal activity. In 2011, Fontainhas dem-

onstrated that microglia motility is regulated by ionotropic glutamatergic neurotransmission and decreased by ionotropic γ -aminobutyric acid (GABA) neurotransmission (Fontainhas et al. 2011). In support of these findings a recent study revealed that in rodent hippocampus, dendritic neuronal N-Methyl-D-aspartate receptor (NMDAR) activation triggers ATP release that in return, induces outgrowth of microglia processes (Dissing-Olesen et al. 2014). These new functions and mechanisms of microglia regulation contribute to the hypothesis that microglia participate in neuronal functions during homeostatic and injury states.

13.6.2 Antigen Presentation

At the periphery, dedicated cells are doing the antigen presentation process, which is to present exogenous antigen to T cells. This constitutes the cellular component of adaptive immunity. Professional antigen presenting cells (APC) such as dendritic cells, macrophages and lymphocytes phagocytose their targets. They will migrate then to lymph nodes where they will present to T cells digested exogenous proteins attached to their cell surface, to specific receptors such as class II proteins of major histocompatibility complex (MHC II). In the CNS, antigen presentation by microglia is debated. In physiological conditions, microglia do not express MHCII. Only a subset of cells, which will be considered as dendritic, cells express co-stimulatory molecules such as CD11c (Bullock et al. 2008). Ford in 1995 showed that microglia to a few extent do an activity of antigen presentation in vivo (Ford et al. 1995). However, after an localized immune challenge with IFN- γ , microglia express high levels of MHCII and CD11c in vivo and will present antigens at their cell surface in vitro (Gottfried-Blackmore et al. 2009). One of the reason of microglia would not be able to present in vivo antigens, would assess that microglia would have to exit the parenchyma to migrate toward lymph nodes. Antigen presentation by microglia in the CNS would not take place in the parenchyma but rather in the meninges and choroid plexus. These structures contain perivascular APCs. Antigen presentation could be assessed by a direct drainage of antigens in the CNS in the cerebro-spinal fluid via the arachnoid space toward channels present at the level of cribriform plate of the ethmoid to finally join lymph nodes in the deep cervical (Galea et al. 2007; Ransohoff and Engelhardt 2012). However, recently, it has been shown that the CNS present a functional and classical lymphatic system, called the glymphatic system (Louveau et al. 2015). Self antigens including CNS antigens do not promote any response since auto-reactive T cells are eliminated by clonal deletion in the thymus. In the case of auto-immune diseases such as MS, reactive T cells against myelin escape from this immune tolerance mechanism and enter the CNS under certain conditions leading to

the demyelination process (Goverman 2011). Microglia seem to play a negative role in EAE progression, animal model of MS since when microglia activity is suppressed and the severity of pathology is reduced (Heppner et al. 2005) (Heppner et al. 2005). However, this deleterious role of microglia could be linked to their defective phagocytic activity or antigen presentation function in disease (Ransohoff and Engelhardt 2012).

13.6.3 Migration (chemotaxis)

Microglia is able to migrate toward a lesional site where they can eliminate debris and contribute to tissue repair. Microglia migration is dependent on gradients of chemokines and cell surface receptor expression and their localization from the injured site (Flynn et al. 2003; Dijkstra et al. 2004; Wang et al. 2008). For example in MS, secretion of CCL2/MCP1 and CXCL10/IP10 and the expression of their receptors is increased and has been associated to an activation of microglial cells (Simpson et al. 2000; Tanuma et al. 2006). CCL2/MCP1 protein is part of the chemokine family CC. CCL2 contains four residues cysteine highly conserved characteristic of the CC chemokine family. In mouse, CCL2 is a protein containing 125 amino acids. A few studies revealed that CCL2 is constitutively expressed by neurons in the CNS and more specifically in inflammatory conditions (Reaux-Le Goazigo et al. 2013). CCL2 is also produced by glial cells (Barna et al. 1994; Berman et al. 1996; Glabinski et al. 1996; Hanisch 2002). Perivascular astrocytes are the main source of CCL2 in the brain and also during inflammatory conditions, Microglia express CCR2 which is the receptor of CCL2 (Conductier et al. 2010). CCL2 was first described for its chemoattractant activity of monocytes toward inflammation sites. Its expression in the CNS is increased in several inflammatory pathologies and notably in Alzheimer's disease. Numerous studies showed that CCL2 is increased at the level of aggregated amyloid plaques associated to a microgliosis (Ishizuka et al. 1997).

13.6.4 Phagocytosis

Endocytosis is defined by internalization of extracellular material into vesicles that are transferred into the cytosol (Napoli and Neumann 2009). This process consists in three mechanisms. Microglia are able to bind extracellular particles through specific receptors which lead to engulfment of these particles. This process is named endocytosis mediated by receptors implying membrane invaginations that are responsible for the formation of the vesicles (Goldstein et al. 1979). A second process is known to engulf extracellular fluids containing single proteins or little molecules and

is named pinocytosis (Glenn et al. 1991). Macropinocytosis has been shown to be responsible of soluble Abeta protein engulfment in a model of AD (Mandrekar et al. 2009). Phagocytosis is the third mechanism and is known to engulf solid particules (Kinchin et al. 2008). The word phagocytosis comes from Ancient Greek meaning eat for phagein and cell for kutos. Pathogen phagocytosis has been discovered by the Russian embryologist Ilya Metchnikoff in the 80s showing that white blood cells could engulf bacteria. Metchnikoff showed that phagocytosis allowed the host defense, that was beneficial for tissue homeostasis and linked to inflammation in humans (Metchnikoff and Binnie 1905). In mammals, professional phagocytes derive from a common myeloid lineage. Phagocytosis is the main function of microglia cells with the actin-dependent engulfment of exogenous particules and debris (Aderem and Underhill 1999) and digest the taken-up material by a professional lysosomal machinery. Depending on the type of the phagocytic receptor microglia responds differently in their downstream cytokine signaling, either pro- or anti-inflammatory. In the presence of apoptotic cells, microglia phagocytose without inflammation and by production of anti-inflammatory cytokines such as transforming growth factor β (TGF β) (Moller et al. 2000; Wu et al. 2002). Also adenosine triphosphate (ATP) released from injured/necrotic neurons can activate microglia through binding to purinergic receptors and convert them to the neurotoxic phenotype (Koutsilieri et al. 2002). Clearance of apoptotic cells, accumulated proteins and exogenous pathogens by microglia is one of the important action to maintain CNS (Chan et al. 2001). Clearance of cell debris following programmed cell death or apoptosis is necessary to limit damages to neighboring cells that are still healthy. Indeed, debris will inhibit growth and tissue repair. Thus phagocytosis is essential during development and during repair after a lesion. However, in case of chronic activation, microglia secrete neurotoxic factors such as inflammatory cytokines and NO. If the phagocytic activity is not controlled and well regulated, the neuroprotective and beneficial effect of microglia can inverse leading to chronic neuroinflammation (Aderem and Underhill 1999). Phagocytosis by microglia is supposed to follow a model in three steps proposed by Savill in 2002. There is a step of chemoattraction in which the phagocyte (microglia) has to find its target by following molecular find-me signals secreted by the target. Once the phagocyte reached its target, it will establish a direct membrane contact via ligand-receptor interactions. Apoptotic cells liberate extracellular nucleotides such as ATP or UTP (Elliott et al. 2009). UDP coming from degradation of UTP will act in P2Y6 receptors which facilitate phagocytosis (Koizumi and Fujishita 2007). CX3CL1 or fractalkine is another signal secreted by apoptotic cells (Truman et al. 2008; Noda et al. 2011). Microglia expressing CX3CR1 will phagocytose apoptotic cells. Eat-me signals are secreted by

microglia and will bind the target to eliminate. For example, apoptotic cells are recognized by microglia through scavenger receptors. “Bridge” molecules or eat-me signals bind phosphatidylserines exposed at the cell surface of apoptotic cells and are detected by receptors in microglia, such as milk fat globule-epidermal growth factor (MGFG-E8) and many others. Different types of phagocytosis exist. Complement cascade play an important role in the phagocytosis of opsonized particules. Complement receptor 3 (CR3 or CD11b/CD18) is expressed by microglia in the CNS and macrophages at the periphery. Complement cascade proteins opsonize targeted pathogens or synapses and participate to their phagocytosis by microglia through CR3 (Aderem and Underhill 1999). Opsonization consists in covering the target with molecules such as C3b or C4b (Brown 1991) in order to facilitate their engulfment by the phagocyte.

Normal microglia phagocytoses injured neurites and myelin debris in a slow process (Gao et al. 2003), whereas activation of microglia increases this ability to phagocytose axonal and myelin debris (Block et al. 2004).

Tremblay and collaborators suggested that microglia would participate to synapse elimination and neuronal circuits remodeling in an experience-dependent manner in a healthy and mature brain (Tremblay et al. 2010). This work also showed that the majority of microglia processes (around 94%) in visual cortex of juvenile mice are juxtaposed to astrocyte and neuronal excitatory synaptic elements including the synaptic cleft. These interactions are done “en passant”. 3D reconstruction of microglia processes shows microglia extending protusions looking like fingers surrounding dendritic spine contacting axons (Tremblay et al. 2010). Using in vivo two-photon imaging of fluorescent-labeled neurons and microglia, it has been demonstrated that the resting microglial processes make brief (approximately 5 min) and direct contacts with neuronal synapses at a frequency of about once per hour (Wake et al. 2009). Microglia preferentially contact small size dendritic spines and remove them (Tremblay et al. 2010). These contacts are activity-dependent, and are reduced in frequency by decreased neuronal activity.

13.6.5 Synaptic Plasticity

Synaptic plasticity corresponds to the ability of synapses to change the strength of their connectivity. These changes can occur during short periods of time, leading to the addition or removal of connections between neurons (Bear 1999; Bliss and Collingridge 1993; Holtmaat and Svoboda 2009; Katz and Shatz 1996; Luscher and Malenka 2012). The main cellular and molecular mechanisms underlying learning and memory abilities rely on neuronal plasticity including synaptic plasticity, which can be studied by looking at the long-

term potentiation (LTP) phenomenon, corresponding to adaptation that occurs when synapses are strengthened following tonic activity. For instance, LTP is induced through high-frequency stimulation (HFS) of neuronal afferents resulting in persistent augmentation of their strength (Bliss and Collingridge 1993; Cooke et al. 2006). Interestingly, recent evidence suggests that microglia can directly modulate synaptic plasticity. Indirect evidences through the effect of inflammatory factors such as interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF α), also attest of the modulatory effect of microglia on synaptic plasticity. It is known that IL-1 β gene expression is increased after LTP induction *ex vivo* but also *in vivo* in freely moving rats (Balschun et al. 2003; Schneider et al. 1998). This elevation in IL-1 β expression seems to play a role not only during induction but also during LTP maintenance. Indeed, intracerebroventricular administration of IL-1ra (IL-1 receptor antagonist) after induction of LTP impaired its maintenance. Administration of IL-1ra before LTP induction also seems to be able to impair induction and maintenance of the LTP (Loscher et al. 2003; Schmid et al. 2009). Using transgenic mice, Goshen and collaborators confirmed the involvement of IL-1 β in LTP since IL-1raKO mice do not show LTP induction in the hippocampus after HFS stimulation (Goshen and Yirmiya 2009). TNF α also appears to be involved in synaptic plasticity since *ex vivo* and *in vitro* studies demonstrated that TNF α enhanced synaptic efficacy by increasing surface expression of glutamatergic 2-amino-3-(5-methyl-3-hydroxy-1,2-oxazol-4-yl) propanoic acid (AMPA) receptors and that blockade of TNF α signaling reduced synaptic strength and AMPA receptors expression (Beattie et al. 2002). TNF α also plays a clear role in a form of long-term plasticity called synaptic scaling, a plasticity mechanism able to adjust the strengths in synapses through increased AMPA receptors expression in response to an episode of strong cell activation. This leads to neuronal network stabilization. Indeed it has been shown that TNF α is necessary for increased surface AMPA receptors and synaptic strength after chronic blockade of neuronal activity (Stellwagen and Malenka 2006). Despite the involvement of TNF α in this form of synaptic plasticity, it seems that TNF α is not involved in acute plasticity since most of the studies looking at the role of TNF α in LTP did not find any effect after manipulating TNF α signaling (Albensi and Mattson 2000; Kaneko et al. 2008; Stellwagen and Malenka 2006). Recently CX₃CR1/CX₃CL1 microglial signaling pathway has been investigated and seems to play a role in the modulation of synaptic plasticity, even if contradictory results have been described. Indeed, neuronal activity is known to contribute to synaptic maturation (Hua and Smith 2004; Huberman et al. 2008; Katz and Shatz 1996). *Ex vivo* manipulations demonstrated that administration of fractalkine inhibits LTP induction. This effect was not found in CX₃CR1^{KO} mice (Maggi et al. 2009).

Oppositely, Rogers and collaborators reported complete absence of LTP *ex vivo* in CX₃CR1^{KO} mice (Rogers et al. 2011). No difference were found in LTP in these mice at adulthood whereas during development CX₃CR1^{KO} mice exhibited an increased long-term depression (LTD) and decreased duration and latency of epileptic crises induced by pentylenetetrazol (PTZ), experimental model of epilepsy. Thus, microglial CX₃CR1 is involved in the maturation of hippocampal neurons since CX₃CR1^{KO} mice display increased spine density and PSD95 expression, a post-synaptic protein. The immature synaptic phenotype characterized in CX₃CR1^{KO} mice may correspond to impaired hippocampal development previously discussed (Paolicelli et al. 2011). In the end, these data suggest that CX₃CL1-CX₃CR1 signaling in microglia could play a different role during development and at adulthood in the healthy brain. Less recent *ex vivo* studies demonstrate the role of microglia in maturation of hippocampal synapses. On hippocampal slices from mice lacking KARAP/DAP12 (transmembrane receptor expressed by microglia and known to activate innate immune system) (Hamerman et al. 2005), synaptic maturation is altered (Roumier et al. 2004, 2008). On postnatal day 22, expression of the NR2B subunit of NMDA receptors was increased and Glur2 subunit from AMPA receptors expression was decreased, characteristic of immature synapses (Roumier et al. 2004). Finally, recent results reported the involvement of microglia in synaptic plasticity using the CNS-TGF β -1 transgenic mice, which have a reduced number of microglia cells without affecting neuronal density. Indeed, Koeglspenger and collaborators demonstrated that hippocampal function was altered in these mice (Koeglspenger et al. 2013). The reduced microglial density also was associated with a decreased LTP and increased LTD in the hippocampus. The mechanisms thought to be involved in these effects relied on enhancement of NMDA receptor activation and reduced glutamate recycling. These results are in agreement with the changes observed in mice deficient in CX₃CR1/CX₃CL1 displaying a lack of LTP and hippocampal-dependent learning and memory (Rogers et al. 2011). Altogether, these data emphasized the idea of the key role of microglia in neuronal plasticity.

13.6.6 Exosome

Glia cells and specially microglia require intense cell to cell communication between all partners of the synapse and also intercommunication between structures or even with the periphery. Intercellular communication can be mediated through direct cell to cell contact or paracrine action of secreted molecules. A novel mode of communication relying on the exchange of extracellular vesicles (EVs) between cells has become evident. Various cell types release EVs of

different origin into their environment, which have the potential to transfer a collection of biomolecules between cells locally or over longer distances (Thery 2011; Raposo and Stoorvogel 2013). Extracellular vesicles comprise shedding microvesicles (MVs), exosomes, and apoptotic bodies, which differ in size, cargo, membrane composition, and origin. Apoptotic bodies are released during apoptosis, whereas the other types of vesicles are derived from healthy cells (Thery 2011; Cocucci et al. 2009). While MVs directly pinch off from the plasma membrane and are heterogeneous in size (up to 1000 nm in diameter), exosomes originate from the endosomal system and exhibit a regular shape (50–100 nm in diameter). Exosomes correspond to the intraluminal vesicles of multivesicular bodies (MVBs). Hence their generation involves sorting at the level of the endosomal limiting membrane mediated by the ESCRT (endosomal sorting complex required for transport) machinery (Simons and Raposo 2009; Baietti et al. 2012) and is mediated extracellular ATP (Bianco et al. 2005). Exosomes carry characteristic bioactive lipids, nucleic acids, biogenesis-related proteins (Tsg101 and Alix are classic markers), through which they can influence recipient cells (Tetta et al. 2013). Exosome influence is particularly relevant on immune system (Robbins and Morelli 2014). Reactive microglia were shown to release exosomes and MVs carrying inflammatory cytokines, caspases and P2X7 receptors which may induce and propagate inflammatory reactions throughout the CNS (Prada et al. 2013). Microglia-derived MVs transmit inflammatory signals to recipient microglia which in return upregulate co-stimulatory CD86 molecule and inflammatory factors (iNOS, Cox2 etc) (Verderio et al. 2012). Microglia-derived MVs interact with neurons and can stimulate neurotransmission in vitro and after injection in vivo (Turola et al. 2012). MHCII can be packed into exosomes and its amount is increasing upon stimulation with interferon- γ . It has been suggested that microglia does not release exosomes constitutively (Hooper et al. 2012).

13.7 Role in Neural Development

13.7.1 Elimination of Migrating Stem Cells and Apoptotic Cells

Microglial phagocytosis of newborn neurons has been shown as an important process for the shaping of neural circuitry. During development, programmed cell death occurs among several cells of the neuronal lineage (de la Rosa and de Pablo 2000). These cells undergo apoptosis, triggered by proteolytic cascades mediated by caspases, and characterized by DNA fragmentation and membrane blebbing (Porter and Janicke 1999; Yuan and Yankner 2000). These apoptotic cells include both postmitotic neurons and proliferating neu-

roblasts. In the developing cerebellum, microglia release superoxide anions that trigger cell death in a subset of newborn neurons, and the resulting apoptotic cells are phagocytized by microglia (Marin-Teva et al. 2004). Interestingly, in the adult CNS, microglial-mediated phagocytosis of apoptotic neuroblasts also occurs. In the mammalian brain, two discrete regions exhibit persistent neurogenesis: the subgranular zone (SGZ) in the dentate gyrus of the hippocampus and the SVZ, which lines the walls of the lateral ventricles (LVs) (Whitman and Greer 2009; Ihrie and Alvarez-Buylla 2011). Postnatally generated cells include glutamatergic and GABAergic neurons that populate the dentate gyrus of the hippocampus and are integrated into neural circuitry during learning and memory formation processes. Alternatively, neuroblasts generated in the postnatal SVZ migrate through a long pathway, the rostral migratory stream, towards their final destination in the glomerular and granule cell layers of the olfactory bulb (OB) (Lois et al. 1996). Within the OB, neuroblasts differentiate into GABAergic and to a lesser extent glutamatergic cells, which are integrated as juxtglomerular neurons (Zhao et al. 2008; Brill et al. 2009; Sequerra et al. 2010). These two neurogenic sites both present neural stem cells that in turn are functionally integrated into neuronal circuits (Zhao et al. 2008; Doetsch et al. 1997). Despite the abundant amount of new born neurons, only a small proportion are functionally integrated (Ma et al. 2009). The majority of the new born precursors will undergo apoptosis at early stages and are phagocytosed by microglia (Sierra et al. 2010). Notably, the mechanisms underlying the microglial phagocytosis of newborn cells in this neurogenic niche remain elusive. Tyro3, Axl and Mer (TAM) receptor tyrosine kinases and their ligands Gas6 and protein S have been shown as key elements for the efficient phagocytosis of apoptotic cells in the mature immune, nervous and reproductive systems (Lemke and Burstyn-Cohen 2010). As such, the activity of these signaling pathways might govern microglial-mediated phagocytosis in this adult neurogenic niche. Moreover, whereas several studies have addressed the roles of microglia in shaping adult hippocampal circuitry, only a few have addressed the potentially analogous functions of microglial cells in the neurogenic niches.

13.7.2 Synaptic Pruning

During the initial 2 weeks of post-natal CNS development there is a period of transient synapse formation and elimination dependent on neuronal activity. Synaptic pruning or synapse elimination was initially hypothesized to be dependent on neuronal activity and intrinsic molecular mechanisms (Luo and O'Leary 2005; Katz and Shatz 1996; Huberman et al. 2008; Hua and Smith 2004). This hypothesis was altered when studies showed that during CNS development

microglia play a key role in refinement of neuronal circuits (Marin-Teva et al. 2011). Recent studies demonstrated a role of microglia in the dynamic interactions between microglia processes and synapses resulting in proper pruning (Schafer et al. 2012; Paolicelli et al. 2011). For instance, microglia phagocytose hippocampal synapses during mouse post-natal development (Paolicelli et al. 2011). Immunogold detection of pre- and post-synaptic proteins in microglia confirmed the integral role of microglia in synaptic pruning. Paolicelli and collaborators showed that CX₃CR1 knock-out mice exhibit fewer microglia cells in postnatal hippocampus compared to wild-type mice at the same age. In a pathological context, the CX₃CR1 receptor can modulate microglia in terms of number, activation and recruitment toward lesion sites, by detecting the fractalkine ligand CX₃CL1 expressed on damaged neurons (Jung et al. 2000; Cardona et al. 2006b). In a developing CNS without inflammation, CX₃CL1 would modulate number of microglia, activation and recruitment toward synapses to eliminate. These findings are consistent with previous data suggesting that immune molecules, such as class I molecules of histocompatibility major complex, complement cascade molecules and neuronal pentraxins, contribute to synaptic pruning during development (Stevens et al. 2007; Schafer et al. 2012; Huh et al. 2000; Goddard et al. 2007; Datwani et al. 2009; Corriveau et al. 1998; Boulanger 2009). Recently, immune molecules, including complement proteins C1q and C3, have emerged as critical mediators of synaptic refinement and plasticity (Stevens et al. 2007; Stephan et al. 2012; Schafer et al. 2012). Indeed, pruning of inactive retinal ganglion cells (RGC) synapses in the lateral geniculate nucleus (LGN) is mediated by microglia phagocytosis of the pre- and post-synaptic elements through the complement receptor 3 (CR3) which is activated by the complement cascade beginning with C1q and the opsonization is mediated by C3 (Stevens et al. 2007; Schafer et al. 2012). Recently, retinal TGF- β was identified as a key regulator of neuronal C1q expression and synaptic pruning in the developing visual system. Mice lacking TGF- β receptor II (TGF β RII) in retinal neurons have reduced C1q expression in RGCs and reduced synaptic localization of complement, and pruning defects were observed in complement-deficient mice. TGF- β is implicated in the regulation of neuronal C1q expression in order to initiate complement- and microglia-mediated synaptic pruning.

13.7.3 Synaptogenesis

During CNS development, several studies showed that microglia participate to synaptogenesis regulation. Microglia can stimulate synaptogenesis through secretion of thrombospondins (TSP) (Chamak et al. 1995; Moller

et al. 1996) or extracellular matrix proteins (Christopherson et al. 2005). The absence of these proteins induces a robust decrease of synapses during post-natal period (Christopherson et al. 2005). SIRP α and CD47 are co-localized at synaptic level in retinas for example (Mi et al. 2000). Genetic deletion of CD47 results in a loss of SIRP α localization suggesting that CD47 is necessary for the recruitment and localization of SIRP α . In neuronal hippocampal cultures, SIRP α is localized at the axonal and dendritic level instead of CD47 which is restrained to dendrites (Ohnishi et al. 2005). CD47 improves neurite formation and neuronal arborization in culture (Murata et al. 2006). Interaction between SIRP α and CD47 promotes filopodium formation and spines (Miyashita et al. 2004). Interestingly, a recent study showed that the anti-inflammatory cytokine IL-10 released by microglia increased number of dendritic spines (Lim et al. 2013a). It also showed that IL-10 receptors are present on hippocampal neurons at early stage of brain development (Lim et al. 2013b) suggesting that developing microglia regulated synaptic functions and neuronal development through the interactions of the IL-10 released from the microglia with IL-10 receptors expressed on the hippocampal neurons.

13.7.4 Synaptic Maturation

Neuronal activity is known to contribute to synaptic maturation (Sanes and Lichtman 1999; Katz and Shatz 1996; Huberman et al. 2008; Hua and Smith 2004). Since 2011, microglia has also been showed to contribute to synaptic maturation (Paolicelli et al. 2011). Excitatory synaptic transmission toward CA1 neurons show an immature phenotype in knock out CX₃CR1 mice indexed by increased in long term depression (LTD) and decreased duration and latency of epileptic crises induced by pentylenetetrazol (PTZ), experimental model of epilepsy. These phenotypic characteristics of CX₃CR1-knockout mice are associated with defect in synaptic pruning and immature synapses (Paolicelli et al. 2011). Microglial CX₃CR1, fractalkine receptor, is involved in maturation of hippocampal neurons since CX₃CR1-knockout mice have increased spine density and increased PSD95 expression (Paolicelli et al. 2011). Less recent ex vivo studies demonstrate the role of microglia in maturation of hippocampal synapses. On hippocampal slices from knock out KARAP/DAP12 (transmembrane receptor expressed by microglia and known to activate innate immune system (Hamerman et al. 2005), synaptic maturation is altered (Roumier et al. 2004, 2008). On postnatal day 22, expression of subunit NR2B of NMDA receptors was increased and Glur2 subunit from AMPA receptors expression was decreased, characteristic of immature synapses.

13.8 Molecular Regulation in Development and Survival

Tissue-resident macrophages have many tissue-specific functional characteristics, which are a reflection of distinct gene-expression programs (Okabe and Medzhitov 2015). Thus, microglia express functional and unique molecular characteristics not seen in other tissue macrophage populations (Hickman et al. 2013; Butovsky et al. 2014; Gautier et al. 2012). The mechanism to establish these organ-selective properties is through environmental influences on RNA expression profiles, exerted at the level of epigenetic histone and DNA modification (Amit et al. 2015).

13.8.1 PU.1-, RUNX1- and IRF8-Dependent Microglia Development

Between embryonic days 8 and 9, EMPs migrate to the forming CNS where their further development is regulated by transcription factors that are essential for regulation of microglia cell development are primarily PU.1, RUNX1, and IRF8. PU.1 is a member of the ETS family of transcription factors (Rosenbauer and Tenen 2007), and its targeted disruption leads to the lack of B cells, monocytes, macrophages, and parenchymal microglia (Beers et al. 2006; McKercher et al. 1996). RUNX1 is a critical regulator of microglia during embryonic development (Ginhoux et al. 2010), as well as a modulator of proliferation and homeostasis in the postnatal stage (Zusso et al. 2012). IRF8, similarly to RUNX1, is involved in the regulation of microglia differentiation during development as a heterodimeric partner of PU.1, as well as in microglial activation in adulthood (Kierdorf et al. 2013). These myeloid lineage-determining transcription factors set the stage for cell differentiation throughout a specific cell lineage by establishing common enhancers. Subsequently, these factors cooperate with environment-dependent transcription factors to promote tissue-resident macrophage-specific gene expression.

13.8.2 IL34-, CSF1r- and Tgfb β 1-Dependent Microglia Maintenance and Survival

Microglia express receptors that trigger essential cellular developmental and survival signals. The receptor for colony stimulating factor 1 (Csfr1) plays a major role in peripheral macrophage development and survival (Cecchini et al. 1994; Chitu and Stanley 2006). Dai et al. showed that targeted disruption of the Csfr1 gene results in mononuclear phagocyte deficiency (Dai et al. 2002). Furthermore, Ginhoux et al. showed that the development of microglia and primitive yolk sac macrophages is dependent on Csfr1

signaling (Ginhoux et al. 2010). However, the Csfr1^{op/op} mouse strain with a natural occurring null mutation in Csfr1 did not reveal the same severe phenotype observed in the Csfr1r-deficient animals (Yoshida et al. 1990). Detailed examination of Csfr1^{op/op} mice revealed the presence of microglia but at reduced numbers (Blevins and Fedoroff 1995; Erlich et al. 2011). Thus, survival and maintenance of microglia are majorly influenced by Csfr1r function. However, Csfr1r may have Csfr1-independent functions in microglial homeostasis, suggesting the existence of another ligand for this receptor. Lin et al. discovered another ligand of the Csfr1r known as IL-34 (Lin et al. 2008). Both, IL-34 and Csfr1 share some similar signaling functions via Csfr1r (Wei et al. 2010). However, both ligands showed a differential expression pattern in vivo. Moreover, IL-34 is required for the development of Langerhans cells and microglia (Greter et al. 2012; Wang et al. 2012). IL-34-deficient animals had reduced numbers of microglia and were consequently more susceptible to viral infections in the CNS (Wang et al. 2012). Greter et al. demonstrated that IL-34 is expressed by specific neuronal subsets in the cortex and hippocampus and that microglial development was not influenced by IL-34 deficiency. In contrast, microglial cell number was decreased in specific regions of the adult brain (Greter et al. 2012). These results indicated that IL-34 plays a role in the adult brain for microglial survival and homeostasis. Additionally, a recent study showed that the signaling of both IL-34 and Csfr1 are important for microglial proliferation upon neurodegeneration during prion infection and AD (Gomez-Nicola et al. 2013). Microglial proliferation was induced by administration of Csfr1 and IL-34, whereas IL-34 showed a stronger proliferative capacity than Csfr1. Interestingly, heterozygous mutations in the Csfr1r locus can be found in patients with hereditary diffuse leukoencephalopathy with spheroids (HDLS), characterized by demyelination of the cerebral white matter and formation of spheroids which lead to progressive cognitive and motor dysfunction (Rademakers et al. 2012). However, it is still unclear, what roles IL-34 and Csfr1 play in microglial development or homeostasis in these patients. Future studies will elucidate the detailed function of these important physiological signaling molecules in microglia.

Microglia have a unique homeostatic molecular and functional signature (M0) in the healthy brain (Hickman et al. 2013; Butovsky et al. 2014; Gautier et al. 2012) which is tightly controlled by TGF β 1 signaling (Butovsky et al. 2014; Gosselin et al. 2014). TGF β 1 signaling is required in vitro for microglia to express a partial microglial molecular signature characteristic of acute ex vivo adult microglia. Furthermore, a loss of microglia has been observed in mice deficient for TGF β 1 in the CNS (Butovsky et al. 2014). In support of the role of TGF β 1 on regulation of homeostatic microglia, Gosselin et al. showed that signal-dependent transcription

factors, such as SMAD3, which is triggered by TGF β in the brain, activated a subset of PU.1-poised enhancers and super-enhancers in microglia (Gosselin et al. 2014). Accordingly, TGF- β 1 was found capable of inducing gene expression and enhancer profiles that appeared much more similar to in vivo microglia than in vitro microglia cultured in its absence (Butovsky et al. 2014; Gosselin et al. 2014). Moreover, Tichauer et al., reported that TGF β 1-Smad3 pathway is impaired in aging (Tichauer et al. 2014). Thus, age-related impairment of TGF β 1-Smad3 can reduce protective activation while facilitating cytotoxic activation of microglia, potentiating microglia-mediated neurodegeneration.

13.9 Molecular Signature

13.9.1 Homeostasis

Recent studies reported that microglia have a unique transcriptomic signature, which distinguishes them from other CNS cells and peripheral macrophages or monocytes (Gautier et al. 2012; Chiu et al. 2013), and express a unique cluster of transcripts encoding proteins for sensing endogenous ligands and microbes, defined as the sensome (Hickman et al. 2013). When compared to different tissue-resident macrophage subsets, microglia showed a specific or enhanced expression of genes including *Hexb*, *Tmem119*, *Siglech*, *Olfml3*, *Cx3cr1*, *Gpr84*, *Trem2*, *Socs3*, and *Fcrls* (Gautier et al. 2012). Chiu et al used ImmGen Data to identify 50 genes enriched in microglia as compared to genes expressed within CNS tissues (Chiu et al. 2013). Among them, *Olfml3*, *Siglech*, and *Tmem119* are highly enriched markers for microglia. Hickman et al., identified over 100 genes enriched in microglia as “the microglial toolset for sensing changes in the brain’s milieu” including *Csf1r*, *Cxcr4*, *Cx3cr1*, *Olfml3*, *P2ry12*, *Cd33*, *Siglech*, *Tmem119*, and *Tyrobp* (Hickman et al. 2013). We analyzed mRNA, microRNA and proteomics in adult microglia and identified 239 genes including *Fcrls*, *P2ry12*, *Tmem119*, *Olfml3*, *Csf1r*, *Gpr34*, *Mertk*, *P2ry12*, *Hexb* and eight microRNAs and 74 proteins are uniquely or highly expressed in microglia as compared to their expression in myeloid and other immune cells (Butovsky et al. 2014). Several of these microglial genes were validated in human microglia (Butovsky et al. 2014). Identification of the homeostatic microglia signature will provide robust tools including microglia specific antibodies and genetic mouse models to investigate microglial biology and to generate a more accurate description of microglia phenotypes in disease (reviewed in (Crotti and Ransohoff 2016)).

13.9.2 Aging and Disease

During aging microglia exhibit deramified and fragmented processes in postmortem human brain subjects (Streit et al. 2004). Hickman et al. analyzed the microglia molecular signature in aging mice and found most of the sensome genes are downregulated (Hickman et al. 2013). Furthermore, aging microglia are characterized by expression of cell surface markers like *Lgals3*, *Axl*, *Clec7a*, *MHC class II*, and *Cxcr4* (Holtman et al. 2015b). A similar observation about aged microglia being “immunologically activated but not polarized to a pro- or anti-inflammatory phenotype” at the transcriptional level has been recently reported (Butovsky et al. 2014). Microglia acutely isolated from a murine model of peroxisomal β -oxidation deficiency (*Mfp2*^{-/-}) shows robust and chronic inflammation with increased expression of inflammatory surface and cytokine markers and suppression of the homeostatic purinergic receptor P2ry12, and other cell surface molecules in *Mfp2*^{-/-} model (Verheijden et al. 2015) similar to what was reported in aged microglia (Hickman et al. 2013). A partial explanation of the loss of homeostatic functional phenotypes in aging microglia could be the downregulation of *Tgbr1* observed in *Mfp2*^{-/-} mice (Verheijden et al. 2015), given that TGF β 1 signaling has been reported to be fundamental for microglia expression signature (Butovsky et al. 2014).

Microglia from AD model mice show the upregulation of *Il1b*, *Clec7a*, *Axl*, and *Itgax*. By contrast, the homeostatic genes including *Cx3cr1*, *Tmem119*, and *Csf1r*, as well as some phagocytosis and/or endocytosis genes, were reduced in microglia in the context of AD (Orre et al. 2014).

Similarly, transcriptomics and microRNA analysis of microglia purified from SOD1G93A mice reveals that microglial signature genes are lost during disease progression in this murine model of ALS (Butovsky et al. 2015). P2ry12, transcription factors *Egr1*, *Atf3*, *Jun*, *Fos*, and *Mafb*, and the upstream regulators *Csf1r*, *Tgfb1*, *Tgfb1r*, and *Cd39*—an ATP catabolic ectoenzyme—are downregulated in spinal cord microglia from SOD1G^{93A} mice as compared to those from control mice. In contrast, upregulation of apolipoproteins, including ApoE and miR-155, in microglia from SOD1 mice and human spinal cord from ALS donors were reciprocally upregulated (Butovsky et al. 2015). In particular, ApoE has been proposed to trigger an inflammatory phenotype in microglia, preferentially in male mice. Genetic ablation of miR-155 restores expression of the microglia-specific target genes, attenuates expression of ApoE, and improves survival (Butovsky et al. 2015). Thus, the unique homeostatic microglia gene signature is progressively lost during aging and in neurodegenerative diseases.

13.10 Molecular Signaling in Immunomodulation

13.10.1 Fractalkine/CX3CR1 Signaling

Fractalkine/CX3CL1 protein is the only known member of the chemokine family CX3C and contains 373 amino acids. The protein is synthesized as an anchored membrane protein. It can be converted to a soluble glycoprotein by cleavage of membrane protein form under the action of metalloproteases (Hundhausen et al. 2003) and is secreted by microglia in spinal cord (Meucci et al. 2000; Ransohoff 2002; Clark et al. 2007). CX3CL1 acts like a conventional chemokine, acting locally by direct contact under its anchored form or indirect under its soluble form (Bazan and Allan 1997). Indeed, its increasing the recruitment and activation of many cell populations expressing the receptor CX3CR1, most of them being monocytes. Membrane CX3CL1 act like an adhesive molecule participating to the leukocyte capture and infiltration (Ransohoff 2009). However, it is established that both forms of CX3CL1 bind to CX3CR1 with the same (Harrison et al. 2001). CX3CL1 is one of the chemokine which is constitutively expressed at high levels in the CNS and acts only on the CX3CR1 receptor. The chemokine was cloned from endothelial activated cells and neurons. CX3CL1 and its receptor have been described to play a role in neuron-microglia communication. CX3CL1 is expressed by neurons (Nishiyori et al. 1998; Schwaeble et al. 1998; Hughes et al. 2002) and CX3CR1 is uniquely expressed by myeloid cells including microglia cells (Nishiyori et al. 1998; Hughes et al. 2002; Mizuno et al. 2003). However, CX3CL1 has been shown to be expressed by astrocytes (Mizuno et al. 2003) and some studies have revealed that CX3CR1 could be expressed on neurons (Meucci et al. 2000). CX3CL1/CX3CR1 signaling plays an important physiological role in neuron-microglia communication and in neuroprotection under inflammatory or lesional conditions (Mizuno et al. 2003; Hughes et al. 2002). In vitro and in vivo studies have shown CX3CL1 interaction to its receptor contributes to the attenuation of microglial activation and neurotoxicity under inflammatory conditions. In vitro, soluble CX3CL1 acts like an anti-inflammatory factor through the inhibition of the IL1b, IL6, TNFa, NO and iNOS expression in microglial and mixed glial cultures (Mizuno et al. 2003; Zujovic et al. 2000). In 2009, Lyons showed that both forms of CX3CL1 decrease the expression of IL1b and MHCII in primary cultures of neuron-microglia (Lyons et al. 2009). In vivo, blockade of endogenous CX3CL1 using an anti-CX3CL1 potentiate the increase of TNFa expression and isoprostane-8 (oxidative stress marker), suggesting that CX3CL1 expression is necessary for the control of microglial (Zujovic et al. 2001). Genetic ablation of CX3CR1 showed that the absence of CX3CL1/CX3CR1 signaling exacerbates microglial responses under inflammatory

conditions. Indeed, the mice present a intensive microglia activation and an increase of IL1b production in the hippocampus following LPS stimulation (Cardona et al. 2006a). Moreover, in neurodegenerative disease models such as Parkinson disease or ALS, the absence of CX3CR1 induces a more robust activation of microglia and an important neuronal loss compared to WT mice (Cardona et al. 2006a). During chronic neurodegenerative pathologies or even in a case of acute inflammation, CX3CL1/CX3CR1 signaling regulates microglial activation. It is also the case in diverse physiopathological conditions in which CX3CL1 expression levels are modulated (Hughes et al. 2002; Kastenbauer et al. 2003). CX3CL1 is decreased in the CNS and in the hippocampus of aged rats (Lyons et al. 2009; Wynne et al. 2010) and contributes to low chronic inflammation characteristic in aging. The decrease of CX3CL1 is linked to the increase of activation markers such MHCII and CD40 in microglia and also to an increase of IL1b expression (Wynne et al. 2010). CX3CL1 administrated in the CNS decreases microglial activation and restores synaptic plasticity deficits caused by aging (Lyons et al. 2009). Regarding the receptor CX3CR1, LPS reduces in vitro its expression on microglia cultures (Boddeke et al. 1999). Recently, it was shown that in vivo, peripheral injection of LPS decreases CX3CR1 gene expression on microglia at adulthood and in aged mice (Wynne et al. 2010). CX3CR1 expression is reduced 4 h after LPS injection and restored 24 h later in adult mice. Instead the expression is still decreased in aged mice. This decrease of CX3CR1 expression in aged mice is matching the prolonged expression of IL1b (Wynne et al. 2010). CX3CL1/CX3CR1 signaling modulates microglia and their functions on the CNS.

13.10.2 CD39-ATP Axis

Nucleotides represent a novel and ubiquitous class of extracellular signaling substances in the CNS. ATP can be released from neural and glial cells (Dunwiddie et al. 1997; Pankratov et al. 2006; Koizumi 2010; George et al. 2015). Type 2 receptors for extracellular nucleotides (P2 receptors) function either as ion channels (P2X receptors, permeable for Na⁺, K⁺ and Ca²⁺) (Buell et al. 1998) or are G-protein-coupled (P2Y receptors) (King et al. 1998). They have a broad distribution in embryonic and adult CNS among most cell types. ATP acts as fast as neurotransmitters (Edwards and Gibb 1993; Bardoni et al. 1997) and is stored in vesicles (George et al. 2015). Moreover, ATP can trigger or inhibit neurotransmission by acting on neurotransmitter release via presynaptic receptors (Gu and MacDermott 1997). Microglia present several nucleotide receptors. They respond to ATP with the release of cytokines (IL1b) and undergo apoptosis following activation of P2X7 receptor (Ferrari et al. 1997) acting as a potent cytolytic stimulus. P2X7 receptor is activated in

diverse brain pathologies (Rodrigues et al. 2015). Microglia modulation of neurotransmission is also mediated by binding of ATP to P2RY receptors located on astrocytes. Microglia rapidly release small amounts of ATP and astrocytes in turn amplify this release and this independently of cytokine release.

Ectonucleotidases play a major role in modulating or terminating P2 receptor functions by hydrolysing extracellular nucleotides. Members of the ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) family hydrolyse nucleoside 5'-triphosphates and nucleoside 5'-diphosphates. Northern hybridization suggests that the three related family members NTPDase1 to NTPDase3 are expressed in mammalian brain (Zimmermann and Braun 1999). NTPDase1 (CD39) hydrolyses nucleoside 5'-triphosphates and -diphosphates equally well (Kaczmarek et al. 1996). CD39 is a major ecto-nucleotidase expressed by microglia. CD39 and its activity of ecto-nucleotidase have been shown to be upregulated after cerebral ischemia in the hippocampus (Finsen et al. 1993). Under these conditions, considerable ATP is released and possibly other nucleotides may come from damaged cells. Microglia present a high capacity for surface-located nucleotide hydrolysis. CD39 may protect microglia from ATP-mediated overstimulation and would facilitate the formation of final hydrolysis product adenosine that has neuromodulatory and immunomodulatory roles (Di Virgilio 1998). CD39 activity is also present under physiological conditions in microglia. CD39 may be implicated in termination of purinergic signaling at the vicinity of the synapse involving astrocyte and neuronal transmission.

13.10.3 GPR34

G protein-coupled receptors (GPCR) form the largest gene family among transmembrane receptors, including more than 900 genes in humans and other mammals (Fredriksson and Schiöth 2005). A great number of stimuli, such as light, hormones, neurotransmitters, peptides, and nucleotides, activate the distinct receptors. About 155 so-called “orphan” GPCR (Wigglesworth et al. 2006) await identification of their physiological relevance. GPCR control almost every physiological function making this receptor family the most frequently used target for therapeutic drugs. Among the five structurally different GPCR families (Fredriksson and Schiöth 2005), the rhodopsin-like receptors form the largest in humans and other vertebrates. The rhodopsin-like family is divided further into subfamilies and groups. The P2RY12-like receptor group includes the ADP receptors P2RY12 and P2RY13, the UDP-glucose receptor P2RY14, and the orphan receptors GPR87, GPR82, and GPR34 (Schöneberg et al. 2007).

GPR34 is an orphan receptor of the P2RY12-like receptor group and was first discovered by mining GenBank™ for

novel GPCR sequences and homology cloning, assigned to the human X chromosome (Schöneberg et al. 1999a). Phylogenetic studies revealed that GPR34 has been highly conserved over the past 450 million years of vertebrate evolution (Schulz and Schöneberg 2003). To date, there is no report of GPR34 deficiency in humans, and sequencing of more than 100 worldwide samples of human genomic DNA revealed no functionally relevant alleles indicating the physiological importance of the gene (Engemaier et al. 2006). GPR34 shows a ubiquitous expression profile in mouse and human tissues (Schöneberg et al. 1999b). More detailed analyses showed GPR34 expression in the myeloid progenitor cell line HL-60 in K562 cells, and WEHI-3B cells, the macrophage cell line RAW 264.1 (Engemaier et al. 2006), and in the murine mast cell line P815.

GPR34 is implicated in cellular chemotaxis and immune response. Recently, several members of the P2RY12-like receptor group have been assigned to agonists, including nucleotide derivatives and lipids as physiological ligands (Sugo et al. 2006). Specifically, GPR34 has been shown to be a receptor for lysophosphatidylserine (lysoPS) (Kitamura et al. 2012). However, a recent study showed by knocking down *Gpr34* in mice, no evidence that lysoPS are natural agonists of receptor (Liebscher et al. 2011). LysoPS are generated by hydrolysis of membrane lipids through phospholipases A₁ and A₂ when apoptotic cells expose phosphatidylserine at the cell surface. LysoPS are potent activators of histamine release from mast cells (Bellini et al. 1990). Furthermore, they have been described as growth inhibitors of T cells and as chemoattractant molecules for fibroblasts and tumor cells (Bellini et al. 1990). Deficiency in *Gpr34* showed no major alterations but led to improper immune response upon antigen and pathogen challenges (Liebscher et al. 2011).

Microglia expressed highly *Gpr34* (Butovsky et al. 2014). GPR34 function in microglia has not yet been fully understood but one study presented *gpr34* as important for microglia phagocytosis (Preissler et al. 2015). Altered microglia morphology and decreased phagocytosis was reported in CD39 deficient mice (Bulavina et al. 2013). This convergent microglial phenotype in CD39- and GPR34 may point towards unidentified endogenous purinergic agonists of the ADP receptor P2RY12-like GPR34. Future studies need to address GPR34 relevance under pathophysiological circumstances such as EAE and Alzheimer's disease.

13.10.4 TAM System

The TAM system was discovered as a family of receptor tyrosine kinases (RTKs) in 1991. This system is known to modulate the immune system and in particular microglia by helping clearance of apoptotic cells and restoration of homeostasis. The TAM family is composed of three recep-

tors, Tyro3, Axl and MerTK (Lemke and Rothlin 2008). TAM system mediates homeostatic phagocytosis of apoptotic cells and membranes in adult tissues, facilitate also the infection of target cells by virus and contribute to some extent to progression and metastasis of cancer (Lemke and Rothlin 2008). The principal TAM receptors are Axl and MerTK. Activation of MerTK or Axl modulate the activation of the immune system.

13.11 Biomarkers

Because of mesodermal origin, microglia share many features with other myeloid cell populations. Immunohistochemical studies showed microglial expression of macrophage markers, including F4/80, Fc receptor, and CD11b, in mouse (Perry et al. 1985) and, later, in human (Akiyama and McGeer 1990).

As the tissue-resident macrophage of the CNS, murine microglia have since been confirmed to express multiple macrophage markers, including the colony-stimulating factor (CSF)-1 receptor (CSF-1R, CD115), the integrin CD11b, the surface glycoproteins F4/80, the inhibitory immune receptor CD200R, the surface enzyme tyrosine-protein phosphatase nonreceptor-type substrate or CD172 α , the fractalkine receptor CX3CR1, and the calcium-binding protein Iba-1. Microglia also express lower levels of the panhematopoietic marker CD45 compared with tissue macrophages, which also permits their discrimination from monocytes in the bloodstream, whereas the hemoglobin scavenger receptor CD163 enables distinction from perivascular macrophages in the steady state (Dijkstra et al. 1985; Serrats et al. 2010). CD169/Siglec-1 is a marker for mature dendritic cells/macrophages (Davies et al. 2013). It has recently been proposed as a marker for invaded monocyte-derived cells in the CNS, as it has been shown not to be expressed in resident CNS microglia (Butovsky et al. 2012; Gao et al. 2015; Zondler et al. 2016).

Two first microglia-specific antibodies have been generated using adult microglia as “cell-live vaccine”. Rats were vaccinated with CD45^{Low}/CD11b⁺ adult mouse microglia to generate mouse anti-rat antibodies against surface microglial molecules. Two microglia-specific mAbs were identified CD39 (ectonucleoside triphosphate diphosphohydrolase) and 4D4 mAbs which distinguish non-overlapping populations of peripheral inflammatory monocytes and resident microglia in mice (Butovsky et al. 2012). Based on the identification of a unique transcriptomic signature microglia including surface molecules (Butovsky et al. 2014; Chiu et al. 2013; Gautier et al. 2012; Hickman et al. 2013), additional microglia-specific antibodies have been developed including FCRLS (Fc receptor-like S, scavenger receptor) (Butovsky et al. 2014), P2ry12 (Purinergic Receptor P2Y, G-Protein Coupled, 12) (Butovsky et al. 2014; Haynes et al.

2006) and Tmem119 (transmembrane protein 119) (Satoh et al. 2016; Bennett et al. 2016).

13.12 Molecular Targeting

13.12.1 Genetic Manipulation

Genetic manipulation of microglia *in vivo* has led to valuable insights about the origin of microglia and their behavior under steady-state conditions and diseases (for review, see (Wieghofer et al. 2015; Wieghofer and Prinz 2016)). The fractalkine receptor CX3CR1 is highly expressed on microglia but also other macrophages, monocytes and dendritic cells (Jung et al. 2000). The Cx3cr1⁺/GFP mouse line therefore strongly labels microglia and is the best-studied model in microglia research. Constitutive Cre deleter strains are more efficient and lead to a high “accumulative” degree of recombination in the population of interest. Nevertheless, the lack of temporal control of the recombination events is a disadvantage of these lines. Here, the tamoxifen-inducible Cre-ER-T2/Mer-Cre-Mer system comes into play. This system allows the induction of recombination within a short time window by pulsing with tamoxifen. Two tamoxifen-inducible knockin Cx3cr1*CreER* mouse models were established to target microglia specifically (Goldmann et al. 2013; Parkhurst et al. 2013; Yona et al. 2013)). Both lines have in common that only long-lived cell populations are stably labeled, whereas short-lived cells, like monocytes, underlie a turnover and are continuously replenished by hematopoietic stem cells in the bone marrow (for review, see (Wieghofer et al. 2015; Wieghofer and Prinz 2016)). Parkhurst et al. and Bruttger et al. used this model as a tool to deplete microglia by crossing them to the Rosa26-fl-stop-fl-DTR (R26-iDTR) transgenic line to induce the expression of the primate diphtheria toxin receptor (DTR) upon Cre-mediated recombination (Bruttger et al. 2015; Buch et al. 2005; Parkhurst et al. 2013). The DT can cross the blood–brain barrier (BBB) and leads to an arrest of translation after receptor-mediated entering of the cell by blocking the phosphorylation of the elongation factor 2 (EF-2) (Wieghofer et al. 2015). After the administration of DT, specifically microglia are virtually absent after 1 day and only slowly recovered in a time-dependent manner over the course of 1 week while CX3CR1⁺ cells in blood and spleen remained unaffected (Bruttger et al. 2015).

13.12.2 Therapeutic Approaches

Microglia, since it plays so much of a key and critical role in neuroinflammation and thereby neurodegeneration progression has been the reason why they have been put in focus as inter-

vention targets in disease treatment. A corollary of neuroinflammation proposes that suppression of microglial production of neurotoxic mediators will result in neuroprotection. Although several drugs alleviate symptoms of neurodegenerative diseases, chronic use of these drugs is often associated with debilitating side effects, and none seems to dampen the progression of these diseases. So far, the development of effective neuroprotective therapies is impeded by our limited knowledge of the pathogenesis of neurodegenerative diseases. Since several studies have demonstrated that the inhibition of microglial activation control the pathogenesis, thus the activation of counter regulatory mechanisms is essential to avoid the escalation of CNS inflammatory processes (McCarty 2006). This may be possible through the identification of agents that target over activated microglial cells and the determination of their anti-inflammatory mechanisms. This may help development of better therapeutic strategies for neurodegenerative diseases.

Some of the well-studied inhibitors of inflammation are glucocorticoids, minocyclines, vitamin E, D, endocannabinoids, transforming growth factor beta1 and several synthetic drugs (Dheen et al. 2007). Though several anti-inflammatory drugs have been shown to diminish neuroinflammation, a very few have direct functional effects on microglial activity (Lleo et al. 2007).

Non steroidal anti-inflammatory drugs (NSAIDS) which include ibuprofen, naproxen and many other generic drugs, have been identified with a tendency to irritate the stomach, with the possibility of more serious complications such as an ulcer, stomach bleeding, colon or small bowel irritation over long term usage (Silverstein et al. 2000). Though synthetic cyclooxygenase 2 (COX-2) selective NSAIDs were brought into the market as alternatives highlighting its association with less gastrointestinal toxicity than nonselective NSAIDs, recent studies have shown that they cause more serious adverse effects like cardiovascular events and other life threatening side effects (Mukherjee et al. 2001). Therefore, there is an urgent need to develop drugs that have wide spectrum anti-inflammatory effects and which are able to slow down or curtail the progression of the degenerative process without causing any debilitating side effects.

13.13 Pharmacological Targeting

Recent evidence supports that CSF1R inhibitors could cause the depletion of the microglia. Thus, injection of a blocking antibody against Csf1r on activated microglia induced a strong reduction of proliferating cells during prion infection and AD (Gomez-Nicola et al. 2013). Moreover, treatment with the CSF1R/c-kit inhibitor PLX3397 decreased brain microglia by 99% after 21 days (Elmore et al. 2014b). Inhibition of the CSF1R at lower levels in 3xTg-AD mice

with a specific CSF1R inhibitor (PLX5622) prevented microglial association with plaques and improved cognition (Dagher et al. 2015). Prolonged inhibition of CSF1R in APP/PS1 mice by an orally administration of tyrosine kinase inhibitor (GW2580) resulted in the blockade of microglial proliferation and the shifting of the microglial inflammatory profile to an anti-inflammatory phenotype (Olmos-Alonso et al. 2016). Pharmacological targeting of CSF1R in AD mice in both studies resulted in improved performance in memory and behavioral tasks and a prevention of synaptic degeneration, although these changes were not correlated with a change in the number of amyloid- β plaques (Olmos-Alonso et al. 2016). These studies support for the efficacy of CSF1R inhibitory strategies in the treatment of Alzheimer's disease-like pathology to reduce microglia numbers and reduce the potentially damaging components of neuroinflammation, thus underpinning the possible evaluation of CSF1R inhibitors in clinical trials for Alzheimer's disease.

13.14 Review Questions

- What is the origin of microglia?
 - Yolk sac erythromyeloid progenitors*
 - recruited myeloid cells
 - neural stem cells
 - hematopoietic stem cells
 - all of the above
 - None of the above
- Neuroinflammation is occurring in the brain in response to:
 - infection
 - neurodegenerative disease
 - neuronal lesion
 - acute injury
 - all of the above*
 - None of the above
- Microglia in homeostasis are:
 - quiescent
 - neurotoxic
 - neuroinflammatory
 - producing inflammatory cytokines
 - none of the above*
- Modulatory factor involved in induction of homeostatic microglia:
 - PU.1
 - TGF β 1*
 - IRF8
 - RUNX1
 - None of the above
- Microglia physiological functions are:
 - cytokine production
 - antigen presentation
 - brain surveillance

- (d) phagocytosis
 - (e) *all of the above*
 - (f) none of the above
6. Neuron-microglia communication is controlled by:
- (a) CX3CL1
 - (b) CX3CR1
 - (c) SDF-1
 - (d) *all of the above*
 - (e) none of the above
7. Unique microglial biomarkers are:
- (a) CD11b
 - (b) Iba1
 - (c) CX3CR1
 - (d) F4/80
 - (e) P2ry12
 - (f) Tmem119
 - (g) a and b
 - (h) c, e and f
 - (i) *e and f*
8. Targets to pharmacologically deplete microglia:
- (a) Iba1
 - (b) *Csf1r*
 - (c) Cox2
 - (d) CX3CR1
 - (e) None of the above
9. Describe the available animal models to target microglia. What are the limitations of the animal models?
10. Describe the common characteristic of microglia in development and aging.

13.15 Answers

9. There are several mouse models to target microglia have been developed. The most popular are *Cx3cr1Cre* and *Cx3cr1CreER* mice crossed to *Rosa26-fl-stop-fl-DTR* or floxed-genes of interest will serve a model to specifically and conditionally ablate microglia or delete genes in microglia. Limitations: *Cx3Cr1* is a broad myeloid receptor, thus the ablation system is not specific to parenchymal CNS microglia. Therefore, treatment of *Cx3cr1CreER* mice with tamoxifen for 5 days will induce Cre expression in all peripheral myeloid cells and microglia. Due to low rate of microglia proliferation, within 30 days, microglia will continue to express Cre-recombinase, but peripheral myeloid cells will be repopulated with new bone-marrow derived myeloid cells and will not express the Cre-recombinase.
10. Based on morphological and molecular aspects, microglia during development and aging show common amoeboid and inflammatory profile. In addition, the molecular homeostatic signature is not present embryonic microglia and suppressed during aging.

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Abstract

This chapter describes lymphocyte maturation and differentiation. Interactions of lymphoid tissues with the central and peripheral nervous systems play a fundamental role in health and disease. Recent advances in xenobiology has opened new approaches to study relationships between peripheral immunity and nervous system development, homeostasis, response to injuries, and disease in animal models. Humanized animal models help to study the complexity of human immune system development and adaptive immune responses. Humanization of a mouse brain with human glial cells provides an additional tool to study hemato-lymphoid brain interactions in small animals.

Keywords

Antibodies • B cells • Effector memory T cells • Follicular dendritic cells (FDCs) • Immune privileged • Regulatory T cells • Stat4 • Stat6 • T-Bet

14.1 Introduction

For many years, the immune system was studied separate from the nervous system and vice versa. This is for several reasons. *First*, under normal physiological conditions peripheral lymphocytes are not found in perivascular compartments and not within the central nervous system (CNS) parenchyma. This supported the notion that the brain was “immune privileged.” However, the presence of infiltrating immune cells, including lymphocytes, in the CNS is present in most neurodegenerative diseases. *Second*, there is no question about ongoing adaptive immune activities

during CNS infections and autoimmune disorders. *Third*, the functional links between the CNS and lymphoid organs are operative in development, cell to cell interactions, signaling, and modeling. Despite the complete segregation and privileged status of the mammalian CNS, immune-neural interactions have shown common developmental paths and share cell types and regulatory molecules. Similarities between the nervous and immune systems show essential homeostatic and coordinated responses to danger. Both organs are able to establish direct and non-direct connections and carry information from and to distant parts of the body by use of a range of chemical signaling molecules. Both also communicate rapidly through chemical and cellular networks by utilizing shared and conserved second messengers and signaling pathways. They form synapses to facilitate information transmission and have threshold sensitivity of cellular receptors for ligands. Nervous and immune organs acquire memory with maturation, training, and exposure to new signals. *Finally*, studies of human immune system and nervous system development and function became possible in severely immunodeficient mice by transplantation of human hematopoietic and neural stem cells, named humanized animal models.

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14.2 Overview of Embryonic Hematopoiesis, Lymphopoiesis and Nervous System Development

Today, the common concept of lymphoid tissue ontogeny relies upon the concept of circulating stem/progenitor cell populations, which migrate from the yolk sack into the aortogonad mesonephros (AGM), then into organ anlagen, initiating hematopoiesis in fetal liver and bone marrow, and lymphopoiesis in thymus [reviewed in (Baron et al. 2012; Jagannathan-Bogdan and Zon 2013; Prinz and Priller 2014; Rodgers and Dizerega 2013)]. Neural crest cells (NCC) are one of the earlier developmental “bridges” between the blood and brain. During early embryonic development, primitive hematopoiesis (erythropoiesis and macrophages) begins in the yolk sac; and the first neuroepithelial and neural crest cells from neural tube provide support by secretion of erythropoietin (Suzuki et al. 2013). NCC are a pluripotent population of cells that migrate from the dorsal neuroepithelium and give rise to multiple cell types including most of the peripheral nervous system (PNS) (neurons of the sensory, sympathetic, parasympathetic and enteric ganglia as well as ganglionic satellite glial cells and Schwann cells lining peripheral nerves), pigment cells and craniofacial bone and cartilage (Takahashi et al. 2013; Theveneau and Mayor 2012).

Similarities between the nervous and immune system development regulation are made accordingly. For example, a regulatory circuit made up of transcriptional factors *Gata2*, *Fli1*, and *Scl/Tal1* and their enhancers, *Gata2-3*, *Fli1+12*, and *Scl+19*, operates during specification of hematopoiesis (Pimanda et al. 2007), as well as *SCL* and *Gata2* are common with midbrain and DA system development (Herberth et al. 2005). Ephrin-B2 is a protein that in humans is encoded by the *EFNB2* gene, a member of the ephrin (EPH) family. The ephrins and EPH-related receptors comprise the largest subfamily of receptor protein-tyrosine kinases and have been implicated in mediating developmental events, especially in the nervous system and in erythropoiesis (Carmeliet and Tessier-Lavigne 2005). During maturation and differentiation of cells and organ structures, some such regulatory genes participate in the regulation of molecules and pathways involved in systemic crosstalk between systems.

14.2.1 T Cells

The thymus is a primary lymphoid organ that is involved in the development of the T cells, which are effectors of adaptive immune responses. The human thymus develops at ~embryonic day 35 from the endoderm with subsequent epithelial–mesenchymal interaction. In regards to the ontogeny of the immune system, most of what is known was derived from rodent studies. Thymic epithelium and surrounding mesenchyme, which derived from the cephalic region of the neural

crest, play an important role in T cell development. At the embryonic stage, lymphomyeloid commitment precedes thymic development in $CD45^+CD117^+CD34^+$ hematopoietic clusters of AGM region. In addition, multilineage progenitors restricted to T, NK (natural killer) and macrophage lineage (pTM) or just T lineage (pT), or B and B plus macrophage (pBM) were identified in the AGM region. At the time of entry into the thymic epithelium, immigrant cells express $CD45^+$, $CD117^+$, $CD44^+$, $CD34^+$, and $\alpha 4$ integrin; but they are negative for $CD62L$, $CD25$, and $Thy-1.2$ in mice. One of the important cytokines that regulates survival of human lymphoid progenitors is interleukin (IL)-7. Before exposure to epithelial inductive influence by thymus epithelial stromal cells (TEC), lymphomyeloid progenitors are IL-7 receptor alpha chain positive ($IL-7R\alpha^+$). Upon differentiation to functionally mature lymphocytes ($CD4$ and $CD8$), $IL-7R\alpha$ appears to be downregulated, and cells lose thymic homing capacity. In knockout models of mice, deletion of *IL-7* gene or related receptors abrogate development of lymphocytes and lymphoid organs (Tsapogas et al. 2011; Liang et al. 2012; Ribeiro et al. 2013). The transmembrane receptor Notch is essential during early T lineage development. In the absence of Notch1-mediated signals, T cell development is arrested at an early stage; and B cells accumulate intrathymically.

14.2.2 B Cells

The precise site of human B cell commitment in ontogenesis is not yet known. The B cell precursors derive from the intraembryonic para-aortic region. B cell commitment might take place in the omentum and fetal liver. These progenitors express $CD117^+AA4.1^+CD34^+CD45^+CD19^+Sca-1^-$. The fetal liver has long been considered the initial site of B cell commitment, and from then on B progenitors expand in a synchronous wave-like pattern reaching a peak in the perinatal stage. Even after birth, B cells may develop at sites other than the bone marrow. The myeloid suppressing transcription factor Pax-5 plays a critical role in B cell commitment, while the chemokine receptor CXCR4 and its ligand SDF-1 α play a pivotal role in B cell development of the fetal liver. Another hypothesis based on mouse development suggests that interleukin-7 receptor- α ($IL-7R\alpha$)-expressing progenitors from mouse fetal liver promotes their development into B cells as well as pre-lymphoid tissue inducers compartment (van de Pavert and Mebius 2010).

14.2.3 Cells Derived from Hematopoietic Precursors are Involved in Immune Responses in the Brain

Brain tissue contains only one type of specialized resident cell of mononuclear phagocyte lineage, which is known as microglia. Several waves of population in the brain by cells of

macrophage lineage have been proposed: primitive macrophages from yolk sac with high proliferation capacity, myeloid precursors from fetal liver with ability to proliferate, and bone marrow derived non-dividing (or very limited division) precursors in adult. Two types of brain macrophage residents exist (microglia and perivascular macrophages) with non-selective but differently expressed human markers. All are CD45^{lo}CD11c⁺CD11b⁺(Mac-1^{+/−})CD64^{+/−}CD68^{+/−}RCA-1^{+/−}MHC-classII^{+/−}Ham56⁺ and belong directly to immune system as effectors of innate immunity. They are major participants in the establishment of adaptive immune reaction and immune privilege in situ in the parenchyma and the meninges. The nature of microglia and other brain residents from monocyte lineage were well explored in mice (Ginhoux et al. 2010; Perdiguero et al. 2014). These cells originate from yolk-sac-derived erythro-myeloid; and the turnover and replacement with bone-marrow derived monocytes/tissue macrophages is extremely limited.

14.3 Postnatal Development of Lymphocytes

14.3.1 Hematopoietic Stem Cells and Lymphoid Cell Lineages

The generation of lymphocytes from adult hematopoietic stem cells (HSCs) is different from the embryonic development. HSCs are defined by their unique capacity to self-renew as well as their multilineage differentiation into all blood cell lineages. Therefore, they are crucial for reconstitution of hematopoiesis on transplantation into recipients with bone marrow (BM) ablation. Although representing approximately only 0.05–0.1 % of total BM cells, virtually all HSC activity has been shown to be contained within the lineage negative or low (Lin^{−/lo}) Sca1⁺c-Kit(CD117)^{lo/hi} (LSK) CD34^{−/lo} HSC compartment. The LSK HSC pool can be further subdivided for short-term repopulating cells using additional markers. As HSCs acquire high levels of CD34 expression, CD27 and another tyrosine kinase type receptor, Flk-2/Flt3, lose long-term repopulating activity.

It is as yet unanswered whether T, B, NK and DCs (dendritic cells) develop from common lymphoid progenitors (CLPs), or whether they are derived from either lymphoid-restricted stem cells or multipotent progenitors. However, at least in the adult murine bone marrow, a population of Lin[−]IL-7R⁺Thy-1[−]Sca-1^{lo}CD117^{lo} cells has been shown to contain CLPs on the basis of their capacity to develop into lymphoid cells, while being unable to support myeloid differentiation. It is possible that between common myeloid precursors (CMP) and granulocyte monocyte precursors (GMP) exist the population of progenitors for monocytes and dendritic cells (MDP), which is positive for CX₃CR1 (fractalkine receptor) (Fogg et al. 2006). The progenitor cell

choice of the T-cell fate is dependent on environmental signals. Identification and characterization of thymus seeding progenitors (TSPs) has been challenging, owing to the rarity of these cells in the BM or blood at any given time; thus, their lineage potential and level of commitment are still largely debated. Potential candidates of TSPs include: lineage marker negative (Lin[−]) Sca-1⁺ cKit/CD117⁺ (LSK) cells; LSK Flt3⁺ multipotent progenitors (MPP) and further subdivided based on the expression of CCR9 and VCAM-1; LSK Flt3^{hi} Rag-1⁺ early lymphoid progenitors (ELP) or lymphoid-primed multipotent progenitors (LMPP); Lin[−] Sca-1^{lo} cKit^{−/lo} Flt3^{hi} IL-7Rα⁺ common lymphoid progenitors (CLP) or their progeny Lin[−] cKit^{−/lo} B220⁺ IL-7Rα⁺ preTα⁺ CLP-2; and, Lin[−] cKit^{lo} CD90⁺ CLP-2-like circulating T-cell progenitors (CTP). However, the identity of the cells that first enter the thymus is generally accepted to be a Lin[−] cKit⁺ Flt3⁺ CD24^{−/lo} PSGL1⁺ CCR9⁺, commonly referred to as the early thymic progenitor (ETP) (Thompson and Zuniga-Pflucker 2011). The generation of B-lymphocytes from HSCs is controlled by two cytokine receptors (Flk2/Flt3 and IL-7R) and six transcription factors (PU.1, Ikaros, E2A, Bcl11a, EBF, and Pax-5). Ikaros and PU.1 act in parallel pathways to control the development of lymphoid progenitors in part by regulating the expression of essential signaling receptors Flt3, CD117, and IL-7Rα. The generation of the earliest B cell progenitors depends on transcriptional factors E2A and EBF that coordinately activate the B cell gene expression program and immunoglobulin heavy-chain gene rearrangements at the onset of B-lymphopoiesis. Pax5 restricts the developmental options of lymphoid progenitors to the B cell lineage by repressing the transcription of lineage-inappropriate genes and by simultaneously activating the expression of B-lymphoid signaling molecules. Two other transcriptional factors LEF1 and Sox4 contribute to the survival and proliferation of pro-B cells in response to extracellular signals. Finally, IRF4 and IRF8 control the termination of pre-B cell receptor signaling, and thus, promote differentiation to small pre-B cells undergoing light-chain gene rearrangements (Singh et al. 2005). Productive variable-region and joining-region rearrangements (VLJL rearrangements) of the immunoglobulin light chain are completed in pre-B cells; expression of the B-cell receptor (BCR) drives pre-B cells to the immature B-cell stage. Newly formed B cells are exported to peripheral lymphoid tissues as functionally immature or transitional intermediates cells.

14.3.2 T Cell, TCR, MHC and CD4–CD8 Development

Intrathymic development of T cells is based on three major sets of genes: promiscuous tissue-specific antigen (TSA) expression on thymic epithelial cells (TEC); MHC class I and II molecules expression on antigen-presenting cells

(APC), such as cortical TEC, medullary TEC, thymic dendritic cells (DCs), and macrophages; and T cell receptor (TCR) molecules.

14.3.2.1 The Thymic Epithelial Cells

The nature of TEC precursors and pathways of maturation is still in debate. The most accepted concept today is the presence of self-renewing precursors of endoderm origin [reviewed in (Anderson et al. 2014)]. These cells undergo a maturation pathway from a cortical TEC (cTEC) without expression of a transcriptional regulator known as an autoimmune regulator (AIRE), to an immature medullary TEC (mTEC) with low levels of AIRE expression, then to mature mTEC AIRE-positive, and finally to Hassall's corpuscles. Expression of TSA is a physiological property of TECs, representing all parenchymal organs, and including up to 5–10 % of all currently known genes, including fetal stage-, tumor- and sex-associated. TSA expression increases with TEC maturation. Brain-, intestine- and liver-related TSA have the highest presentation on mTEC. TECs do not form a static scaffold, but similar to stratified epithelia of the skin and gut have a steady state turnover with a half-life of 3–4 weeks. Together with a turnover rate of 2 weeks for thymic DCs, this makes the APC population in the medulla a highly dynamic compartment (Derbinski and Kyewski 2005; Perniola 2012; Sun et al. 2013).

Interaction with TEC is the major step in T cell education in the thymus and in establishment of self tolerance. The cross-talk between derived early T lineage progenitors (ETPs) with MHC II-positive cTEC give rise to CD4⁺CD8[−] double negative (DN) thymocytes. These cells undergo proliferative expansion and lose B- and NK potential. After that, T cell lineage commitment and the onset of TCR β -chain rearrangement occurs. Then positive selection takes place through interaction of now CD4⁺CD8⁺ double positive cells with cTEC bearing MHC I and II. Then cross-talk between mTEC and DCs expressing TSA and circulating antigens leads to deletion (or induction of anergy) of self-reacting T cells, known as negative selection. These events take place in the corticomedullary junction and medulla; then already single positive CD4 or CD8 cells leave the thymus (Bommhardt et al. 2004; Yui and Rothenberg 2014; Shah and Zuniga-Pflucker 2014).

14.3.2.2 T Cells Receptor and CD4/CD8 Commitment

Signaling through the T cell receptor (TCR) controls key events in the life of T cells: their development in the thymus from common lymphoid progenitors, the survival of naïve T cells following their exit from the thymus, and the differentiation of these cells into effector populations with discrete functional profiles. Mature T cells express either as $\alpha\beta$ - or $\gamma\delta$ - TCRs, which recognize antigens. These receptors are generated by a rearrangement of gene segments that result in

the formation of genes encoding the α -, β -, γ - and δ -chains of the receptors. The choice of $\gamma\delta$ vs. $\alpha\beta$ chains of TCR expression happens at the stage of pre-TCR by selection of cells with productive TCR β rearrangements irrespective of the V β gene segment used. Rearrangement of TCR generates receptors with different affinity and avidity to an unlimited amount of peptides (derived from self and non-self proteins) in the context of MHC molecules expressed on thymic APC/TEC. Thymocyte fate and selection are related to a degree of TCR downregulation and internalization after TCR/peptide/MHC engagement. Maximal TCR downregulation is correlated with negative selection (highest avidity, agonists), and suboptimal TCR down-regulation mediated positive selection (lowest avidity, antagonists). Intermediate stages in development of early thymocytes from triple negative (CD3[−]CD4[−]CD8[−]) into triple positive (CD3⁺CD4⁺CD8⁺) Selection undergoing, in context of MHC class II antigens generates CD4⁺ cells. The Lck protein tyrosine kinase activity upon “strong” TCR signaling favors development of CD4⁺ cells; whereas, “weak” TCR signaling and reduction of Lck activity favors development of CD8⁺ cells. TEC and thymic hematopoietic DCs are able to induce the proliferation and differentiation of CD4⁺CD8[−]CD25[−]T cells into CD4⁺CD25⁺FOXP3⁺ (forkhead box P3) regulatory T cells (Treg). This induction depends on peptide/MHC class II interactions, and the presence of IL-2. Singer et al. summarized the existing models of CD4/CD8 lineage choice (Singer et al. 2008). These mature, but naïve T cells exit peripheral blood and seed lymphoid organs in T-cell specific zones, in order to be acquired by adaptive immune responses for elimination of infected or tumor cells, support for humoral immune responses, formation of immunologic memory, prevention of excessive tissue damage, and facilitation of tissue regeneration.

14.3.2.3 T Cells and Brain

The long living neurons and synapses that provide long-term memory have to be strongly protected from damage by inflammatory/autoreactive CD4⁺ and cytolytic/cytotoxic CD8⁺ T cells. Brain parenchyma does not provide the support for the migration or survival of T cells. The highly specialized nature of the endothelial cell lining, with the cerebral vasculature and tight junctions, forms a physical and functional barrier to lymphocytes. For a short period of time, T cells can be found in perivascular spaces and make parenchymal foci as an inflammatory reaction to acute viral or bacterial infection. However, such physiologic responses in the brain parenchyma are quickly terminated by the down-regulation of MHC class I and II co-stimulatory molecular expression on glial cells (immunosuppressive function of IL-10, TGF- β and prostaglandins), by apoptosis of T cells through Fas-Fas ligand (CD95/CD95L) interaction. Activation of brain microglial cells, DCs, and macrophages

may also be stopped by factors directly produced by neurons, such as CD200 molecules. This protein from the Ig superfamily plays an important role in the downregulation of myeloid cell function, which expresses a receptor to this ligand. Persistence of T cells in the brain is a rare event and is associated with chronic pathology as a tertiary lymphoid structure, with support and activation signals coming from DCs and macrophages.

14.3.3 B Cells

14.3.3.1 Development in Bone Marrow

In adults, B cells are generated from B-lineage committed precursors in the BM. Newly formed B cells express antigen-specific surface antibody (sIgM+ and sIgD+). In the BM, B cells are subjected to multiple selective pressures that purge autoreactive B cells and guide differentiation of the remaining cells into functionally distinct peripheral B cell compartments. The naïve B cells then enter the circulation and migrate to the spleen and lymph nodes. The generation of B-lymphocytes from hematopoietic stem cells (HSCs) is controlled by two cytokine receptors (Flk2/Flt3 and IL-7R) and six transcription factors (PU.1, Ikaros, E2A, Bcl11a, EBF, and Pax-5). Ikaros and PU.1 act in parallel pathways to control the development of lymphoid progenitors in part by regulating the expression of essential signaling receptors Flt3, CD117, and IL-7R α . The generation of the earliest B cell progenitors depends on transcriptional factors E2A and EBF, which coordinately activate the B cell gene expression program and immunoglobulin heavy-chain gene rearrangements at the onset of B-lymphopoiesis. Pax5 restricts the developmental options of lymphoid progenitors to the B cell lineage by repressing the transcription of lineage-inappropriate genes and by simultaneously activating the expression of B-lymphoid signaling molecules. Two other transcriptional factors LEF1 and Sox4 contribute to the survival and proliferation of pro-B cells in response to extracellular signals. Finally, IRF4 and IRF8 together control the termination of pre-B cell receptor signaling, and thus, promote differentiation to small pre-B cells undergoing light-chain gene rearrangements. Productive variable-region and joining-region rearrangements (VLJL rearrangements) of the immunoglobulin light chain are completed in pre-B cells, and expression of the B-cell receptor (BCR) drives pre-B cells to the immature B-cell stage. Newly formed B cells are exported to peripheral lymphoid tissues as functionally immature or transitional intermediates cells (Clark et al. 2014).

14.3.3.2 Distribution of B Cells and Function

Selection from newly formed B cells into transitional, follicular and marginal-zone (MZ) B cells depends on integrated signals from several classes of surface receptors,

such as: the BCR and co-signals, the tumor-necrosis factor receptor (TNFR) family, and the G-protein-coupled receptors (GPCRs). Follicular and MZ long-lived compartments participate differentially in B-cell effector functions such as the germinal-centre (GC) reaction, long-term memory, antigen presentation, and antibody and plasma-cell (PC) generation. Different strengths of BCR signaling are required for the development of the three mature B-cell subsets: peritoneal B cells require the strongest BCR signal, follicular B cells require an intermediate BCR signal, and marginal-zone B cells require a weaker BCR signal. During B lymphocyte development, antibodies are assembled by random gene segment re-assortment to produce a vast number of specificities. A potential disadvantage of this process is that some of the antibodies produced are self-reactive; and in humans, the majority (55–75 %) of all antibodies expressed by early immature B cells displayed self-reactivity, including polyclonal and anti-nuclear specificities. Most autoantibodies are removed from the population at discrete checkpoints during B cell development. Inefficient checkpoint regulation would lead to substantial increases in circulating auto-antibodies. So, an important role of the BCR at the immature B-cell stage is to induce efficient elimination of these potentially harmful cells. This can be achieved in three ways: immature B cells are eliminated through negative selection (BCR-induced cell death), they are inactivated (anergy), or they revise the specificity of their BCRs (receptor editing). Finally, secretion of IgM, IgG1, and IgG2c represents Th1-dependent immune responses; IgG2 and IgE represents Th2-dependent; while IgG3 represents T cell independent immune responses (Hua and Hou 2013).

14.3.3.3 Brain and B Cells

In contrast to T cells, in different immunopathologies, the brain provides a fostering environment to B cells. Primary central nervous system (CNS) lymphomas are usually of B cell origin. The cerebrospinal fluid (CSF) of patients with chronic infectious and autoimmune diseases of the CNS typically contains remarkably stable oligoclonal Ig bands. In the CNS of multiple sclerosis patients, clonally expanded B cells and plasma cells persist, while ectopic B cell follicles develop in the meninges of patients with secondary progressive multiple sclerosis (MS), and B cell differentiation may be recapitulated in the CNS of MS patients (Krumbholz et al. 2006).

14.3.4 Natural Killer and Other Innate Lymphoid Cells

Human NK cells comprise ~15 % of all lymphocytes and are defined phenotypically by their expression of CD56 and lack of CD3 expression. Two distinct populations of human NK

cells could be identified based upon their cell-surface density of CD56. The majority (~90 %) of human NK cells has low-density expression of CD56 (CD56^{dim}) and express high levels of Fcγ receptor III (FcγRIII, CD16); whereas, ~10 % of NK cells are CD56^{bright}CD16^{dim} or CD56^{bright}CD16⁻. Over the past decade a number of phenotypic and functional properties of human NK cells have been characterized. There is now good evidence to suggest that distinct immunoregulatory roles can be assigned to the CD56^{bright} and CD56^{dim} NK-cell subsets. Peripheral NK cells are expressing in addition to CD16 and CD56, CD161 (NKR-P1A). The low-affinity FcγRIII on the surface of NK cells binds to Ab-coated (opsonized) targets and signals through associated subunits containing an immunoreceptor tyrosine-based activation motif (ITAM) to direct antibody-dependent cellular cytotoxicity (ADCC). Of significance, the CD56^{bright} NK-cell subset expresses the high-affinity IL-2R constitutively and can produce interferon gamma (IFN-γ) in response to picomolar concentrations of IL-2. Because IL-2 is produced only by T cells and not NK cells, the expression of high-affinity IL-2Rαβγ on CD56^{bright} NK cells is necessary to promote cytokine cross-talk between NK cells and T cells in secondary lymphoid organs. NK cells constitutively express several receptors for monocyte-derived cytokines (monokines), including IL-1, IL-10, IL-12, IL-15, and IL-18, and most likely receive some of their earliest activation signals from monocytes during the innate immune response. Conventional NK cells now could be classified as population of innate lymphoid cells (ILCs), which belong to group 1 with two other populations that not carry described above receptors but produce IFN-γ. Group 2 ILCs secrete IL-5 and IL-13 following stimulation by IL-25 or IL-33 ("alarmin") released by damaged epithelial cells or activated myeloid cells. Group 3 ILCs include lymphoid tissue inducer (LTi) cells, natural cytotoxicity receptor (NCR)+ and NCR- retinoic acid-related orphan receptor (ROR)γt+ subsets that produce cytokines IL-17A and/or IL-22. Functionally, the ILC family can be considered as "innate" equivalents of T effector cells (cytotoxic CD8 T cells and differentiated CD4 Th subsets), as they secrete a similar array of cytokines and activate an analogous set of target cells (Xu and Di Santo 2013).

14.4 Organization of the Secondary Lymphoid Tissues

14.4.1 Embryonic Development of Lymphoid Tissues

Development of the lymphatic system begins with the invagination of endothelial cells from veins and the formation of lymphoid sacs (Fig. 14.1). At the location of the lymph sacs, connective tissue protrudes into these lymph sacs, forming

the very first anlagen of the lymph nodes. At this moment, differentiation of mesenchymal cells into specialized cells, known as lymphoid organizer cells, which can initiate the formation of lymph nodes, is expected. The platelet-derived growth factor (PDGF), fibroblast growth factor, as well as TGF superfamily of growth factors, which are crucial for the differentiation of mesenchymal cells, are likely to be involved in the earliest phases of lymphoid organ formation. However, no molecules have been identified that direct the early specification of mesenchymal cells into specific lymph node organizer cells. The mesenchymal specification of lymph node organizer cells is expected to be independent from lymphotoxin beta receptor (LTβR) signaling. The stromal organizer cells mediate attraction and retention of hemopoietic cells (lymphoid tissue inducers, LTi), resulting in accumulation and clustering of cells (Lu and Browning 2014; Cupedo and Mebius 2005; Benezech et al. 2010).

For the generation of LTi cells, which are derived from IL-7R-expressing hematopoietic precursors, expression of retinoic-acid-receptor-related orphan receptor-γ (RORγ) by LTi cells is mandatory. LTi cells mediate triggering of the LTβR on stromal cells through expression of LTα1β2, which is induced by ligation of either the TNF-related activation-induced cytokine (TRANCE), which signals through TRANCE receptor (TRANCE) and/or IL-7R. Functional LTi cells need to express CXCR5 as well as CCR7 to respond to CXCL13, CCL19, and CCL21, chemokines involved in lymph node formation. To generate functional lymphoid tissue organizers, LTβR signaling leads to expression of mucosal addressin cell adhesion molecule (MAdCAM-1), intercellular adhesion molecule 1 (ICAM-1), and vascular cell-adhesion molecule 1 (VCAM-1) on these cells, as well as the production of the chemokines CXCL13, CCL19, and CCL21. The mesenchymal cells produce more chemokines, express adhesion molecules and establish the first positive-feedback loop for the recruitment of LTi cells.

Days before birth, lymphocytes and dendritic cells (DCs) are recruited to the lymph node and Peyer's-patch anlagen. Because T cells and B cells themselves express LT-α1β2, recruitment of lymphocytes is amplified by a second positive-feedback loop and culminates in the formation of a mature lymph node or Peyer's patch, consisting of segregated T-cell zones and B-cell follicles, high endothelial venules (HEVs) and additional specialized mesenchymal cells.

IL-7R signaling has been reported to be essential for the development of Peyer's patches, but this pathway is also involved in the generation of some lymph nodes. In the absence of a functional IL-2R common γ-chain (γc) and Janus kinase 3 (Jak3), which are both components of the IL-7R signaling pathway or in the absence of IL-7, no peripheral lymph nodes develop. Although lymphopenia has been suggested as an explanation for the inability to detect lymph nodes in IL-7R-deficient and γc-deficient mice, lymph nodes

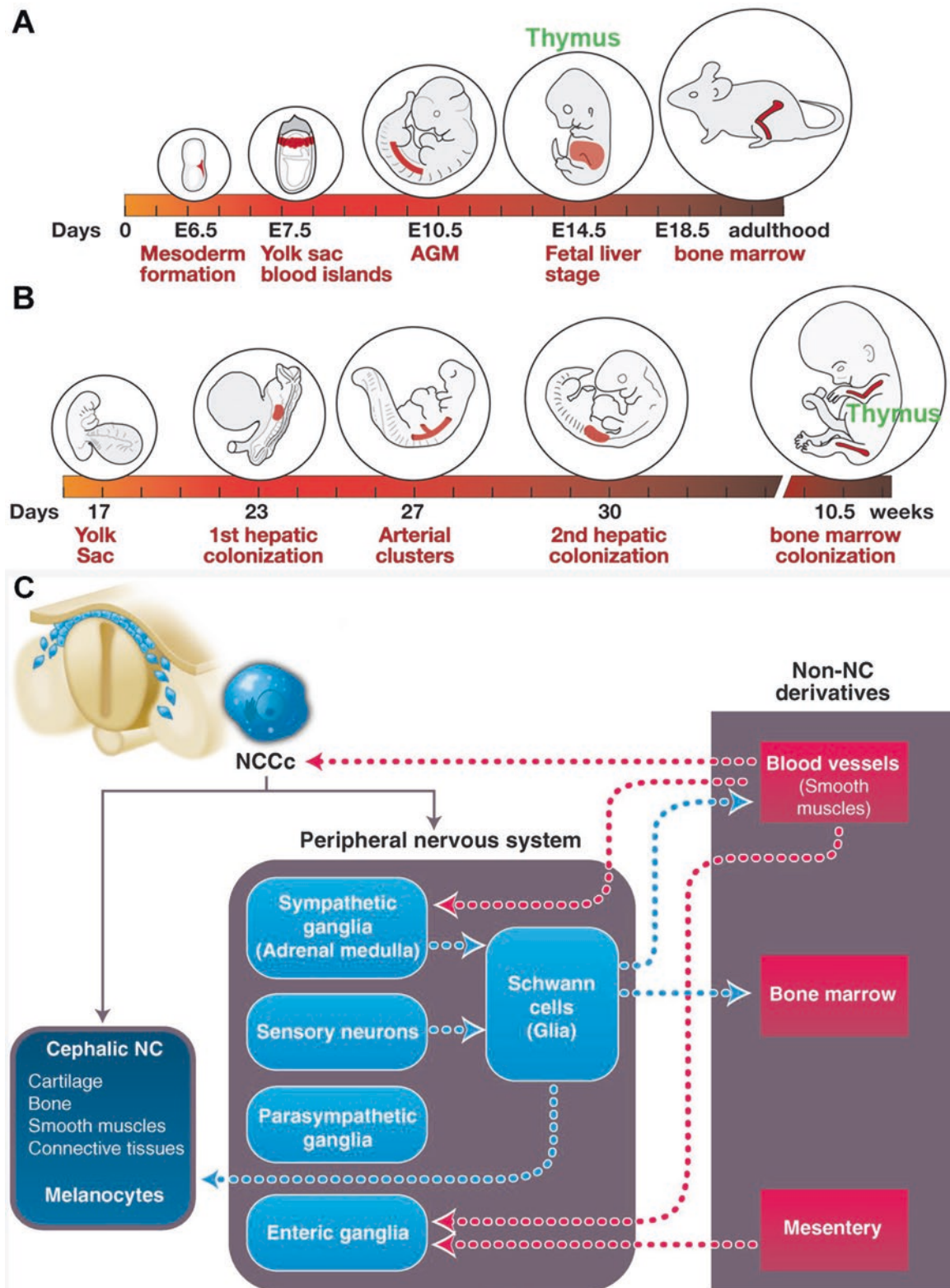


Fig. 14.1 Hematopoiesis during development and neural crest cells as a link to nervous system components. **(a)** Hematopoietic development in the mouse. Formation of mesoderm during gastrulation (around E6.5), development of yolk sac blood islands (~ E7.5), emergence of HSCs in the aorta-gonad-mesonephros region (E10.5; other sites such as large arteries and placenta not shown), active fetal liver hematopoiesis (E14.5), and hematopoiesis in the bone marrow of the late gestation fetus (E18.5) and adult animal. Active circulation begins at approximately E9.0. **(b)** Hematopoietic development in the human embryo. An embryo at yolk sac stage of hematopoiesis (day 17), at the time of the

first hepatic colonization by HSCs (day 23), arterial cluster formation (day 27), second hepatic colonization (day 30), and bone marrow colonization (10.5 weeks). Active circulation begins at approximately day 21. Used with permission and modified from Baron et al. (2012). **(c)** Schematics showing interactions between neural crest- and non-neural crest derivatives. Neural crest-derived cells are presented in blue and light blue boxes. Cell-cell interactions are shown by dotted arrows (light blue and red). Used with permission and modified from Takahashi et al. (2013)

can easily be found in severe combined immunodeficient (SCID), recombination-activating gene (Rag) 1 product and Rag2-deficient mice, which also lack mature lymphocytes.

Inducers CD45⁺CD4⁺CD3⁻ cells that form a precursor population can differentiate to antigen-presenting cells, as well as NK cells, but not to B or T cells. Detailed phenotyping of adult LT_i (or accessory) CD45⁺CD4⁺CD3⁻ cells has shown that they are similar to a CD4⁺ cell population (Brendolan and Caamano 2012).

14.4.2 Adult Lymphoid Tissues

14.4.2.1 Lymph Node Architecture

The lymph nodes have two components: the stroma with its fixed cells and the parenchyma with its migrating cell populations. In contrast to non-lymphoid parenchymal organs, the stromal network has an indispensable functional role. Fibroblastic reticular cells (FRCs) constitute the main stromal population and form its internal three-dimensional network. FRCs express surface molecules and produce ‘homing’ chemokines for T cells, B cells and DCs. They provide anatomical arrangements that influence the traffic patterns of lymphocytes, facilitates lymphocyte ‘crawling’ along preformed ‘corridors’. Moreover, FRCs typically wrap reticular fibers (mainly made up of type III and IV collagen, elastin, and laminin) to form a set of interconnected channels, called the FRC conduit system. This system allows for the efficient and rapid transfer of soluble molecules, such as antigens, chemokines, and immune complexes from the subcapsular sinus to the deeper cortex, the high endothelial venules (HEVs), and perhaps the follicular compartment. Lymph nodes can undergo rapid and profound hypertrophy during an immune response, and FRCs are deeply involved in matrix reorganization and the remodeling of lymph-node architecture.

The conduit system and sinuses are lined by sinus endothelial cells (SECs). These are flattened cells that do not form a continuous layer, especially in the wall of the medullary sinus, but contain intercellular gaps or pores. Gaps have also been demonstrated in the floor of the subcapsular sinus.

FRCs and SECs might be ontogenically related and serve to filtrate lymph fluid collected from all peripheral tissues, including brain interstitial tissue and cerebrospinal fluid. In the subcapsular sinus, macrophages and DCs sample the lymph and remove microorganisms and debris contained there. These cells also transport and/or process antigenic material for presentation to B and T cells. However, soluble lymph-borne material has restricted access to the lymphocyte compartment and the cortex.

Migrating naïve T and B cells (as well as monocytes, NK and DC) enter lymph node via high endothelial venules (HEVs), migrate through the net of dendritic cells and, if not acquired, exit via single (one) for each nodule efferent lymph

vessel back to circulation. The CCR7 chemokine receptor and two of its ligands CCL-19 and 21 have been attributed, but none of them are strongly confirmed to be expressed by human HEVs. Both ligands might be transported to the luminal surfaces of HEVs (Comerford et al. 2013; Pfeiffer et al. 2008). All secondary lymphoid tissues have some structural similarity and major component is a lymphoid follicle with germinal center (GC), follicular mantle and marginal zone (van de Pavert and Mebius 2010).

14.4.2.2 Antigen-Presenting Cells

The lymph node harbors a composite population of dendritic cells (DCs)—residents and emigrants—capable of selection, stimulation and elimination of effector lymphocytes, e.g. trigger adaptive immunity and/or tolerance. In the human lymph node T-cell compartment two primary subsets of DCs, compared to six in mice, have been identified: monocytoïd CD11b⁺CD11c⁺ and plasmacytoïd interferon-(IFN) α producing CD123⁺CD11b⁺CD11c⁻. The last subset clusters with T cells around HEVs. CD11c⁺ DCs are present as stellate cells whose delicate processes or ‘veils’ extend in many directions; these are called interdigitating DCs. In the B cell compartment, follicular DCs (FDCs) orchestrate B cell function. Whether FDCs originate from hematopoietic progenitors or from stromal elements is still a controversy. New evidence suggests the presence of two types of FDCs within human germinal centers: the classic FDCs that express CD21 and fibroblast-related markers represent stromal cells and the CD3⁻CD4⁻CD11c⁻ germinal center dendritic cells (GCDC), which represent hematopoietic cells that may be analogous to the antigen-transporting cells described in mice. Under specific conditions in vitro, human monocytes can downregulate expression of the hematopoietic marker CD45 and function as FDCs. FDC are recognized as a bridging cell between innate and adaptive (B cell) responses (Aguzzi et al. 2014). For example, FDCs are generally considered to be the major source of CXCL13 (also called B cell-attracting chemokine-1) both in normal and aberrant lymphoid tissue. The most CXCL13-expressing cells in rheumatoid arthritis and ulcerative colitis are of monocyte/macrophage lineage. They are located in irregular lymphoid aggregates within an FDC network, but also within and near smaller collections of B cells in diseased tissue where no FDCs are detected. These cells occupy the outer layers of primary and secondary lymph node follicles—the light zone and the follicular mantle. FDCs constitute a three-dimensional sponge-like network formed by interdigitation of adjacent cells. As shown by ultrastructural studies, these cells present filiform or beaded dendrites with characteristic ‘ball-of-yarn’ convolutions. FDCs play a pivotal role in promoting B-cell proliferation and differentiation in germinal centers. They are very efficient in trapping and retaining antigen–antibody complexes through CD21–CD35 and Fc γ RIIB receptors for long periods and presenting them

as iccosomes (immune-complex-coated bodies) to memory B cells. Spleen also contains lymph node-like organized structures, however this organ is multifunctional. For detailed information read (Kain and Owens 2013).

14.4.3 Lymph Node as the Home for Primary Adaptive Immune Responses and Peripheral Tolerance

14.4.3.1 Common Rules of Adaptive Immune Responses

The major function of the immune system is the control of our biologic individuality. The strength and quality of immune responses is based on highly polymorphic MHC molecules and various recombinations of T and B cell receptors. The specificity of proteins expressed by viruses, bacteria and parasites, along with their interaction and processing through host cell antigen-presenting machinery is the second side of the coin. The same is true for changed self-proteins by oxidation, nitration and other types of chemical alterations.

Cytokine/chemokines induced by external, self and self-modified proteins, loaded into DCs/macrophages and delivered from affected tissues (including the brain) by lymphs into regional draining lymph nodes control the balance between induction of immunity or tolerance. Both the induction of antigen-specific immunity and tolerance rely on the direct interaction of DCs with naïve T cells. These interactions occur in the T cell zone of lymph nodes in the vicinity of high endothelial venules (Siebert and Luther 2012; Baaten et al. 2013).

14.4.3.2 CD4⁺ T Cell Polarization

Depending on the co-stimulatory and cytokine signals that are provided by DCs at the time of priming, naïve CD4⁺CD25⁻ T cells differentiate into T helper (Th) cells such as Th1, Th2, Th17 and T follicular helper (Tfh) cells. Activation of naïve CD4⁺ T cells through the TCR elicits low but detectable transcription of both genes, IL-4 and IFN- γ . The expression of T-box transcription factor, T-bet, plays a critical role in Th1 development and supports transcriptional competence at the IFN- γ locus and selective responsiveness to IL-12. As stimulation progresses in the presence of signaling through the essential IL-12 receptor (via transcription factor STAT4), primed cells have IL-4 expression and become Th1 helper cells.

Another transcriptional factor GATA3 is involved in Th2 differentiation and contributes to chromatin remodeling events that favor IL-4 gene stable demethylation and IL-4 production. Signaling through IL-4 receptor and downstream with STAT6 favors stable production of IL-4 (as well as IL-5, IL-13) and differentiation in Th2 cells. It is unclear whether Tfh cells differentiate as a separate lineage at the time of T-cell priming or whether they emerge at a later stage from non-polarized, primed T cells or even from polarized Th2 or Th1

(less possible) cells. IL-6, IL-23 via STAT3 pathway in the presence of TGF- β facilitate differentiation of naïve Th cells into Th17 phenotype to protect against fungal and bacterial infections. As of today, there are at least four different Th cell subsets (Th1, Th2, Th17, and iTreg) that have distinct functions to regulate immune responses. Each cell subset expresses a unique set of transcription factors as well as hallmark cytokines. The cytokine environment created by activated CD4⁺ T cells themselves, by dendritic cells, and/or other cell types during the course of differentiation plays a critical role in the Th fate decisions (Yamane and Paul 2012, 2013).

14.4.3.3 CD4⁺T⁻ B Cell Interactions

A proportion of newly primed T cells that have upregulated expression of CXCR5 migrate toward the T-cell-zone-follicle boundary, where they might provide cognate help to antigen-specific B cells. In this location, they can interact with B cells and also with CD4⁺CD3⁻ accessory cells (LTi cells analogs for development of lymph nodes). The CD4⁺CD3⁻ accessory cells that are present at the T-cell-zone-follicle boundary, and within the follicles, provide signals through CD134 (also known as OX40) and CD30 at the surface of follicular T cells, which direct localization of these cells, maintenance of germinal-centre reactions and the generation of B-cell and CD4⁺ T-cell memory responses.

These follicular T cells strongly express the CD28-related molecule ICOS (inducible T-cell co-stimulator; also known as CD278) and secrete moderate amounts of IL-10, which are co-stimulatory factors with crucial functions in T helper cell and B-cell responses. After stimulation, they express CD134 and rapidly upregulate CD40 ligand (CD40L; also known as CD154) from pre-formed stores. This is consistent with their capacity to induce germinal-centre B cells to survive, secrete antibody and express activation-induced cytidine deaminase (AID). AID is crucial for immunoglobulin class switching and somatic hypermutation. IL-21 co-stimulation potently induces the expression of both B lymphocyte-induced maturation protein-1 (BLIMP-1) and AID, as well as the production of large amounts of IgG from B cells (Ettinger et al. 2005). Tfh, Th1 and Th2 cells can all participate in antibody responses. Th1 and Th2 cytokines guide class-switch recombination to result in particular immunoglobulin classes and subclasses, thereby skewing antibody responses depending on the nature of the stimulus. For example, IFN- γ promotes switching to IgG2a during antiviral responses, and IL-4 promotes the production of IgE during anti-parasite responses. Both Th1 and Th2 cells are crucial helpers in the extrafollicular pathway of B-cell differentiation, and are potent inducers of class switching to T-cell-dependent antibody classes and subclasses outside germinal centers. CD4⁺ Treg cells that suppress Th1/Th2 cells function, expansion of CD8⁺ cells and B-cell production of antibody have also been described. These are

CD4⁺CD25⁺CD57⁻ T cells that co-express CXCR5 and localize to T cell zone and germinal centers. T cells that express NK1.1 (CD4⁺ NKT cells) as well as NK are there.

14.4.3.4 B Cell Differentiation and Humoral Immune Responses

The help that T cells provide to B cells allows the production of high-affinity memory B cells and long-lived plasma cells that are specific for foreign antigens. The T cells independent immune responses usually occurs after direct activation of B cells by bacterial capsular polysaccharides and by microorganism-derived Toll-like-receptor ligands. This is a rapid antibody response to the pathogens through the generation of short-lived, low-affinity extrafollicular plasma cells.

The essential structure for T cell-dependent responses is a germinal center. Germinal centers arise in lymphoid tissues following antigenic stimulation and provide a milieu for proliferation of B cells (known as centroblast), somatic hypermutation that results in an increase in the affinity for antigens for a minority of germinal-center B cells. This process results in B cells becoming self-reactive.

Centroblasts exit cell cycle to become centrocyte and bind antigen that is associated with FDCs using newly expressed cell-surface immunoglobulin, after processing B cells present antigen in MHC II to T helper cells and elicit help. Interaction with T helper cells has several outcomes: (1) survival and selection of high-affinity centrocytes; (2) class-switch recombination of immunoglobulin and differentiation into long-lived plasma cells and memory B cells; (3) perpetuation of germinal-center reactions by stimulating centrocytes to recycle to become centroblasts. In the absence of antigen-specific T cells, centrocytes are eliminated and germinal centers abort early after induction.

Spontaneous ectopic germinal-center formation have long been recognized to occur in human autoimmune diseases and is a source of somatically mutated high-affinity autoantibodies.

14.4.3.5 CD8⁺ T Cells

A small proportion of follicular T cells in human germinal centers are CD8⁺ (less than 5% in tonsils and 10–15% in lymph nodes). Unlike B cells, which require a significant amount CD4⁺ T cell help in order to achieve high specificity, magnitude and stability in antibody production (humoral immune responses), CD8⁺ cell mediated (cytotoxic/cytolytic) cellular immune responses during the first several days after induction are independent from CD4⁺ help. Only memory formation is dependent of CD4⁺ T cell help. Mainly it depends upon CD40 ligation and IL-2 production by CD4⁺ Th1 helper cells, and is regulated by co-stimulatory molecules expressed by DCs.

The effector CD8⁺ T cells possess an arsenal of cellular weapons and are exquisitely refined in their ability to recognize target cells displaying small peptides on a protein scaffold

of MHC. Upon recognition of a foreign peptide, CD8⁺ T cells can use either cytopathic or non-cytopathic mechanisms to rid a cell of an invading pathogen. These mechanisms include deposition of pore-forming molecules and granzyme release, engagement of the apoptosis-inducing ligands (such as Fas ligand), and the release of cytokines such as IFN- γ and TNF- α . All this machinery is programmed in the lymph node during priming and delivered at the site where CD8⁺ cells perform their function. CTLs will eliminate virally or bacterially infected cells (stromal, macrophages/DCs, or lymphocytes) residing in lymph nodes that would have led to lymphopenia and immune-suppression (HIV/AIDS).

14.4.3.6 Adaptive Immunity and the Brain

Just like for any other tissue, the status of post-capillary vein endothelial cells plays a dominant role in penetration of activated lymphocytes into the brain parenchyma. Systemic activation of such cells by exo- and endotoxins via increased production of TNF- α and other pro-inflammatory cytokines, up-regulation of adhesion molecules allows lymphocytes to enter perivascular spaces in the meninges and choroids plexus, neither of which have an established blood–brain barrier. These two compartments contain potential (macrophages) and actual dendritic cells. Following viral and bacterial infections that might affect APC and induce local immune reactions, memory cells are formed. In healthy individuals at middle age, in the CSF very low number of lymphocytes are present, >80% of which are memory T cells (CD4⁺CD45RO⁺CD27⁺CXCR3⁺). Continuous recirculation of these cells between blood and the CSF is supported by low levels of P-selectin expression on endothelial cells of the deep stromal veins of the choroid plexus and the bridging meningeal veins. Engelhardt and Ransohoff (2005) review lymphocyte migration in the brain in great detail. These memory cells will persist through human life and respond in situ to re-appearing antigen (viral, bacterial, etc). Even so, the memory recall in the brain is delayed due to the immunosuppressive brain environment and activation of pro-apoptotic T-cells-glia interactions via CD95/CD95L (Fas/FasL), CD30/CD153 and other probable TNF-family related factors. New technical advances in real-time imaging of the leukocyte behavior in cerebral infection provide insights into these processes (Pai et al. 2014).

14.5 Neuro-Immune Interaction

14.5.1 Innervation of Lymphoid Tissue

The clinical, epidemiological, and experimental data have suggested that factors, hormones, and neural mediators that link the immune and nervous system might involve in the pathogenesis of multiple immune-mediated disorders of the

nervous system. These molecules are members of the same superfamily, which allow the mutual and bi-directional neural-immune interaction (Procaccini et al. 2014).

In lymphoid organs, similar to blood vessels, sympathetic/noradrenergic and sympathetic/neuropeptide Y (NPY) innervation predominate parasympathetic (cholinergic) innervation and are actually located along the vessels. In the thymus at the ultrastructural level, noradrenergic fibers are seen in proximity to thymocytes, mast cells and fibroblasts (Fig. 14.1). Catecholaminergic nerve fibers also run in close contact to TECs. Since TECs form the blood-thymus barrier in the outer thymic cortex, these cells may be targets for circulating epinephrine from the adrenal gland or norepinephrine (NE) released from the perivascular nerves. In spleen the sympathetic nerve fibers are present among cells in the T-dependent area; macrophages and B cells residing in the marginal zone and the marginal sinus, the site of the lymphocyte entry into the spleen. In lymph nodes noradrenergic fibers supply paracortical and cortical zones (T cell-rich regions) but are absent from nodular regions and germinal centers, the B cell-containing areas. The gut, lung and nasal mucosa have the highest intensity of innervation of associated lymphoid tissues. Nerves predominate in T cell zones of lymphoid aggregates where they contain neuropeptides and the sympathetic neurotransmitter NE.

14.5.2 Regulation of Immune Cells Activity by Neurotransmitters and Neuropeptides

As was mentioned above, antigen-presenting cells starting from thymic TECs, and ending with tissue resident macrophages/DCs, play an indispensable role in lymphocyte function from programming central tolerance and immunity to support of their effector function and peripheral tolerance. Cells of macrophage/DC origin express functional receptors for NE ($\alpha 1$, $\alpha 2$, $\beta 1$, $\beta 2$), dopamine (D1-5), neuropeptide Y (Y1), opioids (μ , δ , κ), and parasympathetic nicotinic acetylcholine receptors. Complex signaling through these receptors will affect activation status of APCs, expression of co-stimulatory/co-inhibitory molecules and profile of secreted cytokines/chemokines. Signaling through Ach receptors suppresses TNF secretion following the activation of lymphocytes, so the receptors play an anti-inflammatory role. Macrophages/DCs and lymphocytes express the variety of adrenergic receptors and the co-signaling during antigen presentation in the presence of increased concentrations of NE or dopamine can modulate T cells polarization toward Th2. The same direction influences histamine, serotonin, neuropeptides such as substance P, vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating polypeptide, calcitonin gene-related peptide, α -melanocyte-stimulating hormone and leptin (Elenkov et al. 2000; Scheiermann et al. 2013) (Fig. 14.1).

14.6 Humanized Animal Models

14.6.1 Hemato-Lymphoid “Humanization” of Mice

The humanized mouse field is evolving, and has received significant attention as an experimental platform to study human hematopoietic and immune system development (Rongvaux et al. 2013), human stem cells and normal tissues growth in the context of regenerative medicine (Isobe et al. 2014), and human tumor stem cells and tissues biology in cancer-related research (Williams et al. 2013). For such models, scientists use immunodeficient mice. The non-obese diabetic mice (NOD) carrying mutation of DNA-dependent protein kinase (*Prkdc*) (Bosma et al. 1983) and deletion of common cytokine receptor gamma chain (γ), complete loss of common cytokine receptor gamma chain (γ .null) (Cao et al. 1995; DiSanto et al. 1995) for NOD.Cg-*Prkdcscid* *Il2rgtm1Wjl/SzJ* (NSG) or deletion of the intracytoplasmic domain for NOD.Cg-*Prkdcscid* *Il2rgtm1Sug/JicTac* (NOG) (Ohbo et al. 1996; Ikebe et al. 1997; Sugamura et al. 1996) are the most popular. These strains were used for studies of human hematopoietic stem cells (HSC) and immune system development and function (Tanner et al. 2014). A marked increase in engraftment was attributed to the low lytic activity of the C5 component of complement [due to two base-pair deletion in a 5' exon of the murine C5 gene (Baxter and Cooke 1993)] with defects of macrophage function (defective regulation of colony-stimulating factor-1 and interferon- γ receptors, and reduced secretion of IL-1 in response to LPS), and NK cell activity (Shultz et al. 1995). The murine polymorphism of signal regulatory protein- α (SIRP- α , CD172 α)—the region in the insulin-dependent diabetes 13 (*Idd13*) locus on chromosome 2 containing a coding sequence polymorphism of *Sirpa*, was responsible for the survival and subsequent development of human HSC in a murine environment. SIRP- α interacts with the widely expressed cell surface transmembrane glycoprotein CD47 (also called integrin-associated protein, IAP). The interaction of these ligands expressed on surface membranes of mouse macrophages and human HSC surfaces provides a negative “stop” signal for destruction of xenogeneic human cells (Takenaka et al. 2007; Takizawa and Manz 2007). The major characteristics of these types of chimeric animals are (1) the high rate of acceptance of human HSC; (2) stable population of mouse bone marrow; (3) occupancy of murine thymus with human thymocytes and their maturation in $\alpha\beta$, $\gamma\delta$ and regulatory T cells (4) complete reconstitution of residual murine lymph nodes with human T, B and dendritic cells; (5) formation of splenic white pulp (Fig. 14.2); (6) distribution of human cells of macrophage lineage throughout brain meninges and perivascular spaces (Fig. 14.3). These “humanized” mice support chronic HIV-1 replication and recapitulate the course of humankind progression with loss

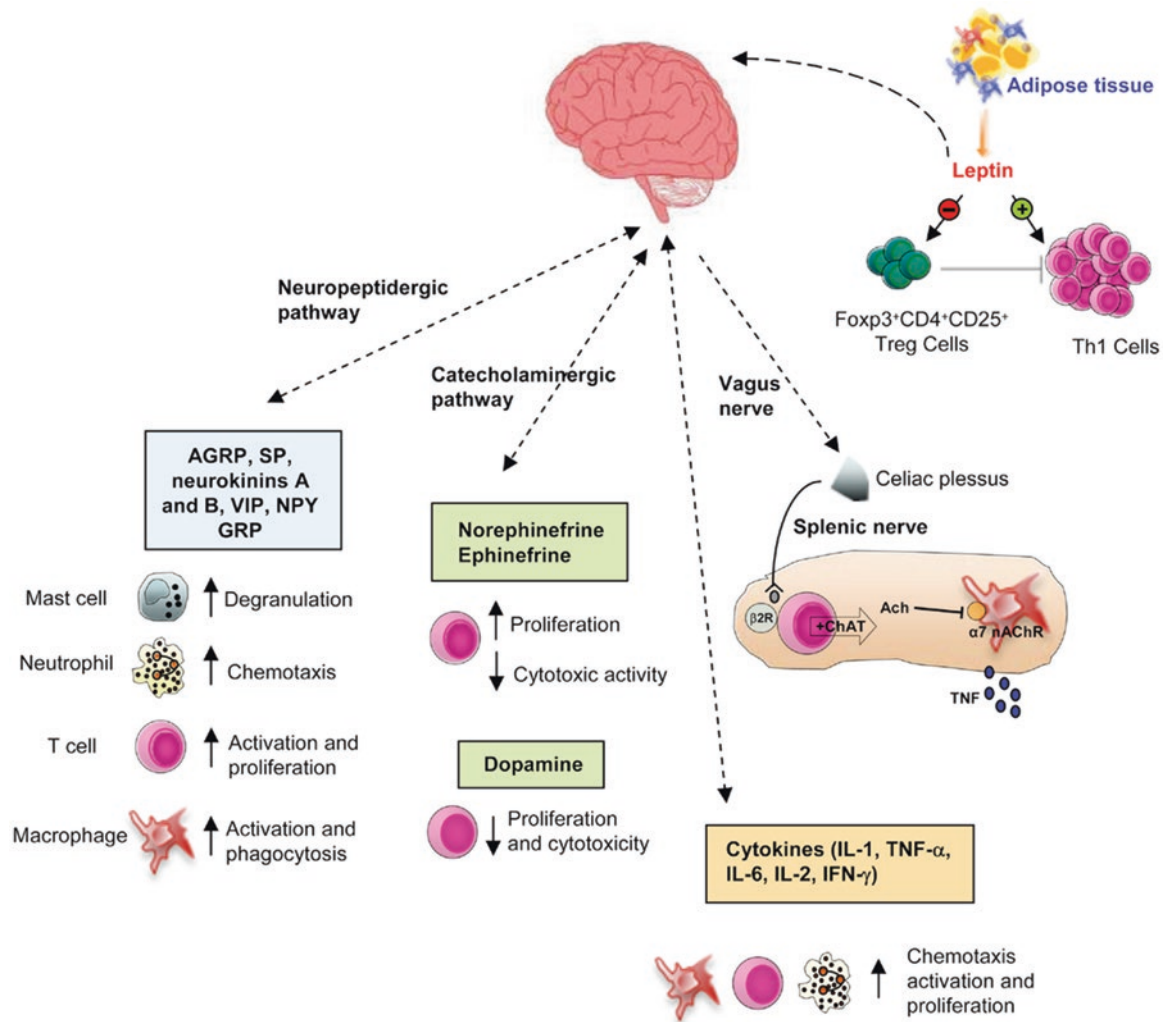


Fig. 14.2 Humanized hematolymphoid organs mouse model. Human CD34⁺ stem cells (HSC) isolated from umbilical cord blood or fetal liver were injected intrahepatically into 1 day-old irradiated pups. A complete human immune system is developed in NSG/NOG mice. The injected HSC reach mouse lymphoid organs including bone marrow, spleen, lymph nodes, gut and develop into a broad range of immune cell lineages. The presence of human CD34⁺ stem cells, myeloblasts, B cell precursors, erythroblasts, promyelocytes, granulocytes and monocytes

in mouse bone marrow were detected. Human T cell development occurred in the mouse thymus, as evidenced by the presence of CD4/CD8 T cells. In lymphoid tissue, distinct follicles filled with human T, B lymphocytes and macrophages were observed. Lymph nodes were also reconstituted with human lymphocytes, macrophages and dendritic cells. A mature human immune system develops in the mouse by 20–22 weeks. From Gorantla et al. (2012)

of CD4⁺, development of HIV-1-specific CTL, and humoral immune responses (Watanabe et al. 2007; Gorantla et al. 2010; Brainard et al. 2009).

14.6.2 Mouse Brain “Humanization”

In contrast to the biological blocks for postnatal reconstitution of mouse brain resident macrophages by human cells, transplantation and development of neurons and glial populations (astrocytes and oligodendrocytes) is possible and highly efficient in immunodeficient mice with NOD/scid or Rag-knockout backgrounds. Human CD133⁺ neuronal progenitor cells transplanted into irradiated at birth pups by 6

months of age populated multiple mouse brain regions with mature neurons with well-established connections within mouse neuronal network (Uchida et al. 2000; Benninger et al. 2000). Transplantation of human glial progenitors isolated from fetal brain with specific phenoty A2B5⁺/PSA–NCAM–showed replacement of significant proportion of mouse astrocytes with human astrocytes and improved mouse cognitive abilities (Han et al. 2013). The same phenotype progenitors transplanted in mice with immunodeficient deficient background and defects in myelinating cell development (*shiverer*, MBP^{shi/shi}) give rise to oligodendrocytes (Windrem et al. 2014). Moreover, these human glia repopulated mouse brains became permissive for human-specific viral infections like human gliotropic JC virus (Kondo et al. 2014).

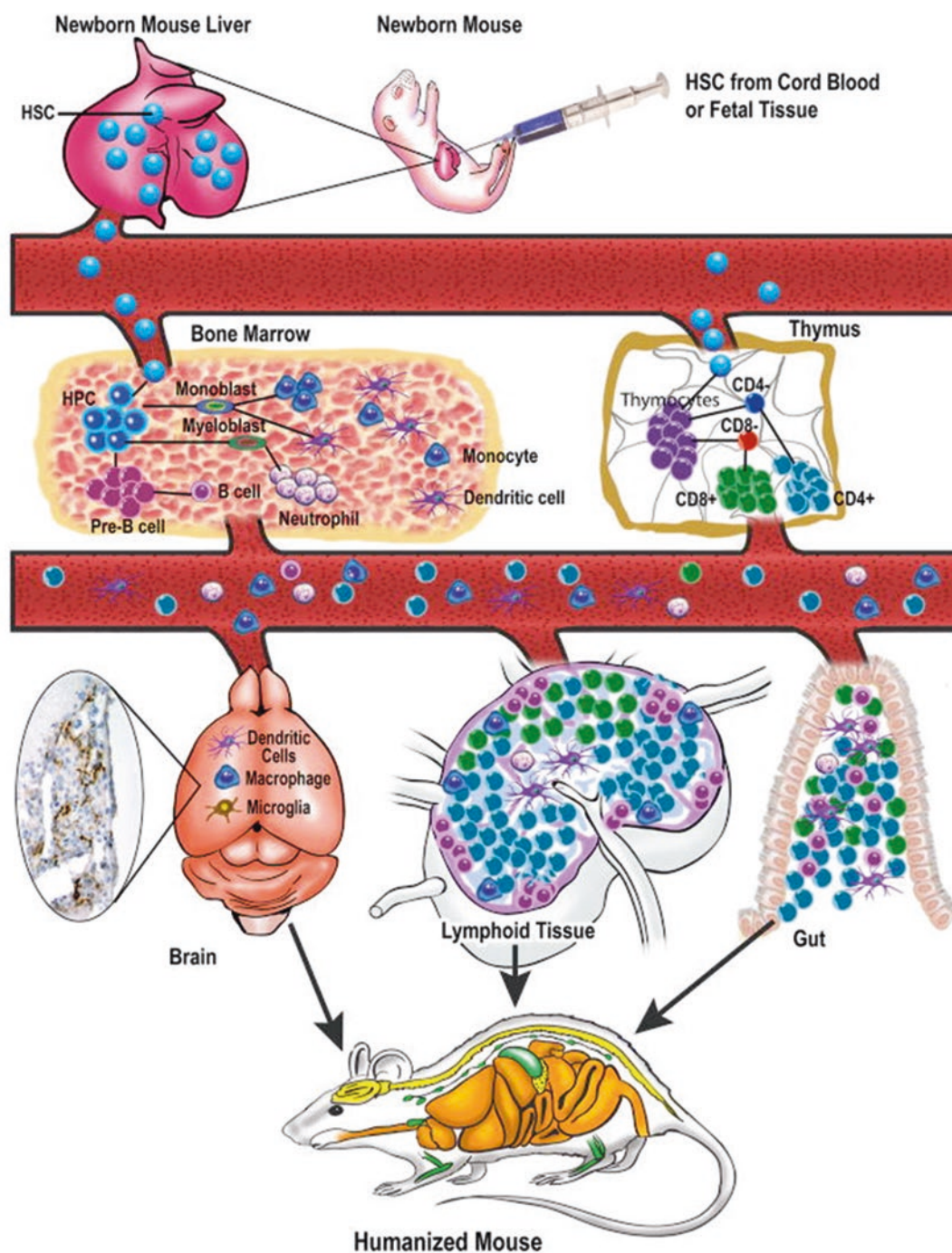


Fig. 14.3 Schematic representation of the CNS-immune system cross-talk. There are bi-directional circuits linking CNS and immune system. The CNS can communicate with the immune system to modulate its activity, through different ways: through the autonomic nervous system (via the sympathetic and vagus nerve innervation), the catecholaminergic pathway, or the neuropeptides and hormones release. They modu-

lates immune system, by increasing the activation of T cells and decreasing Treg cells functions, thus representing a key player in the susceptibility to immune-mediated disorders ($\beta 2R$, $\beta 2$ receptor; $\alpha 7$ nAChR, $\alpha 7$ subunit of the nicotinic acetylcholine receptor). From Procaccini et al. (2014)

14.7 Conclusion

In this chapter, we are reviewing the development of immune system, establishment of organs of the immune system and their function as a collector of information about status

(normal or inflamed) of any tissues including brain. There is a strong relationship between development of both systems and separation of nervous and immune system *post partum*. However, innervation of hemato-lymphoid organs regulates development of inflammation in these compartments.

The efferent information actively is coming from the brain, but development of effector reactions is limited by strong central tolerance during education of lymphocyte by TEC expressing brain antigens as well as peripheral tolerance supported by soluble brain antigens filtered through regional lymph nodes and presented by resident DCs in the absence of co-stimulatory signals. Humanized animal models helped to study the complexity of human immune system development and adaptive immune responses *in vivo*. Humanization of a mouse brain with human glial cells provided an additional tool to study hemato-lymphoid tissue and brain interaction in small animal models. The presented review will help to understand brain immunopathology described in the following chapters.

14.8 Review Questions

1. Briefly summarize the major steps in lymphocyte embryonic development.
2. Briefly summarize the major steps in T lymphocyte development.
3. Briefly summarize the major steps in B lymphocyte development.
4. Briefly summarize the distribution of B cells and their function.
5. Briefly summarize the major steps in helper cells polarization.
6. Briefly summarize the major factors that limit T cells survival in the brain.
7. Briefly summarize the major steps in adaptive immune responses.
8. Briefly summarize the major requirements for B cells differentiation and antibody production.
3. The fetal liver has long been considered the initial site of B cell commitment, and from then on B progenitors expand in a synchronous wave-like pattern reaching a peak in the perinatal stage. Even after birth, B cells may develop at sites other than the bone marrow.
4. Selection from newly formed B cells into transitional, follicular and marginal-zone (MZ) B cells depends on integrated signals from several classes of surface receptors, such as: the BCR and co-signals, the tumor-necrosis factor receptor (TNFR) family, and the G-protein-coupled receptors (GPCRs).
5. Depending on the co-stimulatory and cytokine signals that are provided by DCs at the time of priming, naïve CD4⁺CD25⁻ T cells differentiate into T helper (Th) cells such as Th1, Th2, Th17 and T follicular helper (Tfh) cells. As stimulation progresses in the presence of signaling through the essential IL-12 receptor (via transcription factor STAT4), primed cells have IL-4 expression and become Th1 helper cells. Macrophages/DCs and lymphocytes express the variety of adrenergic receptors and the co-signaling during antigen presentation in the presence of increased concentrations of NE or dopamine can modulate T cells polarization toward Th2.
6. The highly specialized nature of the endothelial cell lining, with the cerebral vasculature and tight junctions, forms a physical and functional barrier to lymphocytes. For a short period of time, T cells can be found in perivascular spaces and make parenchymal foci as an inflammatory reaction to acute viral or bacterial infection. However, such physiologic responses in the brain parenchyma are quickly terminated by the downregulation of MHC class I and II co-stimulatory molecular expression on glial cells (immunosuppressive function of IL-10, TGF- β and prostaglandins), by apoptosis of T cells through Fas-Fas ligand (CD95/CD95L) interaction.
7. Cytokine/chemokines induced by external, self and self-modified proteins, loaded into DCs/macrophages and delivered from affected tissues (including the brain) by lymphs into regional draining lymph nodes control the balance between induction of immunity or tolerance. Both the induction of antigen-specific immunity and tolerance rely on the direct interaction of DCs with naïve T cells.
8. B cells require a significant amount CD4⁺ T cell help in order to achieve high specificity, magnitude and stability in antibody production (humoral immune responses).

14.9 Answers

1. The common concept of lymphoid tissue ontogeny relies upon the concept of circulating stem/progenitor cell populations, which migrate from the yolk sack into the aortogonad mesonephros (AGM), then into organ anlagen, initiating hematopoiesis in fetal liver and bone marrow, and lymphopoiesis in thymus.
2. The human thymus develops at ~embryonic day 35 from the endoderm with subsequent epithelial–mesenchymal interaction. At the embryonic stage, lymphomyeloid commitment precedes thymic development in CD45⁺CD117⁺CD34⁺ hematopoietic clusters of AGM region. In addition, multilineage progenitors restricted to T, NK (natural killer) and macrophage lineage (pTM) or just T lineage (pT), or B and B plus macrophage (pBM) were identified in the AGM region.

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Abstract

Neural stem cells and neurogenesis are a constitutive part of brain development and homeostasis. Importantly, function of neural stem cells and neurogenesis is how a healthy brain is defined. Decline number of neural stem cells and impaired neurogenesis are observed during aging and neurodegenerative disorders. Therefore, research in the past decades has generated great interest in the understanding of neural stem cells and neurogenesis. Many immunological and pharmacological therapeutic approaches are developed based on this research. In the current chapter, we introduce different types of stem cells relevant to the health and diseases of the nervous system. We discuss critical molecules and cell types involved in adult neurogenesis and how this research can be used to promote neural regeneration and repair during diseases. Additionally, we introduce an exciting new avenue of stem cell therapy based on somatic cell reprogramming strategy and discuss its potential applications in various neurological diseases.

Keywords

Adult stem cell • Chemokines • CXCR4 receptor • Differentiation • Embryonic germ cell (EG) • Embryonic stem cell (ES) • Microglia • Neurogenesis • Neurosphere • Progenitor/precursor • Proliferation • Self-renewal • Stem cell • Stromal cell-derived factor 1 (SDF-1/CXCL12)

15.1 Introduction

Neurogenesis continues throughout life and contrary to the previously held dogma that no new neurons are produced in the adult human brain. Neurogenesis during development and throughout adult life is a result of the proliferation, migration, and differentiation of neural stem cells (NSCs). NSCs are cells that can differentiate into all central nervous system (CNS) cells and self-renew while retaining their

multipotential capabilities. Numerous factors contribute to NSC survival, proliferation, migration and differentiation. These factors include, but are not limited to, cytokines, chemokines, growth factors, hormones, morphogens, cell adhesion molecules, and neurotransmitters. New neurons and supporting cells are produced in the adult brain on a daily basis. It is estimated that in the human hippocampus, approximately 700 new neurons are added every day (Deng et al. 2010; Spalding et al. 2013). However, it remains unclear how long these new cells survive, how well they integrate into circuitry of the CNS, whether they have any functional impact on memory formation or cognition, and whether alterations in this neurogenesis process could underlie neurological diseases. Notably, considering that human brain has approximately 100 billion neurons, the rate of neurogenesis is extremely low. Therefore, much research has focused on replacing damaged neurons by either transplantation of

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embryonic, fetal, adult stem cells, or by stimulation of endogenous NSCs to undergo growth and differentiation. Future research into the mechanisms by which neurogenesis is regulated and investigations on the functional impact of the newly generated neurons are imperative for understanding brain development as well as in treating neurodegenerative disorders.

15.2 Stem Cells, Neural Stem Cells and Neural Progenitor Cells

A stem cell is a special kind of cell that has the unique capacity to continually renew itself and also to give rise to additional specialized cell types. It is now known that stem cells, in various forms, can be obtained from the embryo, fetus, and adult. Stem cell plasticity, the ability to differentiate into more than one kind of cell, may vary depending on where the stem cell originates. Most of what is known about these cells has been learned by studying rodent stem cells. However, emphasis of current research is on the utilization of human stem cells in therapy.

15.2.1 Embryonic Stem Cells

Human embryonic stem cells were first obtained in 1998 by two different research teams. The cells obtained from the inner cell mass of the blastocyst (4 to 5-day embryo) are embryonic stem cells (ESC). In contrast, cells cultured from the primordial germ cells of 5- to 9-week fetuses are embryonic germ cells (EGC). In the laboratory, ES or EG cells can proliferate indefinitely in an undifferentiated state but can also be manipulated to become specialized or partially specialized cell types, a process known as directed differentiation. Both ES and EG cells are pluripotent, meaning they have the potential to develop into more than 200 different known cell types. This class of human stem cells holds the promise of being able to repair or replace cells or tissues that are damaged or destroyed by many of the most devastating human diseases.

15.2.2 Adult Stem Cells

An adult stem cell is an undifferentiated cell that is found in an otherwise differentiated (specialized) tissue in the adult. It can renew itself for the lifetime of the organism and yield the specialized cell types of the tissue from which it originated. Adult stem cells usually divide to generate progenitor or precursor cells, which then differentiate and develop into mature cell types that have characteristic shapes and specialized functions. Adult stem cells have been found in tissues that

develop from all three embryonic germ layers, but these cells are rare. Often they are difficult to identify, isolate, and purify. Most adult stem cells grown in a culture dish are unable to proliferate in an unspecialized state for long periods of time. The plasticity of adult stem cells appears to be less than that of ES cells; to date there is no isolated population of adult stem cells capable of forming multiple kinds of cells of the body.

15.2.3 Neural Stem Cells and Neural Progenitor/Precursor Cells

NSCs are self-renewing, multipotent cells that generate neurons, astrocytes, and oligodendrocytes (Gage 2000). During embryonic development, NSCs arise from generative zones derived from the inner lining of the neural tube that extend from periventricular regions of the telencephalon to the spinal cord within the mammalian CNS. NSCs can be isolated from the embryonic or fetal CNS, including basal forebrain, cerebral cortex, hippocampus, and spinal cord. In the adult nervous system, NSCs can be isolated from neurogenic zones (the subventricular zone and hippocampal dentate gyrus). In addition, more recent evidence suggests that stem cells can be isolated from non-neurogenic areas, e.g., the spinal cord. NSCs can be grown in culture and retain both their pluripotency and ability to self-renew.

Neural progenitor cells (NPCs), in contrast, are multipotent and proliferative cells with only limited self-renewal that can differentiate into at least two different cell lineages ("multipotency," but not "pluripotency", Fig. 15.1) (Gage et al. 1995; Weiss et al. 1996; McKay 1997). Lineage-specific precursors or progenitors are cells restricted to one distinct lineage (e.g., neuronal, astroglial, and oligodendroglial). The term "precursors" is used to encompass both stem and progenitor cells" (Emsley et al. 2005).

Other lineage-specific progenitor cells exist in the adult CNS. For example, oligodendrocyte progenitor cells (OPCs), also known as NG2-glia, are glial precursors to oligodendrocytes but may also be able to differentiate into neurons and astrocytes (Nishiyama et al. 2009). Interestingly, although OPCs account for 5 % of cells, it may be the major dividing population in the adult CNS (Dawson et al. 2003).

15.2.4 Induced Pluripotent Stem Cells (iPSCs)

Through exposure to a specific set of transcription factors adult somatic cells can be reprogrammed into pluripotent stem cells. These iPSCs were first created in 2006 by exposure of adult fibroblast cells to four transcription factors, Oct4, Sox2, c-Myc, and Klf4, via a viral vector (Takahashi and Yamanaka 2006). Since then, many other transcription

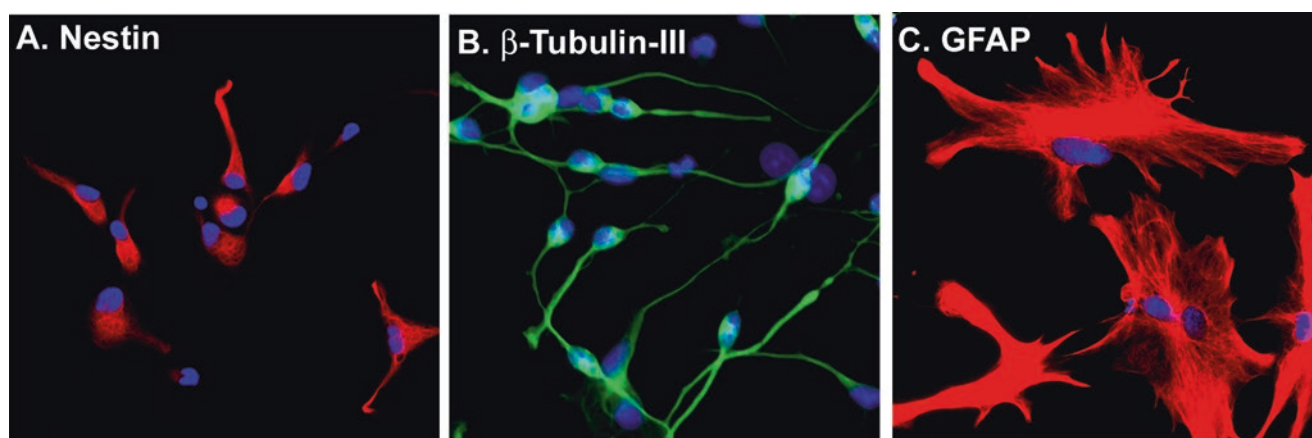


Fig. 15.1 Characterization of human cortical neural progenitor cells. (a) Cells were isolated from human fetal cortex and culture in NPIM containing EGF, bFGF, and LIF. Cells expressed Nestin, a neural stem/

progenitor cell marker. (b–c) After switching to a differentiation medium, NPCs were differentiated into neurons (β -tubulin, b) and astrocytes (GFAP, c)

factors have been discovered that alter the epigenetic markers of cells to mimic a stem cell phenotype.

iPSCs have a tremendous capacity to replace injured or defective tissues if they are exposed to the proper transcription factors for differentiation. As they can be derived from the host, the immune system does not respond strongly to them as it would to standard tissue grafts. However, iPSCs are difficult to produce, and both iPSCs and their differentiated progenies have a tendency to be tumorigenic. This had led to further research to reduce tumorigenicity of these cell types (Ruggieri et al. 2014).

The development of iPSCs has tremendously advanced the field of stem cell research. First, it is now possible to create iPSCs cell lines from specific patient tissues. These patient-derived iPSCs cell lines could then be used to model diseases or used for drug screening, which will further the understanding of disease pathogenesis and therapy. Second, iPSCs technology has also been actively used to create tissue-specific stem cells for cell replacement therapies. Both of these advances spark interest in achieving NSCs, NPCs, and lineage-specific NPCs through reprogramming strategies. See Table 15.1 for detailed features of different stem cell types.

15.3 Stem Cells and Neurogenesis During Brain Development

Neurogenesis is primarily a process that involves proliferation, migration, differentiation, and survival of neural stem cells (Palmer et al. 1997; Gage 2000). Multiple factors have been shown to regulate neurogenesis in the developing and adult nervous system including hormones, neurotransmitters, trophic factors, and chemokines (Cameron et al. 1998; Ferguson and Slack 2003).

15.3.1 Determination and Formation on Neural Tube

Developmental neurogenesis begins when a portion of the mesoderm gives rise to the notochord along the anterior-posterior axis of the embryo. This notochord releases factors that cause primary ectoderm multipotent stem cells to differentiate into more fate-restricted stem cells of the neuroectoderm, forming the neuronal plate. The neuronal plate develops along the dorsal surface at the anterior end of the embryo. As the edges of the neuronal plate fold over its center, fuse, and detach from the ectoderm the neuronal plate forms a hollow neural tube with an outer mantle layer and an inner proliferative zone. This neural tube will become the CNS, while a group of cells termed the neural crest on the dorsal side of the neural tube will give rise to the peripheral nervous system.

Upon formation of the neural tube, regionally distinct transcription factors are expressed, designating the configuration of discrete brain regions that will eventually develop into the forebrain, midbrain, cerebellum, hindbrain, and spinal cord (Hatten 1999; Shimamura et al. 1997). Factors from the microenvironment surrounding NPCs in the neural tube direct the differentiation of these cells to regionally specific arrays of neurons. These distinct differentiation events include dorsal-ventral polarization in the spinal cord, segmentation in the hindbrain, and lamination in the cerebral cortex (McConnell 1995).

15.3.2 Neural Stem Cells in CNS Development

The proliferating cells during CNS development reside in the ventricular zone of the neural tube. These cells are a heterogeneous population that exhibits complex patterns of gene expression (Temple 2001; Florio and Huttner 2014). This diversity of gene expression allows differentiation of

Table 15.1 Features of different stem cell types

Cell type	Location	Self-renewal capacity	Differential capacity
Embryonic stem cells (ESCs)	Inner cell mass of the blastocyst (4 to 5-day embryo)	Infinite	Pluripotent (can differentiate into every cell of the organism except for the trophoblast)
Induced pluripotent stem cells (iPSCs)	Somatic cells	Infinite	Pluripotent (can differentiate into every cell of the organism except for the trophoblast)
Embryonic germ cells (EGCs)	Primordial germ cells of 5 to 9-week fetuses	Infinite	Totipotent (can differentiate into every cell of the organism)
Adult stem cell	Specific niches in adult tissue (location varies for each tissue/organ)	Infinite	Multipotent (can differentiate into most cells of the tissue in which they reside. Also, they may have plasticity, which allows them to differentiated into cells of other tissues)
	Embryo: Inner lining of the neuronal tube		
Neural stem cell (NSCs)	Fetus: Basal forebrain, cerebral cortex, hippocampus, and spinal cord Adult: Subventricular zone and hippocampal dentate gyrus	Infinite	Multipotent (can differentiate neurons, astrocytes, and oligodendrocytes)
Neural progenitor cell (NPCs)	Fetus: Basal forebrain, cerebral cortex, hippocampus, and spinal cord Adult: Subventricular zone and hippocampal dentate gyrus	Limited (can self-renew, but only for a limited number of generations)	Multipotent (can differentiate into at least two cell types of the tissue in which they reside)
Lineage-specific precursor cell	Specific niches in adult tissue (Location varies for each tissue or organ)	1. Limited (can self-renew, but only for a limited number of generations)OR..... 2. Cannot self-renew (It no longer retains the ability to differentiate into more than one cell type), but can amplify in cell number	1. Multipotent (Can differentiate along one specific lineage [e.g. neuronal or astroglial])OR..... 2. Unipotent (Can only differentiate into one cell type)

NSCs to astrocytes, oligodendrocytes, and various types of neurons, depending on the signals present in the microenvironment within the developing CNS. As the development of the embryo progresses, multipotent stem cells differentiate into more fate-restricted progenitors, which give rise to macroglial cells and regionally distinct neuron types. These progenitors first give rise to neurons, then to glial cells during embryonic and fetal development, and finally to astrocytes during postnatal growth. Numerous factors have been identified to regulate these mechanisms, including growth factors, neurotransmitters, and chemokines; these are discussed in detail in later sections.

15.3.3 Stem Cells in Adult Neurogenesis

In the adult human brain, neurogenesis is limited to two regions, where two distinct populations of NPCs have been identified: (1) one in the subventricular zone (SVZ) of the

lateral ventricles and (2) another in the subgranular zone (SGZ) of the dentate gyrus of hippocampus. In these neurogenesis areas, primary progenitors give rise to heterogeneous intermediate progenitor cells, which in turn generate large numbers of neuroblasts that migrate and integrate into the neural networks. For example, in the SVZ of the lateral ventricles, type B1 NSCs give rise to type C transit amplifying cells and type A mature neuroblastic cell progenies, which migrate to reach olfactory bulb, differentiate into neurons, and integrate into the existing neural structures. This active neurogenesis are apparently coordinated by a complex interplay of growth factors, morphogens, cell-cell interactions, neurotransmitters, and endothelial signals (Tong and Alvarez-Buylla 2014). Other, but possibly less significant, populations of NPCs may exist throughout the adult CNS. The physiological role of adult neurogenesis and potential to control it for therapeutic benefit are critical areas of current research for the treatment of neurodegenerative disorders and neural damage.

15.4 Stem Cell Signaling Pathways for Migration, Proliferation and Differentiation

Neurogenesis includes proliferation, neuronal fate specification of NPCs (differentiation), migration, maturation, and functional integration of neuronal progeny into neuronal circuits (Ming and Song 2005). A full understanding of the molecular mechanisms regulating proliferation, migration and differentiation of these cells is essential if these cells are to be used for therapeutic applications.

15.4.1 Proliferation

Proliferation and survival of stem cells are important for the maintenance of the neural stem cell pool for neurogenesis. The signals involved include members of the fibroblast growth factor (FGF) family, the transforming growth factor- β (TGF- β) superfamily such as the bone morphogenic proteins (BMPs), the growth and differentiation factors (GDFs), and the Hedgehog family. In addition, many other cues are crucial for neural development.

FGFs constitute a large family of structurally related polypeptide growth factors that signal through receptor tyrosine kinases. It has been suggested that cyclin D2 (intimately involved in driving cells through the G1 phase of the cell cycle) is an effector of the FGF-dependent maintenance of the caudal stem zone (Lobjois et al. 2004). Receptor-mediated regulation of proliferation involves calcium signaling and the cAMP and PKA pathway. Activation of the Ras/Raf/Mek/Erk pathway is coupled to the cell cycle machinery via interactions with p53 retinoblastoma tumor suppressor protein (Rb) and E2F families of proteins.

Wnts are a large family of highly conserved secreted signaling proteins related to the *Drosophila* wingless protein that regulates cell-to-cell interactions during embryogenesis (Willert et al. 2003). In vitro and in vivo studies have shown that Wnt signaling is required to expand and maintain neural precursor populations in the brain and the spinal cord. As currently understood, Wnt proteins bind to cell surface receptors of the Frizzled family and act on multiple disparate signaling pathways. Three major pathways have been identified: (i) the Wnt/ β -catenin pathway also referred to as the canonical Wnt signaling pathway, (ii) the Frizzled/planar cell polarity (Fz/PCP) pathway, and (iii) the Ca²⁺ pathway. The details of these signaling pathways have been reviewed recently (Cayuso and Marti 2005; Ille and Sommer 2005). For the canonical Wnt signaling pathway, extracellular Wnt molecules bind to Fz seven transmembrane receptors on the cell surface; these Fz receptors are structurally similar to G-protein coupled receptors (GPCRs). Through several cytoplasmic relay components, the signal is transduced to

β -catenin, which then enters the nucleus and forms a complex with TCFs (from T-cell factor) to activate transcription of Wnt target genes (Logan and Nusse 2004).

Sonic hedgehog (Shh) is one of the members of the hedgehog family of secreted proteins required for multiple aspects of development in a wide range of tissues including the CNS (Machold et al. 2003). In addition to its fundamental role played in pattern formation of the ventral CNS, the Shh-Gli pathway has been demonstrated to play a major mitogenic role in the development of dorsal brain structures, including the cerebellum, neocortex, and tectum (Dahmane and Ruiz i Altaba 1999; Pons et al. 2001; Wallace 1999; Wechsler-Reya and Scott 1999; Dahmane et al. 2001). Shh mediated proliferation is also required for maintenance of neural stem cells in late development and adult. Shh activity is triggered by the binding of the ligand to its receptor Patched (Ptc), an 11-pass transmembrane receptor, and nuclear translocation of Zinc-finger proteins of the Gli family that activate or repress transcription of specific target genes (Jacobs et al. 2003).

Many of those crucial growth factors and morphogens are provided by the endothelial cells in the neurogenic vasculature. However, dietary restriction, exercise/enriched environment are known to positively modulate NPC proliferation within the neurogenic niche, whereas increased levels of glucocorticoids inhibit NPC proliferation. More recently, systemic factors with immunomodulatory property have also been demonstrated to affect NPC functions in the active neurogenesis areas (Villeda et al. 2011; Katsimpardi et al. 2014).

15.4.2 Differentiation

Differentiation is the process by which unspecialized cells (such as a stem cell) become one of the many highly specialized cells that make up the body. There are two directions of differentiation for neural stem cell: neurogenesis and gliogenesis. Neurogenesis requires a stepwise change of neuronal potential and lineage determination through a network of neurogenic factors (Morrison 2001; Florio and Huttner 2014). Neurogenic factors such as BMPs promote neurogenesis by inducing the expression of proneural basic helix-loop-helix (bHLH) transcription factors to activate the expression of a cascade of neuronal genes. Moreover, the activity of proneural genes inhibits gliogenesis in the CNS. In contrast, mammalian “hairy and enhancer of split homolog” (HES) gene expression promotes a glial fate. It is likely that other genes also influence multipotent stem cells to become glial precursor cells. Olig1 and Olig2 promote the development of oligodendrocytes. Notch activation acts in neural stem cells as a lateral inhibitory switch that terminates neurogenesis and initiates gliogenesis, even in the continued presence of neurogenic growth factors.

It has been shown that multiple bHLH genes play a critical role in regulation of neural stem cell differentiation (Bertrand et al. 2002; Ross et al. 2003). The activator-type bHLH genes *Mash1*, *Math* and *Ngn* are expressed by differentiating neurons. These bHLH factors form a heterodimer with a ubiquitously expressed bHLH factor E47 and activate gene expression by binding to the E box, promoting the neuronal subtype specification. bHLH genes promote neuronal subtype specification later in differentiation as well, as reviewed (Kageyama et al. 2005).

Factors that promote self-renewal include but are not limited to fibroblast growth factor (FGF), the transforming growth factor- β (TGF- β), Wnt signaling, and Notch signaling. NSCs are directed towards differentiating along the neuronal lineage by a decrease in Notch signaling and an increase in basic helix-loop-helix (bHLH) transcription factors. As these immature neurons mature they express activator-type bHLH genes *Mash1*, *Math* and *Ngn*. NSCs are directed towards the glial lineage by a low level of bHLH and a higher level of factors such as Notch, ciliary neurotrophic factor (CNTF), and Nrg-1. As these glial progenitors mature to astrocytes they express *Hes* and as they differentiate into oligodendrocytes they express *Oligo1* and *Oligo 2*. Specific cell markers can identify each of these cell types. Nestin and GFAP are expressed by NSCs, PSA-NCAM, DCX, and Tuj-1 are expressed by NPCs, NeuN and Map-2 are expressed by maturing neurons, GFAP is expressed by glial progenitors and astrocytes, and O4 and Gal-C are expressed by oligodendrocytes.

While *Mash1*, *Math* and *Ngn* promote neurogenesis, the repressor-type bHLH genes, including *Hes* genes, regulate maintenance of neural stem cells and promote gliogenesis. There are seven members in the *Hes* family, from which *Hes1*, *Hes3*, and *Hes5* are highly expressed by neural stem cells. *Hes* factors have a conserved bHLH domain; they form dimers and bind to DNA. The target genes for *Hes* factors include the activator-type bHLH genes such as *Mash1*. *Hes1* represses *Mash1* expression by directly binding to the promoter. The activator-type bHLH factors form a heterodimer with another bHLH activator E47 and promote neuronal differentiation from neural stem cells. *Hes1* forms a non-functional heterodimer with E47 and inhibits formation of *Mash1*-E47 heterodimer. Thus, *Hes1* antagonizes *Mash1* by two different mechanisms: repressing the expression at the transcriptional level and inhibiting activity at the protein-protein level.

It has been suggested that Notch signaling is important for maintaining multipotency in some neural stem cells but promotes glial differentiation in others or at different times during development. The Notch signaling pathway is best characterized as mediating cell-cell signaling between adjacent cells. The ligands are members of the Delta and Jagged gene families and the receptors are single-pass transmem-

brane proteins. Upon ligand binding, the intracellular domain of Notch (NICD) is released from the plasma membrane and translocates into the nucleus, where it converts the CBF1 repressor complex into an activator complex. The NICD/CBF1 activator complex upregulates targets such as the *Hes* and *Herp* (*Hes*-related protein) genes, which block neurogenesis. This is the central mechanism to the inhibition of neuronal differentiation by Notch (Yoon and Gaiano 2005). A proposed mechanism for NSCs/NPCs self-renewal and differentiation has been summarized in Fig. 15.2 to help understand the cell fate decision of NSCs/NPCs.

15.4.3 Migration Signaling

A remarkable feature of the developing CNS is the extensive migration of cells. As specific classes of cells come to reside in specific layers, migration also reflects the programmed control of neuronal fate. Control of these movements is rather complex, involving multiple regulatory genes and several extracellular molecules. Slit, reelin, and doublecortin may all regulate neuronal cell migration (Nadarajah and Parnavelas 2002). Slit and its receptor Robos mediate migration of NPCs crossing the midline in the developing brain (Orgogozo 2004). Doublecortin and reelin regulate cortical neuron migration (Feng and Walsh 2001; Nadarajah and Parnavelas 2002). In addition, chemokines such as CXCL12, also known as stromal cell-derived factor 1 (SDF-1 α), play an important role for NPCs migration during development, as discussed in Sect. 5.1.

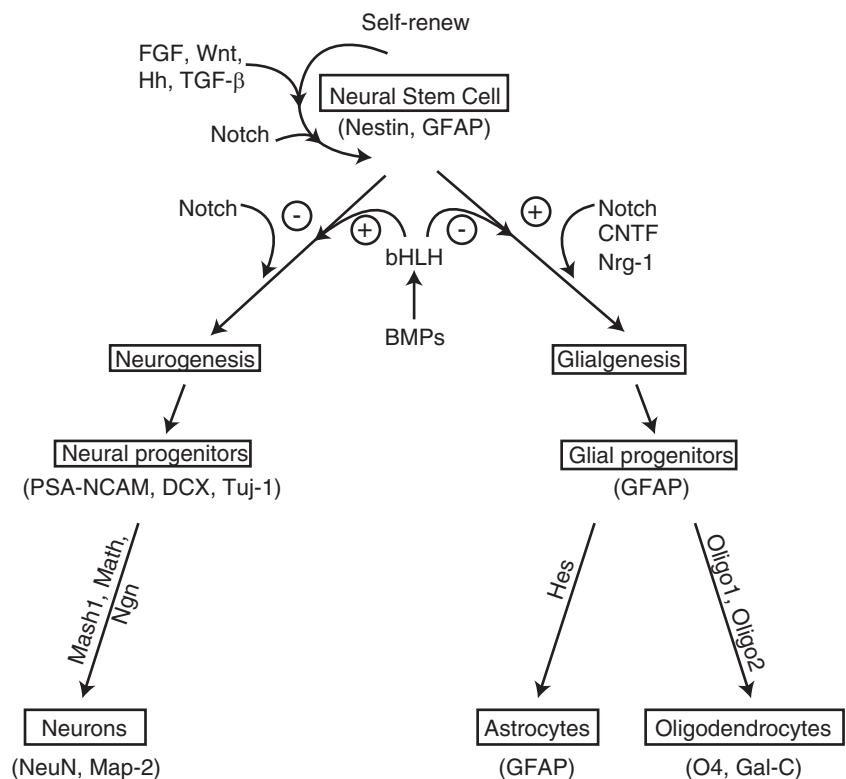
15.5 Chemokines and Neurogenesis

Chemokines constitute a family of structurally related low molecular mass (8–11 kDa) proteins with diverse immune and neural functions. In addition to their well-established roles in the immune system, chemokines play a role in the migration, proliferation, differentiation, and survival of neural stem/progenitor cells (Refer to Chap. 17.1 regarding chemokine and chemokine receptor classification and signaling pathways).

15.5.1 CXCL12 and Its Receptor CXCR4

Studies have shown that chemokine receptors are widely expressed in embryonic and adult neural stem/progenitor cells, including CXCR4, CXCR7, CCR2, CCR5, and CX3CR1 (Ji et al. 2004; Tran et al. 2004; Peng et al. 2004; Widera et al. 2004; Chen et al. 2015). CXCR4 is one of the most highly expressed chemokine receptors. Deletion of the gene for the CXCR4 receptor or of its only known ligand,

Fig. 15.2 A proposed mechanism for a select number of factors that regulate self-renew and differentiation of NSCs/NPCs



CXCL12, results in embryonic lethality. The brains of mouse embryos homozygous for the absence of CXCR4 or CXCL12 have severe abnormalities in the development of the cerebellum (Zou et al. 1998; Ma et al. 1998), hippocampal dentate gyrus (Lu et al. 2001; Bagri et al. 2002) and neocortex (Stumm et al. 2003). In the cerebellum, CXCL12 is highly expressed in the leptomeninges and is the major chemoattractant for external germinal layer cells in the developing cerebellum (Klein et al. 2001; Zhu et al. 2002). In mice lacking CXCL12 or CXCR4, migration of granule cell precursors out of the external germinal layer occurs prematurely, resulting in abnormal development of the cerebellum (Ma et al. 1998). In agreement with a key role for CXCR4, CXCR4 mRNA is expressed at sites of neuronal and progenitor cell migration in the hippocampus at late embryonic and early postnatal ages. The absence of CXCR4 leads to a reduction in the number of dividing cells in the migratory stream and in the dentate gyrus itself. In addition, neurons appear to differentiate prematurely before reaching their target (Lu et al. 2001; Bagri et al. 2002). In the cortex, CXCL12 is highly expressed in the embryonic leptomeninges and is a potent chemoattractant for isolated striatal precursors, while CXCR4 is present in early generated Cajal-Retzius cells of the cortical marginal zone (Stumm et al. 2003). Mice with a null mutation in CXCR4 or CXCL12 show severe disruption of interneuron placement and proliferation, while the submeningeal positioning of Cajal-Retzius cells remains unaffected

(Stumm et al. 2003). In conclusion, CXCL12 likely acts both as a chemoattractant and a mitogenic stimulus for neural stem/progenitor cells in the development of the cerebellum, hippocampus and neocortex.

CXCL12 may also affect spinal cord development. CXCL12/CXCR4 expression is increased in developing spinal cord progenitors. SDF-1 induced chemotaxis in both neural and glial progenitor cell, suggesting that CXCL12/CXCR4 may affect spinal cord development through modulating progenitor cell migration (Luo et al. 2005).

The expression of CXCL12/CXCR4 in the adult CNS suggests that CXCL12/CXCR4 signaling is also important in adult neurogenesis (Tran and Miller 2005; Bagri et al. 2002). In the adult, neurogenesis continues in the dentate gyrus. Adult NPCs in the subgranular zone, which produce granule neurons, express CXCR4 and other chemokine receptors, and granule neurons express CXCL12. The expression of CXCL12 and CXCR4 in adults differs from the embryonic patterns but remains consistent with continued functions in granule cell mitogenesis and/or chemotaxis.

Neural stem cell survival is an imperative issue during neurogenesis. Whether CXCL12-CXCR4 interaction is involved in all three steps of neurogenesis is not clear; however, CXCL12-CXCR4 signaling regulates survival of both NPCs and oligodendrocyte progenitors in vitro (Krathwohl and Kaiser 2004; Dziembowska et al. 2005). In addition to CXCR4, NPC survival also involves CXCR7, which is a

second receptor for CXCL12. Interestingly, although CXCR7 does not mediate classic GPCR signaling, it promotes NPC survival through endocytotic signaling (Zhu et al. 2012).

15.5.2 Other Chemokines and Their Receptors

The role of other chemokines and their receptors in neurogenesis has only recently become realized. For example, it has been suggested that CXCR2 plays a vital role in patterning the developing spinal cord; CXCR2 and its ligand, growth-related oncogene alpha (GRO- α , CXCL1) are crucial in arresting embryonic oligodendrocyte precursor migration (Tsai et al. 2002). Spinal cord oligodendrocytes originate in the ventricular zone and subsequently migrate to white matter, then stop, proliferate, and differentiate (Tsai et al. 2002). Without CXCR2 signaling, a widespread dispersal of postnatal precursors has been seen (Tsai et al. 2002). This shows that GRO- α -CXCR2 interaction plays an important role in holding a population of presumptive white matter (Tsai et al. 2002), thus, creating an organized and functional spinal cord. In young adult mice, increasing peripheral CCL11 chemokine levels in vivo decreased adult neurogenesis and impaired learning and memory, suggesting systemic inflammatory factors may negatively regulate neurogenesis (Villeda et al. 2011). Monocyte chemoattractant protein-1 (MCP-1, CCL2) can also activate the migration capacity of rat-derived neural stem cells. With this in mind, it can be assumed that numerous other chemokines and their receptors will be found to play an important role in regulating NPC function, similar to CXCR4.

15.6 Growth Factors and Neurogenesis

Numerous growth factors regulate neurogenesis in the CNS and are valuable tools in the maintenance and differentiation of NPCs in culture. Ciliary neurotrophic factor (CNTF) released by astrocytes promotes proliferation of NPCs (Emsley and Hagg 2003). Vascular endothelial growth factor (VEGF) in the subventricular zone (SVZ) promotes survival, differentiation, and release of brain-derived neurotrophic factor (BDNF), which will be discussed in detail in Sect. 6.2 (Hagg 2005; Louissaint et al. 2002). Insulin-like growth factor (IGF) produced endogenously in the brain and from circulating blood stimulates proliferation and differentiation of NPCs to a neuronal lineage (Anderson et al. 2002). Transforming growth factor (TGF α) produced by astrocytes promotes proliferation by the activation of epidermal growth factor (EGF) receptor (Hagg 2005; Enwere et al. 2004). Glial-derived neurotrophic factor (GDNF) has been shown

to promote survival of dopaminergic neurons in culture (Nakajima et al. 2001). Platelet-derived growth factor (PDGF) may contribute to survival, differentiation, and migration of NPCs (Kwon 2002). Nerve growth factor (NGF) promotes survival and differentiation (Plendl et al. 1999). Although numerous growth factors are significant in neurogenesis, this section will focus on three growth factors: EGF, basic fibroblast growth factor (bFGF), and BDNF.

15.6.1 Epidermal Growth Factor and Basic Fibroblast Growth Factor

EGF and bFGF regulate survival, proliferation, and differentiation of NPCs. Regeneration of neurons after ischemic brain injury in rats was significantly increased by infusion of EGF and bFGF (Nakatomi et al. 2002). NPC culture systems use EGF and bFGF to maintain the cell pool by increasing proliferation and survival and by preventing differentiation. After NPCs are stimulated to differentiate into neurons, EGF has neurotrophic properties on the newly forming neurons to promote survival. EGF collaborates with sonic hedgehog (SHH) and non-amyloidogenic amyloid precursor protein to promote proliferation (Hagg 2005; Machold et al. 2003; Palma et al. 2005; Caille et al. 2004).

15.6.2 Brain-Derived Neurotrophic Factor

Brain-derived neurotrophic factor (BDNF) promotes survival and differentiation of NPCs to a neuronal lineage (Hagg 2005; Mattson et al. 2004). Infusion of BDNF into the brain of rats has been shown to have antidepressant-like properties, as demonstrated by the learned helplessness test and forced swim test (Siuciak et al. 1997). The antidepressant-like activity may be the result of BDNF-stimulated increase in survival and differentiation of NPCs to neurons in the hippocampus. This is supported by the finding that BDNF infusion into the lateral ventricle increases the number of newly generated neurons in various regions of the rat brain (Pencea et al. 2001). Neurodegenerative disorders like bacterial meningitis may cause an increase in BDNF levels, which correlates with an increase in neurogenesis (Tauber et al. 2005). These support the idea that BDNF is a crucial factor in the regulation of neurogenesis, specifically for persuading NPC cultures to differentiate into neurons. BDNF can come from multiple sources, including but not limited to astrocytes, vascular endothelial cells, neurons, and NPCs. Other growth factors, hormones, and neurotransmitters may use BDNF as a downstream factor to regulate neurogenesis. BDNF expression in astrocytes can be increased by noradrenaline, serotonin, and glutamate (Zafra et al. 1992; Mattson et al. 2004). Testosterone increases the expression of VEGF, which then

stimulates release of BDNF (Hagg 2005; Palmer et al. 2000). Another regulator of BDNF is nitric oxide production by NPCs, which can act in a positive feedback loop with BDNF to shift NPCs from proliferation to differentiation (Cheng et al. 2003).

15.7 The Role of Neurotransmitters in the Regulation of Neurogenesis

Neurotransmitters such as acetylcholine (Mohapel et al. 2005; Harrist et al. 2004; Paez-Gonzalez et al. 2014), dopamine (Hoglinger et al. 2004; Baker et al. 2004; Van Kampen and Robertson 2005), serotonin (Banasr et al. 2004), norepinephrine (Kulkarni et al. 2002), GABA (Wang et al. 2005; Bolteus and Bordey 2004; Overstreet Wadiche et al. 2005), and glutamate (Kitamura et al. 2003; Luk et al. 2003; Brazel et al. 2005), contribute to the regulation of neurogenesis in the dentate gyrus (DG) and the subventricular zone (SVZ) of the lateral ventricles. These neurotransmitters regulate proliferation, differentiation, migration, survival, and synaptic plasticity.

15.7.1 Dopamine in Neurogenesis

Dopamine projections from the midbrain innervate the SVZ and have been shown to promote proliferation and differentiation through the activation of dopamine D2 and D3 receptors (Hagg 2005; Van Kampen and Robertson 2005; Hoglinger et al. 2004; Baker et al. 2004); however, other evidence suggests that activation of D3 receptors does not affect neurogenesis and D2 receptor activation may inhibit neurogenesis (Baker et al. 2005; Kippin et al. 2005). These apparent contradictions reflect the fact that neurogenesis is complex and is dependent on balance among signals that simulate and inhibit neurogenesis (Hagg 2005). Specific D3 activation stimulated proliferation and showed preferential differentiation to neuronal phenotype (Van Kampen and Robertson 2005; Van Kampen et al. 2004), and dopamine D3 activation has been shown to have neuroprotective effects (Carvey et al. 2001; Anderson et al. 2001; Vu et al. 2000).

15.7.2 Serotonin in Neurogenesis

Serotonin projections innervating the SVZ and DG have been shown to influence proliferation and survival (Simpson et al. 1998; Banasr et al. 2004). Serotonin stimulation of 5-HT_{1a} receptors enhances NPC proliferation and increases levels of BDNF, which promotes survival and stimulates differentiation of NPCs to a neuronal lineage (Hagg 2005; Banasr et al. 2004; Mattson et al. 2004).

15.7.3 Norepinephrine in Neurogenesis

Norepinephrine has been shown to influence proliferation, but it has not been shown to affect survival or differentiation in the DG (Kulkarni et al. 2002). Adrenergic receptors are abundant in the CNS; however, their significance in neurogenesis has not been well established.

15.7.4 GABA in Neurogenesis

Maturing neurons receive GABAergic input for 1–2 weeks before they form glutamatergic synapses (Overstreet Wadiche et al. 2005). GABA contributes to the stimulation of proliferation, migration, and neurite outgrowth (Bolteus and Bordey 2004; Owens and Kriegstein 2002). GABA is essential for the maturation of neurons and formation of functional synapses. GABA's role is not simply the creation of new neurons, but also the formation of functional synapses that ultimately lead to repair or improved neurological function.

15.7.5 Glutamate in Neurogenesis

Glutamate is the primary excitatory neurotransmitter in the mammalian CNS, where it has been shown to mediate synaptic transmission, synaptic plasticity, neuronal toxicity, and proliferation and differentiation of NPCs (Dingledine et al. 1999; Arundine and Tymianski 2004). During many neurodegenerative disorders, extracellular glutamate levels are increased in the CNS. This increased glutamate leads to excitotoxicity of neurons, but some of the negative effects may be counterbalanced by increasing neurogenesis and activation of survival pathways in select cells, including NPCs. Glutamate acts on several receptor types, which are classified into “metabotropic” and three “ionotropic” groups: kainate, *N*-methyl-D-aspartate (NMDA), and α -amino-3hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors (Hollmann and Heinemann 1994; Pin and Duvoisin 1995; Monaghan et al. 1989). Metabotropic glutamate receptors are GPCRs whose activation leads to release of Ca²⁺ from intracellular stores or inhibition of adenylyl cyclase (Pin and Duvoisin 1995). Ionotropic receptors are ion channels that open upon binding of glutamate, leading to the influx of sodium and/or calcium and the efflux of potassium, resulting in changes in membrane potential and diverse cellular processes (Arundine and Tymianski 2004).

NMDA receptor antagonist reduced proliferation of striatal neurons, demonstrating that NMDA receptor activation may induce proliferation (Sadikot et al. 1998; Luk et al. 2003). The metabotropic glutamate receptor subtype, mGluR5, is expressed early in development in

regions of active proliferation, suggesting that mGluR5 may play a role in proliferation and/or differentiation (Di Giorgi Gerevini et al. 2004).

Numerous studies have shown beneficial effects to neurogenesis by activating and/or potentiating AMPA receptors. By decreasing the rate of desensitization of AMPA receptors, proliferation of NPCs increases in rat hippocampus (Bai et al. 2003). Multiple mechanisms have been suggested to explain this AMPA receptor-mediated neurogenesis. The increased expression of genes that stimulate proliferation and/or differentiation is one possible method for AMPA-mediated neurogenesis. Activation of AMPA receptors leads to increased expression of immediate early genes, such as: NGFI-A, c-fos, c-jun, and jun-b in oligodendrocyte progenitors (Pende et al. 1994).

Potentiation of AMPA receptors can also increase BDNF expression in the rat hippocampus, especially in the dentate gyrus (Mackowiak et al. 2002). AMPA receptor activation can lead to upregulation of BDNF in a calcium-dependent and calcium-independent manner. Calcium can bind to calcium response elements on the BDNF promoter, resulting in increased BDNF transcription. Also, AMPA receptors can physically associate with the tyrosine kinase Lyn. Lyn activation through AMPA receptors can stimulate mitogen-activated protein kinases (MAPK), leading to increased BDNF expression (O'Neill et al. 2004). As previously described, BDNF promotes survival and differentiation of NPCs to a neuronal lineage (Hagg 2005; Mattson et al. 2004).

15.7.6 Interaction of Neurotransmitters in Neurogenesis

One factor for the creation of the SVZ and DG niches for NPCs and neurogenesis could be the overlap of neurotransmitter systems in these two areas (Hagg 2005; Simpson et al. 1998). Serotonergic and dopaminergic projections converge almost exclusively over the SVZ. Similarly, serotonergic and norepinephrinergic projections congregate over the DG (Hagg 2005). None of these neurotransmitters may be sufficient to stimulate neurogenesis on its own; rather, it is the multitude of neurotransmitters, growth factors, and chemokines that combine to create the niche for NPCs and to stimulate successful neurogenesis.

15.8 Brain Inflammation and Neurogenesis

Previously, the brain was thought to be an immune-privileged organ. Now it is clear that the brain does respond to peripheral inflammatory stimuli through both neural and humoral afferent signals. The concept that the microglia act as the brain's immune system has also become widely accepted.

The inflammatory responses in the brain have different features from those of non-neuronal tissues. Microglia activation and elevated levels of cytokines and adhesion molecules are hallmarks of CNS inflammation (Matyszak 1998).

The likely role of immune cells in regulating neurogenesis is of great interest. Their effects may depend on the timing, dynamics and severity of the inflammation. On the positive side, it has been suggested that the immune system may be protective and even assist regeneration; microglia (Streit 2002) and macrophages (Rapalino et al. 1998) have been shown to facilitate tissue repair after CNS injury. On the negative side, the immune system may also cause "bystander damage" in the inflamed brain. The limited regeneration capacity and extreme vulnerability of CNS neurons to inflammatory conditions make the inflamed CNS a hostile environment for neurogenesis. Pharmacologic approaches to control neurogenesis and/or neuroinflammation are likely to become important areas of research for treatment of CNS injury and neurodegeneration (Whitney et al. 2009; Borsini et al. 2015).

15.8.1 Inflammation Can Suppress Neurogenesis

Accumulating evidence suggests that brain inflammation plays an important role in the pathogenesis of chronic neurodegenerative disease such as Alzheimer's disease, Parkinson's disease and multiple sclerosis (Marchetti and Abbracchio 2005). Acute brain injury from stroke and status epilepticus is also linked to inflammation. Although it has been reported that these insults trigger increased neurogenesis in the subgranular zone of the dentate gyrus, it has also been shown that newly formed dentate neurons were severely lost, which may be associated with brain inflammation. Arvidsson et al. showed that more than 80% of new striatal neurons that are generated from the subventricular zone after stroke in rats die within the first week after the insult (Arvidsson et al. 2002). Systemic inflammation triggered by peripheral infection can cause activation of microglia in the brain of Alzheimer's disease patients, leading to aggravation of the cognition decline. Epidemiological studies suggest that patients on long-term anti-inflammatory treatments have a significantly reduced risk of Alzheimer's disease (Zandi and Breitner 2001).

An invariant feature of damage to the CNS is the migration of microglia to the site of injury and their subsequent activation. However, the specific role of microglia in neurogenesis is still controversial. The activation of microglia can result in either neuroprotective or neurotoxic effects, or both. Previous studies have indicated that microglia are capable of secreting neurotrophic survival factors upon activation, and they can direct neural precursor cell migration and differentiation

(Kim 2005). Yet several publications (Ekdahl et al. 2003; Monje et al. 2003; Hoehn et al. 2005) report pathogenic roles of microglia on neurogenesis, as well as strengthening the traditional view that immune cells in the CNS have an adverse effect on neurogenesis. In lipopolysaccharide-induced inflammation of the rat CNS, basal hippocampal neurogenesis is strongly impaired and the increased neurogenesis triggered by this brain insult is also attenuated. This impairment is associated with microglial activation. Activated microglia were localized in close proximity to the newly formed cells, and the impairment of neurogenesis depended on the degree of microglia activation. Systemic administration of the microglial activation inhibitor minocycline effectively restored neurogenesis, presumably by suppressing inflammation without affecting neurogenesis (Ekdahl et al. 2003).

Using a coculture system, Monje et al. found that activated, but not resting microglia, decreased the differentiation of NPCs to approximately half of control levels. Furthermore, in vivo models of acute and chronic inflammation also demonstrated that inflammation itself suppresses neurogenesis. Neurogenesis and activated microglia show a striking negative correlation, and decreased microglial activation by inflammatory blockade with the nonsteroidal anti-inflammatory drug indomethacin likely accounts for part of its restoration of neurogenesis (Monje et al. 2003). The deleterious effect of activated microglia on neurogenesis is most likely mediated through the action of various factors, such as IL-1 β or IL-6, TNF α , IFN- γ , NO, and reactive oxygen species; all of these factors can be released from microglia and are neurotoxic in vitro (Hoehn et al. 2005). For example, during inflammation, NO is released by microglia and astrocytes at pathological levels via induction of inducible nitric oxide synthase (Bo et al. 1994). Exposure of NO to SVZ NSCs as well as human NSCs has presented a detrimental outcome to neurogenesis while still activating gliogenesis. However, NO has also been shown to promote NSC self-renewal by up-regulating neuron restrictive silencer factor/RE1-silencing transcription factor (NRSF/REST) (Bergsland et al. 2014). The role of NRSF/REST is to maintain the pool of NSCs by repressing neural genes. When homeostasis is lost and NRSF/REST is up-regulated, NSC differentiation is inhibited, leading to diminished neurogenesis (Gao et al. 2011). Therefore, inflammatory response can lead to the inhibition of neurogenesis by pathological upregulation of NRSF/REST (Perez Estrada et al. 2014).

When human neural stem cells were transplanted into the ischemic cortex of rats after distal middle cerebral artery occlusion, transplanted stem cells survived robustly in naive and ischemic brains 4 weeks post-transplant. Survival was influenced by proximity of the graft to the stroke lesion and was negatively correlated with the number of IB4-positive inflammatory cells. The negative correlation of cell survival to lesion size and IB4-positive cells suggests that

inflammatory cytokines are detrimental to be the transplanted cells, in accordance with other studies (Arvidsson et al. 2002). Given that the magnitude of the inflammatory response and the types of cytokines present change with time after ischemia, these data imply that the timing of stem cell transplant could significantly influence cell survival, with greater survival predicted if cells are transplanted after inflammation has subsided (Kelly et al. 2004).

15.8.2 Proregenerative Role of Microglia and Brain Immunity

Microglia have unique characteristic of being both supportive glia and immunocompetent defense cells. Many observations strongly support a neuroprotective and proregenerative role of microglia in the injured CNS. In the facial nerve axotomy model of neuron-microglia interaction and neural regeneration (Streit 2002), microglial cells are found to contact the membrane of neurons that will not undergo apoptosis and will eventually regenerate their axons. Their data suggested that microglia may somehow orchestrate the recovery of these cells by assuming a protective role. Microglia activation after brain injury can also benefit regeneration through the release of neurotrophic molecules and some cytokines.

Schwartz and colleagues bring forward a concept of protective autoimmunity (Moalem et al. 1999). Although an uncontrolled immune response impairs neuronal survival and neurogenesis, a local immune response that is properly controlled can support survival and promote recovery. The immune response that causes cell loss under neurodegenerative conditions also blocks neurogenesis in the adult CNS, while the immune response that protects against cell loss also support neurogenesis (Schwartz 2003).

The mechanisms by which inflammation regulates neurogenesis remain inconclusive. Recently, investigators have begun to dissect out detrimental aspects of the immune system that may be responsible for unnecessary tissue loss and restrictions on axonal regeneration. Early inhibition of TNF or TGF- β 2 significantly decreases scarring and tissue loss, and can lead to improvement in functional outcome after CNS injury (Brewer et al. 1999; Logan et al. 1999). Further investigation of the roles of individual inflammatory factors, growth factors and neurotransmitters in neurogenesis will help to shed light on this important research field.

15.9 Induced Pluripotency and Induced Neural Stem Cells

Because specific neuronal subtypes are irreversibly lost during neurodegenerative diseases, the use of stem cells for cell replacement treatment known as cell therapy has become a

large focus within the research community. The development of iPSCs represents a breakthrough in broadening the feasible cell source for cell therapy. iPSCs were developed by introducing pluripotent associated genes or reprogramming factors into a given cell type, typically somatic cells. To convert these cells, four reprogramming transcription factors were initially used, including Oct4, Sox2, c-Myc, and Klf4, which are commonly referred to as the Yamanaka factors (Takahashi and Yamanaka 2006). These four transcription factors were originally identified for their ability to maintain pluripotency in the development of ES cells. The expression of these transcription factors, through a retrovirus gene delivery system, induces the pluripotency in adult somatic cells. Generating iPSCs through reprogramming strategies avoids the need to destroy embryos for ES cells. Therefore, iPSCs techniques have become a promising alternative strategy for regenerative medicine.

iPSCs and ES cells maintain pivotal features of pluripotency through a network of gene expressions systems and genome wide histone complex modifications (Guenther et al. 2010). There has been active research to use the pluripotency of iPSCs in preclinical animal models and future patient therapy models. In early models, low frequency of reprogramming yielded less than 1 % of somatic cell transformation into iPSCs. Possible reprogramming efficiency issues could be related to expressions levels as well as timing of reprogramming genes (Takahashi and Yamanaka 2006). Avenues to improve reprogramming efficiency toward pluripotency have been explored. For example, the inhibition of Mdb3, a subunit of a nucleosome remodeling and deacetylation (NuRD) complex which is responsible for silencing embryonic stem cell marker genes, has shown to increase reprogramming efficiency while promoting pluripotent stem cells differentiation (Luo et al. 2013). Investigations on epigenetic changes from source somatic cells to iPSCs have identified different combinations of transcription factor(s) for reprogramming (Kim et al. 2009). It is important to note that molecular mechanisms for epigenetic changes of somatic cells into pluripotent states remain unknown. In addition, progenitor cells have been found within fibroblast cultures, cautioning the result of iPSCs from pre-existing progenitor cells.

Somatic reprogramming with transcription factors has been scrutinized for the use of oncogenes. Investigations on the Yamanaka factors have shown that many transcription factors are dispensable when other oncogenes are used. Different sets of transcription factors have shown a reduction in the amount of immature progenitors, improvement of self-renewal, and reduction of allotted time for both proliferation and differentiation; these transcription factors often include Sox2, FoxG1, and Brn2 (Lujan et al. 2012). It is noteworthy that none of these cocktails is free from viral (retroviral and lentiviral) infection systems. The viral vectors may increase the tumorigenicity of iPSCs. Interestingly, increase in efficiency and yield can be achieved through the infection

expressing short hairpin RNA against p53, known as a cellular tumor antigen (Marion et al. 2009). Because Klf4 was previously found to repress p53, the indispensable role of Klf4 on iPSCs generation may be through p53. Overall, tumorigenicity represents the most pressing challenge for the clinical application of iPSCs. To avoid cancerous complications, the new iPSCs generation strategies require reduction of reprogramming transcription factors as well as altering to the source of somatic cells. These new strategies sometimes circumvent the pluripotent state in favor for a direct reprogram to specific phenotypes (Ruggieri et al. 2014).

15.9.1 Induced Neural Stem Cells

Induced neural stem cells (iNSCs) bypass pluripotency to avoid pluripotency-derived complications. However, iNSCs are closer to the post-mitotic cells that have differentiated phenotypes, which may cause problems with cellular proliferation. This has been addressed by using similar reprogramming transcription factors (Oct4, Sox2, Klf4, c-Myc) under different overexpression cultural conditions; iPSC states can be bypassed to direct neural identity (Han et al. 2011). To accumulate large differentiated cultures, patient-specific fibroblast extractions could be utilized and then transformed directly into neural fated stem cells. These iNSCs hold intrinsic potential to differentiate into various lineages such as astrocytes, oligodendrocytes, as well as other neural subtypes. However, since the mechanism behind the differentiation is relatively unknown, direct reprogramming of patient neurodegenerative insults may face numerous challenges. As such, iNPCs with restricted neural identity may be better suited for clinical application (Smith et al., Progress in Neurobiology, In Press).

Comparatively, cultivation of iNPCs opposed to iPSCs, using the four identical iPSC reprogramming factors and a doxycycline induced system, takes 11–12 days from induction to obtain sufficient Pax6+ colonies with similar efficiency. Neuronal markers indicated that transdifferentiation of iNPCs followed a parallel pathway as pluripotent cells (Kim et al. 2011). To explain heterogeneous fates of these induced cells, it has been hypothesized that even though iNPCs were negative for pluripotent markers, they still enter a pluripotent pathway undetectable with current technological means during differentiation. It is possible that cultured iNPCs proliferate in growth factors that promote intermediate progenitors with pluripotency (Ruggieri et al. 2014).

Specific application of transcriptional factors during regulating stages of inductions is able to change the cell lineage and efficacy (Tian et al. 2012). Generation of self-renewal iNPCs has implicated the roles of neuronal specific transcription factors such as FoxG1 and Sox2. FoxG1 and Sox2 are able to reprogram somatic cells into neural precursor states, highlighting the importance of these factors in achieving NPC states. Sox2, first thought as a dispensable factor,

has been shown to be essential for neuronal maturity in a reprogramming system. In the absence of Sox2, FoxG1 and Brn2 will promote differentiation of NPCs into multiple neural lineages but lack maturity (Ruggieri et al. 2014).

It is possible to achieve multipotent NSCs through the direct reprogramming of mice fibroblast cells. Both in vitro and in vivo studies show increase of transdifferentiation efficiency with the help of continued research of molecular mechanistic of pluripotent pathways, using combination of culture stem cells with neural progenitors (Han et al. 2012) or limit expression of Oct4 while regulating Sox2 (Thier et al. 2012). However, these both rely on viral infection system, which are not applicable for clinical treatment of patients. While still addressing the issue of efficiency from proliferation and differentiation, studies have indicated the possibility of reprogramming through messenger RNA to avoid innate antiviral responses. RNA-induced pluripotent stem cells exhibit similar identity of ES cells making them a possible alternative (Warren et al. 2010). Even with similar plasticity, before entering patient treatment, reduction of time between induction and cultivation increase in efficiency yields will have to continue to improve.

15.10 Stem Cell and Neuronal Repair During Brain Injury and Neurodegenerative Disorders

A variety of insults have been shown to be induce neurogenesis within the adult brain (Parent 2003), either in regions that have known neurogenic activity, or even in regions where neurogenesis normally does not occur (Yamamoto et al. 2001).

Damage induced by mechanical injury, prolonged seizures, trauma or stroke increases dentate gyrus cell proliferation, where a majority of the newly generated cells differentiate into granule neurons (Parent 2003). It also has been shown that progenitor cells are capable of proliferation and differentiation into mature myelinating oligodendrocytes in models of acute demyelination (Gensert and Goldman 1997). In various animal models of seizure, such as kainate and pilocarpine models of temporal lobe epilepsy, chemoconvulsant-induced status epilepticus, or electrical kindling models of epileptogenesis, increased dentate granule cell neurogenesis has been observed after stimulation (Parent 2003). Roles in other specific diseases are elaborated below.

15.10.1 Huntington's and Alzheimer's Diseases

Increased neurogenesis has been reported in patients with Huntington's disease and Alzheimer's disease (Jin et al. 2004; Curtis et al. 2003). Compared to control patients,

Alzheimer's disease brains showed increased expression of the immature neuronal marker doublecortin and the early neuronal differentiation protein TUC-4. Expression of doublecortin and TUC-4 was associated with neurons in the dentate gyrus, the physiological destination of these neurons, and in the CA1 region, which is the principal site of hippocampal pathology in Alzheimer's disease. Previously, it has been shown that (Jin et al. 2004) enhanced neurogenesis in Alzheimer's disease transgenic mice, in contrast to other reports (Haughey et al. 2002; Wen et al. 2002). Following those reports, it has been shown that induction of astrocytes in transgenic mice with Neurod-1 encoding retrovirus transformed reactive astrocytes and N2 glia into synaptic responsive neurons. Interesting, reactive astrocytes favored conversion to excitatory glutamatergic neurons, while N2 glia favored heterogeneous conversion to glutamatergic and GABAergic neurons (Guo et al. 2014). This demonstrates the possibility of recovery of neural networks in onset Alzheimer's disease neurodegeneration (Smith et al., Progress in Neurobiology, New Press). Similarly, there is a significant increase in cell proliferation in human subependymal layer in response to neurodegeneration of the caudate nucleus of Huntington's patients compared with control brain. These findings indicate that neuron damage or loss can trigger neurogenesis, and this may represent a mechanism directed toward the replacement of dead or damaged neurons.

15.10.2 Cerebral Ischemia

Recent findings in rodents show that cerebral ischemia is another injury model that stimulates neurogenesis in adult brain. There is evidence that the proliferating cells found after ischemia are NPCs (Yagita et al. 2001). Ischemia can lead to the proliferation of newborn cells that migrate into the zone of ischemic injury, differentiate, and express markers of mature striatal neurons (Arvidsson et al. 2002). In general, global cerebral ischemia induces neurogenesis in the dentate gyrus, whereas focal cerebral ischemia additionally increases new neurons in the SVZ (Zhang et al. 2005). Stroke selectively and significantly increases the number of type A and type C cells, which are more actively proliferating neural stem cells. Infusion with the antimetabolic agent cytosine-arabino-furanoside to the brain almost completely ablates type A and type C cells in the ischemic subventricular zone, thus indicating that neural stem cells contribute to cerebral ischemia-induced neurogenesis (Chen et al. 2004). In a middle cerebral artery occlusion ischemia model, BrdU labeling was increased in the ipsilateral SVZ and rostral migratory stream, indicating directed migration of neural progenitors toward the infarct area from their sites of origin.

15.10.3 Parkinson's Disease

In contrast to cerebral ischemia, Alzheimer's disease and Huntington's disease, which have clear evidence of increased neurogenesis, the question of whether adult brain generates new dopamine neurons is still controversial. Using confocal laser scanning microscope, Zhao et al. found dopaminergic neurons with BrdU-positive nuclei in the substantia nigra (Zhao et al. 2003). But a different conclusion was reported in another study (Frielingsdorf et al. 2004). Kay and Blum also reported the presence of BrdU-positive proliferative cells in the substantia nigra. Some of cells were identified as microglia, and none of them differentiated into dopaminergic neurons (Kay and Blum 2000). Another study (Yoshimi et al. 2005) revealed lack of neurogenesis of TH-positive neurons from proliferative stem cells in the substantia nigra. Most recently, α -Synuclein has been identified for its role in neuroinflammation as well as impairment to nerve repair process in Parkinson's disease. Fan et al. reproduced a transgenic mice model using A53T mutant α -synuclein as well as a caspase-1 knockout model to identify the mechanism of inflammasome related impairment of subventricular zone neurogenesis in mice. Stereotactic injections of microRNA-7 inhibited Nlrp3 inflammasome activation, which lead to improved neurogenesis in the SVZ (Fan et al. 2015). While much is still unknown about α -syn inflammasome mechanism, it has provided a gateway for possible treatment by way of enabling neurogenesis.

15.10.4 Mechanisms of Injury Induced Neurogenesis

The mechanisms that underlie the injury-induced neurogenesis mostly remain unclear. Growth factors and neurotrophic factors are likely candidates. In the case of forebrain ischemia, it has been shown that expression of bFGF and BDNF increase after injury. Normal neurodevelopment is guided by the spatial and temporal expression of trophic and growth factors, so these factors may represent the brain's attempt to protect injured neurons by activating developmental programs. In addition to protecting neurons, trophic and growth factors have also been shown to stimulate proliferation of adult neural stem cells and to direct their differentiation (Pencea et al. 2001; Aberg et al. 2000).

Activated glial cells can direct ischemia-induced neurogenesis. Astrocytes are implicated in the regulation of adult neurogenesis in various settings (Song et al. 2002). Microglia activated during inflammation can be a source of trophic factors and chemokines that influence endogenous stem cell behavior. Endothelial cells are another source of neurotrophic factors, including VEGF, BDNF, bFGF and chemokines that promote the survival and migration of

NSCs. Palmer et al. investigated the relationship of angiogenesis and neurogenesis and referred to the survival coupling of neuroblasts and endothelial cells as the "vascular niche" (Palmer et al. 2000). Treatment with VEGF 24 hours after stroke enhances angiogenesis and neurogenesis, indicating that VEGF plays an important role in brain repair (Zhang et al. 2000).

15.10.5 Functional Significance of Neurogenesis After Injury

Stem cells within the adult brain can be stimulated to replace damaged neurons (as illustrated in Fig. 15.3). Important questions remain: do these newly formed neurons in the adult brain establish appropriate synaptic connections and are they functionally integrated into the existed network? It is still uncertain to what extent neurogenesis contributes to functional recovery. Newly born neurons can establish synaptic contacts and are electrophysiologically functional (van Praag et al. 2002; Carlen et al. 2002). When neurogenesis was blocked, mice exhibited an impaired performance of a hippocampus-dependent memory task, suggesting that the newly born neurons are involved in the formation of certain types of memories (Shors et al. 2001). Using ionizing radiation to decrease neural regeneration after global ischemia, Raber et al. demonstrated that reduction of neurogenesis reduces functional recovery (Raber et al. 2004). Consistent with these data, Stilley et al. reported that transplantation of neuronal cells enhanced cognitive function in patients with basal ganglia stroke (Stilley et al. 2004).

An elaborate study by Ramer et al. finally documented a functional regeneration of sensory axon (Ramer et al. 2000). Not only were specific behavioral tests corresponding to the regenerated fiber phenotypes used, but they also confirmed their findings by re-injury of the regenerated fibers to demonstrate their capability for participation in functional recovery.

15.11 Neural Stem Cells and Their Potential Role in Transplant Therapy for Neurodegenerative Disorders

The use of stem cells to generate replacement tissues for treating neurological diseases is a major focus of stem cell research. Spinal cord injury, stroke, multiple sclerosis, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis and Alzheimer's disease would benefit from replacing destroyed or dysfunctional cells in the brain or spinal cord. The potential of several promising cell types to serve as an effective transplant has been evaluated in terms of

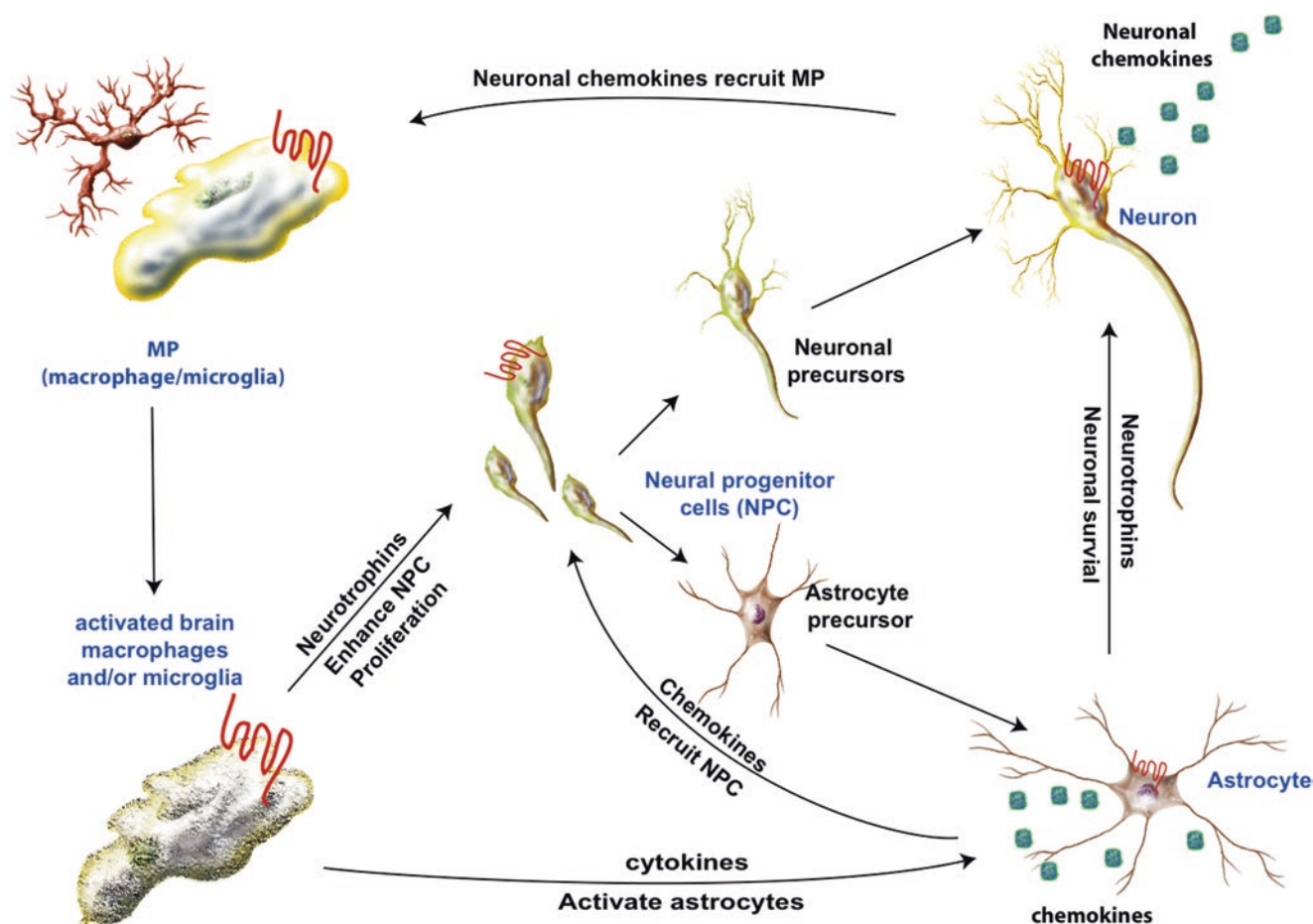


Fig. 15.3 A proposed mechanism for the simulation of neurogenesis during brain injury and neurodegenerative disorders. During neuronal injury, neurons produce chemokines, which recruit mononuclear phagocytes (MP) into the brain and to the site of injury. As these MP enter an environment of injury or inflammation, they become activated, subsequently releasing factors that might promote neurogenesis. These factors include but are not limited to neurotrophins, which may act directly to enhance the survival and proliferation of NPCs, and cyto-

kines that might activate astrocytes. These activated astrocytes produce chemokines that could promote the migration of NPCs to the site of injury and they can release neurotrophins that can promote neuronal survival. Once the NPCs receive these migratory and neurotrophic signals, the NPCs can migrate, proliferate, and differentiate into neuronal or astrocyte precursors, which then mature into neurons and astrocytes that may integrate into the CNS circuitry

the cell's ability to survive, differentiate, and migrate in an inflammatory milieu of the adult CNS (Magnus and Rao 2005). Many different cell types, including mesenchymal stem cells (Andre et al. 2016), neural stem cells, precursors, and embryonic stem cells, have been suggested as candidate cells for therapy (Rao and Mayer-Proschel 2000). In recent years, in vitro and in vivo stem cells transplantation studies were performed in cellular models, animal models including non-human primate model which has demonstrated the value of pre-clinical modeling with iPSCs and highlighted the feasibility of therapeutic neuron transplantation (Nguyen et al. 2011; Jiang et al. 2012; Liu et al. 2012; Hallett et al. 2015). Several neurological disorders that show some progress in stem cell therapy are discussed below.

15.11.1 Stem Cells for Transplant Therapy

Embryonic stem cells can proliferate indefinitely and under certain condition they can differentiate to neurons and glia in the laboratory. Even though they hold the promise of being able to repair or replace cells or tissues that are damaged or destroyed in neurodegenerative disorders, transplantation of embryonic stem cells is not feasible. The lines of unaltered human embryonic stem cells that exist will not be suitable for direct use in patients. These cells will need to be differentiated or otherwise modified before they can be used clinically. Simply injecting freshly isolated stem cells into the patient would lead to the formation of teratomas, as it occurs when stem cells are injected into mice. These current problems

have led researchers away from embryonic stem cells and towards NSCs harvested from various somatic locations.

NSCs isolated from fetal nervous tissue have the potential to differentiate into all types of nervous system cells, including neurons, oligodendrocytes, and astrocytes, so NSCs also have the capacity to replace damaged tissue in both the CNS and PNS. NSCs will restore functional neurons and glia and regenerate injured tissue. It is this characteristic of neural stem cells that makes them a potentially valuable transplantation material in a host of disorders.

Although adult neural stem cells might provide medical solutions that avoid the ethical and legal problems of cloning and fetal stem cell approaches, the limiting factor for the use of adult stem cells in future cell-replacement strategies is that there are insufficient numbers of cells available for transplantation. Previously, proliferation, as well as differentiation, presented low yields of unspecialized states that could not be properly directed to neuronal fates. New data on the plasticity of adult stem cells are very encouraging. Several studies have shown that adult stem cells from various organs have the plasticity to differentiate not only into their original source tissue, but also into cells of unrelated tissue. A clear example of such plasticity is mesenchymal stem cells (MSCs, Table 15.2).

MSCs include bone marrow derived MSCs (BM-MSCs) and human umbilical cord derived MSCs (hUC-MSCs). It is accepted that MSCs can differentiate into mature mesoderm cells such as bone, cartilage and fat (Pittenger et al. 1999). MSCs transplantation is applied well in the field of hematology, most often performed for patients with hematologic malignancies (Gong et al. 2012; Ball et al. 2007; Le Blanc and Ringden 2006). Some studies in vitro and in vivo reported that MSCs showed a minimum potential for trans-differentiation into neurons (Subbarao et al. 2015; Bossio et al. 2013). In addition, MSCs can inhibit the proliferation of T cells both in vitro and in vivo (Bowles et al. 2014; Kassis et al. 2008). Despite low differentiated efficacy into neurons,

MSCs transplantation has been performed in patients who suffered from neurological disorders such as multiple sclerosis and spinal cord injury in clinical trials, and almost all trials reported the safety and tolerability of MSCs, as well as stabilization/mild improvement of the disease (Diez-Tejedor et al. 2014; Qiao et al. 2014; Chen et al. 2014; Prasad et al. 2014; Frolov and Bryukhovetskiy 2012; Mendonca et al. 2014; Connick et al. 2012; Bonab et al. 2012; Li et al. 2014; Mancardi et al. 2015).

It has been demonstrated that somatic cells such as human fibroblasts can be directly converted to iPSCs and iNPCs by direct reprogramming (Liu et al. 2016; Colasante et al. 2015; Takahashi et al. 2007; Ring et al. 2012; Tian et al. 2012). With the development of direct reprogramming techniques, transplantations of iPSCs or iNPCs become attractive therapy approaches for various neurodegenerative diseases and brain injuries. iNPCs have the abilities to differentiate into region-specific and subtype-specific neurons. It has been reported that transplantations of iNPCs into the brain contribute to the improvement of the diseases in animal models (Kikuchi et al. 2011; Grealish et al. 2014; Hallett et al. 2015). As discussed in prior sections, low reprogramming efficacy needs to be overcome before clinical application.

15.11.2 Progress in Stem Cell Therapy in Neurological Disorders

15.11.2.1 Parkinson's Disease

Among all the CNS disorders, Parkinson's disease (PD) has the longest history of stem cell transplantation therapy. In Parkinson's disease, the loss of dopaminergic neurons in the substantia nigra is the major pathological change; thus, the major transplant therapeutic strategy has been to restore dopaminergic neurons by fetal tissue throughout the 1990s (Mehta et al. 1998; Dunnett and Bjorklund 1999; Lindvall et al. 1990). However, fetal tissue grafts showed short graft

Table 15.2 Stem cells-based therapies for neurological disorders

Stem cell types	Sources	Advantages for clinical treatment	Limitations
NSCs	Fetal brain	Low immunogenicity	Immune rejection
		Low tumorigenicity	Ethical issues
			Difficult to get enough cells
MSCs	Bone marrow	Low immunogenicity	Low differentiated efficacy into neurons
	Human umbilical cord blood	No ethical issues	Very limited source of hUCB-MSCs
iPSCs	Somatic cells	No immunogenicity	Tumorigenicity
		No ethical issues	Low reprogramming efficacy
		High capacity of pluripotency	Low differentiation efficacy into specific neurons
iNPCs	Somatic cells	No immunogenicity	Low reprogramming efficacy
		No ethical issues	
		Abilities to differentiate into region- and subtypes-specific neurons	

survival and limited integration of the grafts, which appeared to reduce the usefulness of fetal tissue. The use of stem cells has increased recently, since they appear to be far superior to fetal tissue grafts. Several groups have generated highly enriched populations of dopamine neurons from mesencephalic progenitors. Transplantation of expanded mesencephalic precursors resulted in spontaneous transformation into dopaminergic neurons, and functional recovery has been achieved in Parkinsonian rats (Studer et al. 1998; Sawamoto et al. 2001).

Since only limited numbers of NSCs can be purified from the midbrain, stem cells may be another candidate for transplantation in PD (Storch et al. 2004; Snyder and Olanow 2005). McKay's group first generated dopaminergic neurons from mouse embryonic stem cells, with greater enrichment achieved by mimicking the oxygen tension of the developing midbrain. When transplanted into 6-OHDA-lesioned rats, embryonic stem cell-derived dopamine neurons showed functional recovery in this dopamine-depleted animal model (Lee et al. 2000; Studer et al. 2000; Kim et al. 2002). Takagi et al. (2005) generated large numbers of dopaminergic neurons from monkey ES cells in vitro (Takagi et al. 2005). Behavioral studies and functional imaging revealed that the transplanted cells functioned as dopaminergic neurons and attenuated MPTP-induced neurological symptoms. Further in vitro study has shown that a minimal cocktail of transcription factors: ASCL1, NR4A2, and LMX1A are sufficient to convert mice and human fibroblasts into functional dopaminergic neuronal cells (Caiazzo et al. 2011). This same group has shown that this lentivirus system not only can develop induced dopaminergic neurons but they express normal action potentials and regular dopamine release within 21 days of transduction. This indicates possible high throughput screening applications (Theka et al. 2013).

To reach ultimate functional recovery in Parkinson's disease, transplanted dopaminergic neurons must survive, reinnervate the striatum, and integrate into the host nigrostriatal system. Many reports in recent years are encouraging, with advancements in co-transduction protocols such as ones with dominant negative TP53 lentivirus construct which have shown to improve transdifferentiation at least four-fold (Liu et al. 2014).

In 2014, hESCs-derived dopaminergic neurons were grafted in a rat model of PD successfully as a preclinical testing (Grealish et al. 2014). In addition, cynomolgus monkey iPSC-derived dopaminergic neurons were transplanted into the putamen of a non-human primate Parkinsonian brain (Hallett et al. 2015). These in vitro and in vivo studies highlighted the feasibility of stem cells therapy in PD and stem cell-based dopamine replacement therapies are close to clinical trials (Hargus et al. 2010; Kikuchi et al. 2011; Grealish et al. 2014; Hallett et al. 2015).

While the current research focuses on dopaminergic neurons, non-dopaminergic degeneration is also an important part of Parkinson's disease pathology. It remains to be established whether it is favorable to implant a pure population of dopaminergic neurons or whether the graft should also contain a specific mix of other neuron types and glial cells to induce maximum symptomatic relief.

15.11.2.2 Spinal Cord Injury

Spinal cord injury is associated with the loss of both neurons and glial cells; thus, stem cell-based therapy for reconstituting the injured spinal cord could be used to replace multiple cell types.

The extent of spinal cord injury depends on the severity of the initial trauma as well as the level of subsequent injury. The primary impact of contusion injury triggers a cascade of secondary events including hemorrhage, ischemia, excitotoxicity and inflammation, which lead to apoptotic neuronal and oligodendroglial death (Beattie et al. 2002). Transplantation will depend on a thorough understanding of the lesion environment and specific deficits associated with the individual injury. To maximize integration of transplanted cells, a variety of strategies such as reducing inflammation, inhibiting apoptosis of transplanted cells by in vitro preconditioning, and modifying the glial scar are being tested (Ramer et al. 2004).

The potential application of neural stem cells for spinal cord injury has been investigated by numerous studies. It has been reported that neural stem cells induced to neuronal differentiation by neurogenin-2 provided significant functional benefit following transplantation after contusion injury (Hofstetter et al. 2005). Further, undifferentiated cells can achieve the regional appropriate phenotype specification in response to local signals in exclusive niches (Gage 2000).

Embryonic stem cells also have been tested. Myelination in the injured spinal cord by implanted mouse embryonic stem cells was reported (Brustle et al. 1999). It has been reported that oligodendrocytes derived from human embryonic stem cells were able to myelinate demyelinated foci in spinal cord contusions (Nistor et al. 2005). The discovery of NG2-expressing progenitors within the spinal cord stirred the hope for using endogenous stem cells in spinal cord injury (Horner and Gage 2000). Recently, mesenchymal stem cells have been tested with an intrathecal cell delivery system, which has shown neurological improvements in clinical research. Further studies to increase efficacy of treatment are required (Derakhshanrad et al. 2015).

15.11.2.3 Other Diseases

Cholinergic neurons, the major site of degeneration in Alzheimer's disease and motor neuron disease, have become the target for cellular reprogramming in many in vivo experiments. Wu et al. demonstrate that a "priming" procedure,

which involves culturing the fetal human neural stem cells on laminin in the presence of a cocktail of bFGF and heparin *in vitro*, generates a nearly pure population of neurons after transplantation into adult rat brain. Furthermore, adult fibroblasts were induced with a combination of factors Neurog2 and Sox11 and high-efficiency small molecules, forskolin and dorsomorphin, to rapidly generated cholinergic neurons (Liu et al. 2013). Previously, stem cell-derived cholinergic neurons were found following implantation in regions that normally contain many cholinergic neurons, such as the spinal cord and medial septum, but were not seen in regions normally lacking these cells, such as the hippocampus and cortex. In contrast, the human neural stem cells that did not undergo *in vitro* priming produced primarily glia or undifferentiated cells following implantation (Wu et al. 2002).

Stem cell-based therapy for stroke will be more complicated, because the extensive cell death and massive inflammatory response make these brains a more hostile environment for cell grafts. Various sources of cells have

been tested for their ability to reconstruct the forebrain and improve function after transplantation in animal models of stroke (Lindvall et al. 2004). Further studies have shown to alleviate cerebral inflammation and neural degeneration by reducing intracerebral infiltrations in animal intracerebral hemorrhage models (Qin et al. 2015). In most cases, only a few grafted cells could survive. Some recent exciting findings in rodents suggest that stroke can induce an increase in neurogenesis; thus, a new therapeutic approach based on self-repair has been brought forth, as discussed in Sect. 11.1.

Clinical trials, such as those listed in Table 15.3, have entered a stage of using exogenous stem or progenitor cells to treat cerebral ischemic patients (George and Steinberg 2015). The strategies thus far have encouraging results, but much work is still required to achieve a better understanding therapeutic benefits. Taking advantage of treatments in terms of multiple cell injury pathways may be required to improve clinical results.

Table 15.3 Stem cells that have been trialed in neurological disorders

Reference(s)	Disease	Type of trial	Method	Number of patients	General outcome
Mancardi et al. (2015)	MS	Phase II trial	Autologous hematopoietic stem cells transplantation (AHSCT)	21	Improvement in reducing new T2 lesions, Gd+ lesions, as well as the annualized relapse rate. No improvement in the progression of disability
Li et al. (2014)	MS	Clinical trial	Human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) transplantation	23	Improvement in the overall symptoms, the EDSS scores and relapse occurrence lower than those of the control patients
Bonab et al. (2012)	MS	Open label study	Autologous bone marrow derived mesenchymal stem cells (BM-MSCs) transplantation	25	Improvement/stabilization in the course of the disease in progressive MS in the first year after injection with no serious adverse effects
Connick et al. (2012)	MS	Open label phase IIa study	Autologous bone marrow derived mesenchymal stem cells (BM-MSCs) transplantation	10	Improvement in visual acuity and visual evoked response latency, with an increase in optic nerve area
Mendonca et al. (2014)	Chronic SCI	Phase I, non-controlled study	Autologous bone marrow mesenchymal stem cells (BM-MSCs) transplantation	14	Safe, feasible, and may promote neurological improvements
Frolov and Bryukhovetskiy (2012)	Chronic SCI	Clinical trial	Autologous hematopoietic stem cells transplantation (AHSCT)	20	Improvement in electrophysiology
Prasad et al. (2014)	Subacute ischemic stroke	Phase II, randomized trial	Autologous bone marrow mononuclear stem cells (BMSCs) transplantation	120	Safety, but no beneficial effect of treatment on stroke outcome
Chen et al. (2014)	Chronic ischemic stroke	Phase II study	Autologous peripheral blood stem cells (PBSCs) transplantation	30	Safe, feasible, and effective in improving functional outcome
Qiao et al. (2014)	Ischemic stroke	Clinical trial	Neural stem/progenitor cells (NSPCs) and mesenchymal stromal cells (MSCs) transplantation	8	Improvement in the neurological functions, disability levels, and daily living abilities of the patients
Diez-Tejedor et al. (2014)	Acute ischemic stroke	Phase II, double-blind pilot clinical trial	Allogeneic mesenchymal stem cells (MSCs) transplantation	20	Not reported

MS multiple sclerosis, SCI spinal cord injury

15.12 Future Directions in Neurogenesis for Neurodegenerative Disorders

Although current research still focuses on cell-based therapies by transplantation of exogenous stem cells or differentiated neurons for neurodegenerative disorders such as Parkinson's disease, Huntington's disease or amyotrophic lateral sclerosis, which all result from a loss of relatively defined phenotypes of cells, accumulating evidence indicates that endogenous neurogenesis may occur as part of an intrinsic brain self-repair process. This raises the possibility of developing therapeutic strategies based on activation of dormant capacities of endogenous neural stem cells.

15.12.1 Endogenous Adult Stem Cells as a Therapeutic Strategy

Recent evidence confirms the widespread occurrence of neural stem cells and the production of new neurons in many parts of adult brain. Although the regeneration of new neurons has been observed only at a very low frequency and can only replace a small fraction of neurons (Magavi et al. 2000; Fallon et al. 2000), these findings encourage work toward a therapeutic strategy based on neurogenesis, especially in neurodegenerative disease where persistent stimuli for neurogenesis exist or where additional stimulation and regulation are possible. For those neural injuries and neurodegenerative disorders that involve relatively broad areas and many types of neurons, this will be an especially attractive direction. With appropriate manipulation, perhaps many types of neurons that have been considered irreplaceable can be regenerated under the right circumstances (Kruger and Morrison 2002).

Distinct environmental cues may instruct endogenous progenitors to differentiate into specific neuronal subtypes depending on their locations. Adult stem cells can regenerate specific neuronal subtypes appropriate for the sites of damage. For example, targeted degeneration of corticothalamic neurons in the neocortex and dopaminergic neurons innervating the striatum has been shown to induce regeneration of the same cells types.

The strategy of manipulating endogenous stem cell for repairing relies on finding the appropriate factors and signaling molecules that help the patient's own stem cells survive and grow. Previous studies have shown that exogenous growth factors can increase the rate of neurogenesis in the adult brain and a certain combination of exogenous factors and endogenous stimulation may be necessary for boosting the regeneration of neurons at lesion sites (Weiss et al. 1996). By intraventricular infusion of growth factors, CA1 hippocampal pyramidal neurons that were lost to ischemic injury were remarkably regenerated by endogenous NPCs. These new neurons not only survived for a long period of time but

also established synaptic connections and showed functional contributions by ameliorating deficits in hippocampal-dependent spatial cognition in ischemic rat models (Nakatomi et al. 2002).

Although newly born neurons are able to project over relatively long distances to targets, questions remain about whether and how the projections incorporate into local circuits. Given the complexity of the adult brain network, it is no surprise that the synapses of newly born neurons remain immature and exhibited altered electrophysiological properties even 3 months after ischemia. Moreover, not all incorporation of new cells into the brain circuitry is beneficial (Nakatomi et al. 2002). For example, prolonged seizures lead to the generation of ectopic neurons or abnormal projections in the striatum and hippocampus, so abnormal neurogenesis may even worsen seizure damage by its pro-epileptogenic nature (Parent 2003).

While a growing number of studies report injury-induced enhancement of neurogenesis, in most cases endogenous stem cells are unable to replace neurons lost to injury. There is little evidence supporting a major role of endogenous neurogenesis to improve neural functions or slow disease progression in neurodegenerative disease. Indeed, although hippocampus is a known site of active neurogenesis throughout adulthood, newly generated cells beyond the dentate gyrus appear to be very limited (Rietze et al. 2000). Therefore, achieving functional neuronal regeneration is still a challenging task for neuroscientist. It is very important to understand how the brain microenvironment changes as a result of injury, and how stem cells respond to the spatial and temporal signals presented. The successful development of therapeutic applications based on endogenous neurogenesis will depend on our ability to manage the proliferation, migration, differentiation, and functional integration of recruited cells. Much work is still required to fulfill such a strategy.

15.12.2 Other Potential Therapeutic Value of NSCs

The potential therapeutic value of NSCs may be in their basic biology. NSCs are potent vehicles for gene therapy, so NSCs may be useful to carry copies of genes into tissue that will benefit the disease therapy. NSCs are migratory; thus, transplanted NSCs will migrate from the site of delivery to distant areas of active neurogenesis or to the damaged tissue, where they reconstruct both neural networks and glia support. Because NSCs will migrate to distant, multiple, and extensive regions of the nervous system, they may be useful in "global" degeneration, in which all or most of the nervous system is affected. In addition to regenerative capabilities, neural stem cells may have an immunoregulatory potential. Mesenchymal stem cells have been shown to inhibit effector

T-cell activities, reducing autoimmune inflammation of the CNS (Zappia et al. 2005). This immunoregulatory potential of mesenchymal stem cells suggests that NSCs may also possess similar properties. Indeed, transplanted NPCs and their NPC-derived secreted factors such as VEGF are powerful modulators of microglia activation, suggesting that NPCs regulate local immune response (Mosher et al. 2012). These immunoregulatory and trophic effects add to the therapeutic value of NSCs/NPCs.

Much of the biology of regeneration of nervous system tissue has yet to be discovered. Many of the factors that contribute to regeneration and/or that prevent it continue to be discovered. Although neurogenesis in adult brain is limited and may not be sufficient to achieve functional recovery after injury, understanding the mechanism of NPCs, iNPCs, and subtype-specific iNPCs is imperative to promote regeneration of damaged tissue.

15.13 Review Questions

1. What are the two criteria that must be fulfilled in order to identify a cell as a “stem cell”?
2. What is the difference between a stem cell and a progenitor cell?
3. Discuss the positive and negative effects inflammation has on neurogenesis.
4. List some of the roles of stromal cell-derived factor 1 (SDF-1/CXCL12) in CNS development and homeostasis.
5. What are the effects of brain-derived neurotrophic factor (BDNF) on NPCs?
6. List the processes that a neural stem cell must undergo to become a functional neuron.
7. Discuss the role of Notch signaling in regulating stem cell self-renewal versus differentiation.
8. Discuss the advantages and shortcomings of embryonic, fetal, and adult stem cells for transplantation therapies.
9. Discuss the benefits and problems associated with therapies using exogenous stem cells as compared to targeting endogenous stem cells to promote neurogenesis.
10. Discuss the advantages and shortcomings of induced pluripotent stem cells for transplantation therapies.

15.14 Answers

1. The cell must be able to self renew and differentiate into multiple lineages or cell types.
2. Stem cells are capable of self renewing indefinitely, whereas progenitor cells can only divide a limited number of times while retaining identical properties and differential capabilities as the parent cell. Alternatively,

a progenitor cell may be defined as a cell that can self renew, but can only differentiate into one cell type.

3. This area remains a controversial topic with no definite consensus. Many researchers have provided evidence that microglial activation and inflammation have both pro- and anti-neurogenic effects. It is possible that signals from damaged tissues or the factors causing injury would also be the signals that induce repair. This may be the case for acute damage, although, it is less likely that chronic, more widespread, or more severe damage will induce significant neurogenesis. Factors produced during severe inflammation may directly inhibit neurogenesis, or it may induce neurogenesis but provide a harsh environment that is detrimental for the survival of the newly developed neurons as well as existing neurons.
4. Stem and progenitor cell survival, mitogenesis, and migration; neuronal axon migration guidance; peripheral macrophage recruitment into the CNS.
5. Increased survival of NPC and directed differentiation to neurons; neuronal axon migration guidance.
6. The stem cell must migrate, proliferate, differentiate along the neuronal lineage, mature from a precursor to a fully functional neuron, integrate into the neuronal circuit, and survive.
7. Upon binding of a ligand to the Notch receptor, the intracellular portion of Notch is cleaved and translocates into the nucleus where it induces the transcription of genes that prevent differentiation. By preventing differentiation, Notch promotes self-renewal or quiescence.
8. The three cell types face the problem of possible rejection but immune suppression therapies may be used to overcome this. Embryonic stem cells are advantageous due to their great potential to expand their cell numbers and to differentiate into all the cells of the CNS. Embryonic stem cells have many problems, including ethical objections and limitations from society and government and the fact that embryonic stem cells will form teratomas if not differentiated prior to transplantation. Also, the embryonic stem cells currently approved for federal funding can never be used for human transplantation due to contamination by mouse feeder layers used in the earlier culture of these cells. Fetal stem cells can easily be isolated from aborted fetuses, expanded in culture, and transplanted in an undeveloped or fully differentiated state. These fetal stem cells still face the problem of ethical objections, although these objections may be less than the difficulties faced when using embryonic stem cells. Adult stem cells do not share the same ethical objection as do embryonic and fetal stem cells, but they are much more difficult to isolate and it is almost impossible to expand the cell number in culture and successfully direct their differentiation. Perhaps the most promising use of adult stem cells would be to use stems

cells from tissue other than the CNS for example hematopoietic stem cells which can be easily isolated from the patient. These cells can be expanded and possibly trans-differentiated into neurons.

9. The stimulation of endogenous stem to promote neurogenesis is beneficial because it does not have the risks associated with transplantation of exogenous stem cells. These include damage caused by the surgical procedure to implant the exogenous stem cells and donor versus graft rejection. But transplantation of exogenous stem cells may allow for the correction of problems associated with endogenous stem cells. These endogenous stem cells may have some genetic problem which prevents successful neurogenesis, whereas, the exogenous stem cells would not have this same genetic disability.
10. Generating iPSCs through reprogramming strategies avoids the need to destroy embryos for ES cells. Furthermore, iPSCs has low immunogenicity and high capacity for pluripotency that provide advantages for transplantation therapies. However, tumorigenicity represents the most pressing challenge for the clinical application of iPSCs. In addition, reprogramming efficiency toward iPSCs is historically low and the differential efficiency of iPSCs to specific neurons is also low.

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Abstract

Innate immunity response is an ancient self-defence mechanism, which is conserved across a very broad spectrum of species. The innate immune system constitutes the first line of host defense during infection or homeostatic regulatory activities and therefore plays a crucial role in the initial recognition and regulation of immune responses to invading organisms. Peripheral mononuclear phagocytes (MP; monocytes, tissue macrophages and dendritic cells) respond to pathogens but in molecular pattern-specific manners using phagocytosis and clearance mechanisms for intracellular killing. In the central nervous system (CNS), those are perivascular and brain macrophages and microglia. Of relevance they are separated from the antigen specific and long-lasting adaptive immune system, thus MP provide immediate surveillance responses against invading microbial pathogens and cancerous cells in the CNS. Of particular importance to the CNS, innate immunity can be both neuroprotective or neurodestructive contingent on the activation responses and phenotype that has emerged. In reference to phylogeny, MP-governed innate immunity strongly recapitulates ontogeny as it represents evolutionary primitive defense that is self-contained at a single cell level. It is operative in all species from single cell organisms to plants and, insects and inevitably conserved to man.

Keywords

Damage-associated molecular patterns • Domain-like receptors • Nucleotide oligomerization • Pathogen-associated molecular patterns • Phagocytosis • Phosphatidylserine receptors • Scavenger receptors • Toll-like receptors • Triggering receptor expressed on myeloid cells 2 • TYRO protein tyrosine kinase binding protein

16.1 Introduction for the Innate Immune Responses

The innate immune response of the CNS follows a well-characterized signaling pathway in response to tissue injury, cell death, and pathogens (Olson and Miller 2004).

The functions of the innate immune system include phagocytosis, intracellular killing, secretion of biologically active factors, cell migration, barrier protection and mobility. There are a number of factors that are intrinsic to the MP and govern its function. *First*, rests in the MP's ability to secrete chemokine and other chemical factors that enable entry of inflammatory cells into areas of tissue injury. The ability to recruit a repertoire of immunocytes to damaged and infectious tissue sites, activate the complement cascade and promote clearance of injurious microbes and debris of dead cells and of tissue metabolism together with antibody complexes and subsequent removal are part of the MP's demanding

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functional repertoire. *Second*, the cells that also provide chemical barrier to spread of infectious agents are the MP. *Third*, CNS MP's not only function as sentinels but express and engage specific signaling pathways that facilitate the cells' surveillance functions. These include pattern-recognition receptors (PRRs) that recognize signaling pathways initiated by dying or damaged cells referred to as pathogen-associated molecular and damage-associated molecular patterns (PAMPs and DAMPs). PRRs include Toll-like receptors (TLRs), Nucleotide Oligomerization Domain (NOD)-like receptors (NLRs) and Retinoic acid-Inducible Gene 1 (RIG1)-like receptors (RLRs). TLRs are ubiquitously expressed and are often expressed on the plasma membranes of immune cells and glial cells, including microglia, astrocytes, and neurons (Bsibsi et al. 2002). *Fourth*, misfolded and aggregated proteins engaged in common degenerative disorders of the nervous system can affect MP signaling. Indeed, A β itself can act as a PAMP and interact with TLRs (Lotz et al. 2005; Stewart et al. 2010). NLRs are the major component and inflammasomes and found in the cytoplasm. Some NLR ligands, such as muramyl dipeptide (MDP), are also expressed in microglia, astrocytes, and neurons. NLR ligands have been shown to activate inflammasomes the neuronal cells (Chauhan et al. 2009; Ransohoff and Brown 2012). When stimulated by a ligand, such as lipopolysaccharide (LPS, a TLR ligand) or MDP, TLRs or NLRs MP's become activated and initiate transcriptional up-regulation and release of pro-inflammatory cytokines and chemokines. Other major innate immune response in the CNS is phagocytosis, which is rapidly expanding to three distinct categories: (1) phagocytosis of opsonized microbes and particles, by antibodies and complements (2) phagocytosis of non-opsonized molecules, by scavenger receptors and (3) phagocytosis of apoptotic cells, by phosphatidylserine receptors. The first group includes Fc and complement, integrin family and C-type lectin receptors. The second includes newly standardized class A-J scavenger receptors, including SR-A1 (MSR1), SR-B2 (CD36), SR-D1 (CD68), SR-E2 (Dectin-1), SR-F3 (MEGF10), SR-I1 (CD163), SR-J1 (RAGE). The third includes phosphatidylserine receptors, including BAI1, TIM-1, Tyro3, Axl, MerTK, STAB2/SR-H2, α v β 5 integrin, MEGF10, TREM2 and TYROBP. Newly discovered phagocytosis receptor signaling leads to activation of CrkII, Dock180 and Rac1, which are homologue of *C. elegans* CED-2, CED-5, and CED-10. MEGF10 activation of GLUP are homologue of *C. elegans* CED-1 and CED-6. These diverse receptors transmit innate immune response known as antiviral response, inflammatory activation, phagocytosis.

16.2 Pattern-Recognition Receptor Signaling: Receptors for PAMPs and DAMPs and Consisted of TLRs, NLRs, and RLRs

16.2.1 Pathogen-Associated Molecular Patterns (PAMPs) for Innate Immunity Signaling

The innate immune response relies on recognition of evolutionarily conserved structures on pathogens, including viruses, bacteria, fungus and yeast, termed PAMPs, through a limited number of germ line-encoded pattern recognition receptors. PAMPs are characterized by being invariant among entire classes of pathogens, essential for the survival of the pathogen, and distinguishable from "self" (Janeway and Medzhitov 2002). PAMPs are mostly recognized by TLRs through: Glycoproteins, lipoproteins, peptidoglycans, lipoproteic acid, porin, zymozan, β -glycan and protozoa GPI-anchors to TLR2, DNA to TLR9, RNA to TLR3 and TLR7/8, Gram⁻ bacterial lipopolysaccharide (LPS) to TLR4, Gram⁻ bacterial flagellin to TLR5, and fungal mannan to TLR2 and 4 (Table 16.1). An important property of this system is that no single class of pathogen is sensed by only one type of PRR. Rather, a number of different PRRs are engaged by a given pathogen via various PAMPs, hence securing a rapid and potent inflammatory response and also allowing for some specificity of the response. Please refer to Mogensen's review article for more details about PAMPs (Mogensen 2009).

16.2.2 Damage-Associated Molecular Patterns (DAMPs) for Innate Immunity Signaling

The concept of DAMPs was introduced by Matzinger proposed as the "Danger Theory" in which the injured tissues were postulated to release intracellular molecules, DAMPs, that activate the immune system (Matzinger 1994). The "Danger Theory" remained a theoretical model until High Mobility Group Box 1 (HMGB1) and uric acid crystals were recognized as DAMPs (Scaffidi et al. 2002; Shi et al. 2003). Since then, many more DAMPs were identified and their roles in health and disease are now partially understood (Table 16.2). DAMPs are normally invisible to the immune cells, and become visible only when exposed to the extracellular environment. DAMPs can be secreted from not only dead cells but also living cells under the stress. Exemplary

Table 16.1 IPAF; Ice protease-activating factor, MDA5; melanoma differentiation-associated gene 5, NALP; NACHT, LRR and PYD domains-containing protein, PKR; protein kinase-R, RIG-I; retinoid acid-inducible gene I (Adapted from Mogensen 2009)

Organisms	PAMPs	TLRs	NLRs	RLRs	DNA sensors
Viruses	Glycoproteins	TLR4			
	DNA	TLR9	NALP3		
	RNA	TLR3, 7/8		RIG-1/MDA5 (PKR)	+
Gram⁺ bacteria	DNA	TLR9	NALP3		+
	Lipoproteins	TLR2			
	Peptidoglycans		NOD2, NALP1/3		
	Lipoteichoic acid				
Gram⁻ bacteria	DNA	TLR9	NALP3		+
	Porin	TLR2			
	Peptidoglycans		NOD2, NALP1/3		
	Lipopolysaccharides	TLR4			
	Flagellin	TLR5	IPAF		
Fungi	Zymozan	TLR2			
	β -glycan				
	Mannan	TLR2,4			
Protozoa	DNA	TLR9			
	GPI-anchors	TLR2,4			

Table 16.2 Adapted from Vénéreau et al. (2015)

Organisms	DAMPs	Receptors	NLRs	DNA sensors
Nucleus	Histons	TLR2, 4, 9	NALP3	
	DNA	TLR9		+
	HMGB1	TLR2,4 RAGE TIM3		
	IL1 α	IL1R		
	IL33	ST2		
Cytosol	ATP	P2Y2 P2X7		
	F-actin	DNGR1		
	Cyclophilin A	CD147		
	HSPs	CD147 TLR2, 4 SREC1 FEEL1		
	Uric acid		NALP3	
	S100s	TLR2, 4 RAGE		
	A β	TLR4, 6 RAGE	NALP3	
Mitochondria	mtDNA	TLR9		+
	TFAM	RAGE TLR9		
Endoplasmic reticulum	Calreticulin	CD91		

DAMPs include HMGB1 and ATP. As a representative DAMP, HMGB1 is a mobile chromatin protein that acts as a DNA chaperone by binding DNA transiently and bending it reversibly. HMGB1 is released by the activation of inflammasome (Lu et al. 2013). Secreted HMGB1 binds to receptor for advanced glycation endproducts (RAGE), TLR2 and TLR4, which trigger activation of many signaling molecules, such as nuclear factor-kB (NF-kB) pathway, as well as c-jun kinase and p38 mitogen-activated protein kinase (MAPK) pathways (Andersson and Tracey 2011; Kierdorf and Fritz 2013; Urbanaviciute et al. 2008; Wang et al. 2013; Yang et al. 2013; Maroso et al. 2010).

ATP is also a major and time-sensitive DAMP. ATP can be released at nerve terminals and several other cell types under inflammatory, ischemic, and hypoxic conditions (Garg et al. 2012; Junger 2011). ATP acts as a signaling molecule through the activation of purinergic P2 receptors, which are involved in innate and adaptive immune responses (Junger 2011; Eltzschig et al. 2012). P2 receptors can be further subdivided into metabotropic P2Y receptors (P2YRs), which are G-protein-coupled, and ionotropic P2X receptors (P2XRs), which are nucleotide-gated ion channels. P2Y2R signaling causes production of pro-allergic mediators (for example, IL-33, IL-8, eosinophil cationic protein) during allergic

airway disease (Kouzaki et al. 2011). P2XR channels are opened by the binding of ATP, allowing sodium and calcium influx and potassium efflux. Among P2XRs, P2X7R is predominantly expressed on immune cells such as mast cells, macrophages, microglia, and DCs, and its signaling has been linked to inflammatory and infectious disorders (Junger 2011). The binding of extracellular ATP to P2X7R elicits NLRP3 and inflammasome activation (Gombault et al. 2012).

Other known DAMPs are histones, genomic DNA, IL1 α and IL33 as a result of transcriptional induction from nucleus (Schaefer 2014; Bianchi 2007; Lopetuso et al. 2012), F-actin, cyclophilin A, heatshock proteins (HSPs), uric acid crystals, S100s and A β from cytoplasm (Krysko et al. 2012; Bianchi 2007; Stewart et al. 2010), mitochondrial DNA, mitochondrial transcription factor A (MTFA) from mitochondria (Zhang et al. 2010), and calreticulin from endoplasmic reticulum (Krysko et al. 2011, 2012). Please refer to the references for more detailed review.

16.2.3 Toll-Like Receptors (TLRs) for DAMP- and PAMP-Mediated Signaling

One of the most important findings in the effort to understand innate immunity has been the identification of Toll-like receptors (TLRs). TLRs define a major class of PRRs and are critical to the initiation and tailoring of both innate and subsequent adaptive immune responses (Beutler 2004; Iwasaki and Medzhitov 2004). DAMPs and majority of DAMPs signal through TLRs. For example, bacterial cell wall components are recognized by TLR2, and lipopolysaccharide and viral envelope proteins are recognized by TLR4. Double strand RNA, single strand RNA, and unmethylated CpGs are recognized by TLR3, TLR7/8, and TLR9, respectively (for review, see (Akira et al. 2006)). Primary cultured human and mouse microglia express mRNA for TLR1-9, whereas human and mouse astrocytes express high levels of TLR3, and low-level TLR 1, 2, and 4-6 (Jack et al. 2005; Olson and Miller 2004; McKimmie and Fazakerley 2005; Carpentier et al. 2005). A study on aged mouse brains demonstrated that TLR1, TLR2, TLR4, TLR5, TLR7 and CD14 expression were up-regulated in correlation with age, whereas TLR9 was down-regulated (Letiembre et al. 2007). Interestingly, a TLR4 polymorphism was also associated with successful aging (Candore et al. 2006).

TLR-induced signaling pathways can be broadly classified on the basis of their utilization of different adaptor molecules, i.e., dependent on or independent of the adaptor MyD88, Mal or TIR domain-containing adaptor inducing IFN- γ (TRIF), and, additionally, their respective activation of individual kinases and transcription factors (Akira and Takeda 2004; O'Neill and Bowie 2007). Three major signal-

ing pathways responsible for mediating TLR-induced responses include NF- κ B, p38 MAPKs, and IFN regulatory factors (IRFs) (Akira and Takeda 2004; Akira et al. 2006; Kawai and Akira 2007). Whereas NF- κ B and MAPKs play central roles in induction of a proinflammatory response, IRFs are essential for stimulation of IFN production (Akira and Takeda 2004; Kawai and Akira 2007). These receptors are primarily responsible for canonical antiviral response or inflammatory activation of variety of cells.

16.2.4 Nucleotide Oligomerization Domain-Like Receptors (NLRs) for Inflammasome Signaling

NLRs belong to a family of innate immune receptors which have gained increasing interest are now considered key sensors of intracellular microbes and danger signals. NLRs are defined by a centrally located NOD that induces oligomerization, a C-terminal LRR that mediates ligand sensing, and an N-terminal CARD responsible for the initiation of signaling (Kanneganti et al. 2007).

There are two well-characterized members of the NLR family: NOD1 and NOD2. They sense bacterial molecules derived from the synthesis and degradation of peptidoglycan (Kanneganti et al. 2007). NOD1 recognizes diaminopimelic acid produced primarily by Gram⁻ bacteria (Chamaillard et al. 2003; Girardin et al. 2003a), and NOD2 is activated by muramyl dipeptide (MDP), a component of both Gram⁺ and Gram⁻ bacteria (Girardin et al. 2003b). Activation of NODs induces oligomerization and recruitment of signaling molecules and transcriptional activation of inflammatory genes (Kanneganti et al. 2007). NOD1 and NOD2 primarily activate gene expression of proinflammatory, while other NLRs mainly activate of caspases (such as caspase-1), which catalyzes the cleavage of the IL-1 precursor pro-IL-1 β (Mariathasan et al. 2006; Martinon et al. 2002). This IL-1 cleavage complex was termed the inflammasome and consist of the adaptor ASC (apoptosis-associated speck-like protein containing a CARD), pro-caspase-1, and an NLR family member, such as Ipaf (Ice protease-activating factor), NALP1, or NALP3/Cryopyrin (Mariathasan et al. 2006; Martinon et al. 2002). Several families of inflammasomes have been identified, each recognizing different danger signals or PAMPs through their respective NLR (Kanneganti et al. 2007). For instance, NALP3 plays a role in recognition of ATP (Mariathasan et al. 2006), uric acid crystals (Martinon et al. 2006), viral RNA (Kanneganti et al. 2006), and bacterial DNA (Muruve et al. 2008), whereas both NALP3 and NALP1 mediate caspase-1 activation in response to bacterial MDP (Faustin et al. 2007; Martinon et al. 2004). The major signaling output of inflammasome activation is the maturation and secretion of IL-1 β and IL-18.

16.2.5 Retinoic Acid-Inducible Gene 1 (RIG1)-Like Receptors (RLRs) for dsRNA Signaling

RIG-I and melanoma differentiation-associated gene 5 (MDA5) are IFN-inducible RNA helicases and play a pivotal role in sensing of cytoplasmic RNA (Yoneyama et al. 2004, 2005). RIG-I and MDA5 contain an N-terminal caspase recruitment domain (CARD) and a central helicase domain with ATPase activity required for RNA-activated signaling (Yoneyama et al. 2004). Binding of dsRNA or 5'-triphosphate RNA to the C-terminal domains of RLRs triggers signaling via CARD-CARD interactions between the RNA helicase and the adaptor protein IFN- β promoter stimulator 1 (IPS-1), ultimately resulting in an antiviral response mediated by type I IFN production (mainly IFN- α and IFN- β among others) (Yoneyama et al. 2004; Kawai et al. 2005; Kato et al. 2005; Meylan et al. 2005; Xu et al. 2005). RIG-I and MDA5 have viral specificity: RIG-I is essential for the response to paramyxoviruses and influenza virus, whereas MDA5 is critical for the response to picornavirus and norovirus (Kato et al. 2006; McCartney et al. 2008). In addition to viral RNA, RLRs can recognize self-derived small RNAs generated by RNase L, thus amplifying the IFN response (Malathi et al. 2007). The major signaling output of RLRs is the expression and production of type I IFN.

16.2.6 Protein Kinase R (PKR) for dsRNA Signaling

Viral RNA can also be recognized by the IFN-inducible dsRNA-activated PKR, which represents a major mediator of the antiviral and antiproliferative activities of IFN (Nallagatla et al. 2007; Sadler and Williams 2007). Binding of dsRNA, 5'-triphosphate RNA, or poly(I-C) induces conformational changes in PKR, resulting in autophosphorylation, dimerization, and subsequent substrate phosphorylation (Carpick et al. 1997; Nallagatla et al. 2007; Romano et al. 1995). The best-characterized PKR substrate is the eukaryotic initiation factor eIF2 α , the phosphorylation of which leads to inhibition of protein synthesis (Sadler and Williams 2007). Moreover, PKR has been assigned a role in proinflammatory signal transduction as an upstream kinase involved in mediating dsRNA-dependent NF- κ B activation (Zamanian-Daryoush et al. 2000). However, although PKR was originally considered the principal molecule responsible for cellular recognition of dsRNA, the prevailing view is that the major contribution to dsRNA-activated responses is mediated by RLRs, with recent data suggesting that PKR may be able to amplify RLR signaling (McAllister and Samuel 2009; Zhang and Samuel 2008), thus illustrating cross talk between these different cellular dsRNA-sensing systems involved in antiviral defense.

16.2.7 DNA Sensors for dsDNA Signaling

Cytoplasmic localization of DNA is recognized by the innate immune system independently of TLRs, RLRs, and NLRs (Soulat et al. 2006; Stockinger et al. 2004) and involved in a response to both bacteria and DNA viruses (Charrel-Dennis et al. 2008; Leber et al. 2008; Nociari et al. 2007; Rasmussen et al. 2007). The first cytosolic DNA sensor, DAI (DNA-dependent activator of IFN-regulatory factors), was reported in 2007 (Takaoka et al. 2007). DNAs from various sources were demonstrated to bind to DAI, thereby inducing DNA-mediated induction of type I IFN and other inflammatory genes involved in innate immunity (Takaoka et al. 2007). DAI is probably not the only cytosolic DNA receptor triggering the IFN response. AIM2 (absent in melanoma 2) is a recently reported cytosolic DNA receptor stimulating proinflammatory signaling and maturation of pro-IL-1 β (Hornung et al. 2009). Leucine-rich repeat flightless-interacting protein 1 (LRRFIP1) is the third IFN-inducer DNA sensor and can recognize AT-rich B-form dsDNA as well as GC-rich Z-form dsDNA (Yang et al. 2010). LRRFIP1 triggers the production of IFN- β in a β -catenin-dependent manner. β -Catenin binds to the C-terminal domain of IRF3, inducing an increase in IFN- β expression. Considering the large and heterogeneous group of proteins belonging to the family of PRRs, even more cytoplasmic DNA sensors are likely to be identified.

16.3 Phagocytosis: A Principal Innate Immune Response in MPS

Phagocytosis is the fundamental defense mechanism of innate immune system and is one of primary functions of mononuclear phagocytes. It was first described in the late nineteenth century by that mobile phagocytic cells survey tissues for foreign particles and engage in pitched battles with potential pathogens (Gordon 2008). This process can be classified as (1) phagocytosis of opsonized microbes and particles, mostly by antibodies and complements, (2) phagocytosis of non-opsonized molecules, mainly by scavenger receptors, and (3) phagocytosis of apoptotic cells (efferocytosis), mainly by phosphatidylserine receptors. Many of the receptors activate innate immune signalings important for the inflammatory regulation of immune cells. This part will briefly overview the representative molecules and their intracellular signaling to understand the basic of the molecular mechanism of phagocytosis.

16.3.1 Phagocytic Receptors for Microbe Uptake

Phagocytes express a broad spectrum of receptors that participate in particle recognition and internalization. These receptors include Fc-receptors, complement receptors,

Table 16.3 Adapted from Underhill and Ozinsky (2002)

Receptor class	Phagocytic receptors	Ligands
Fc-receptors	FcγRI (CD64)	Opsonized particles with IgG, CRP or SAP
	FcγRII (CD32)	
	FcγRIII (CD16)	
	FcεRI	Opsonized particles with IgE
	FcεRII (CD23)	
	FcαRI (CD89)	Opsonized particles with IgA
Complement receptors	CR1 (CD35)	Opsonized particles with MBL, C1q, C4b or C3b
	CR3 (αMβ2 integrin, CD11b/CD18, or Mac1)	Opsonized particles with iC3b
	CR4 (αXβ2 integrin, CD11c/CD18, or gp150/95)	
Integrin family	α5β1 (CD49e/CD29)	Opsonized particles with fibronectin or vitronectin
	α4β1 (CD49d/CD29)	
	αvβ3 (CD51/CD61)	
C-type lectin receptors	Mannose receptor 1 (CD206)	α-mannan
	Dectin-1	β-glucan
	Dectin-2	

integrin family, scavenger receptors, and mannose receptors (Table 16.3). Full descriptions of every receptor implicated in phagocytosis are beyond the scope of this review.

Fcγ-Receptors recognize Fc region of IgG, which opsonizes particle (Ravetch and Bolland 2001; Daeron 1997). FcγRs fall into two classes: (a) activating receptors that contain ITAM motifs in their intracellular domains that recruit kinases and activate phosphorylation cascades (FcγRI, FcγRIIA and FcγRIIIA), and (b) inhibitory receptors that contain ITIM motifs that recruit phosphatases that inhibit signaling (FcγRIIB) (Ravetch and Bolland 2001; Daeron 1997). Activating receptors bind IgG-opsonized particles and trigger particle engulfment (Aderem and Underhill 1999). Inhibitory receptors regulated this process by coligation that recruits the phosphatase SHIP that blocks phosphoinositide signaling (Ravetch and Bolland 2001). Thus, relative expression level of activating and inhibiting FcγRs can determine the threshold for phagocytosis and inflammatory responses to IgG-opsonized particles.

Complement receptors (CRs) recognize and internalize complement-opsonized particles. CRs include CR1, CR3 (αMβ2 integrin, CD11b/CD18, or Mac1), and CR4 (αXβ2 integrin, CD11c/CD18, or gp150/95) (Ross 2000). CR1 binds a broad spectrum of microbial opsonins including complement components C1q, C4b, and C3b, as well as mannan-binding lectin (MBL) (Ghiran et al. 2000; Klickstein et al. 1997). The integrins CR3 and CR4 are both heterodimers consisting of a shared beta chain (β2, CD18) paired with specific alpha chains, αM/CD11b, and αX/CD11c, respectively, and both receptors recognize iC3b. Both CR1 and CR3 require additional signals in order to mediate internalization of complementopsonized particles. Inflammatory cytokines, microbial products, and adhesion molecules stimulate phagocytosis through CR3, demonstrating the broad spectrum of extracellular molecules to influence phagocytosis (Wright and Griffin 1985; Pommier et al. 1983).

Integrin family recognizes fibronectin and vitronectin-opsonized molecules. These are primarily α5β1 and αvβ3 integrins (Blystone et al. 1994, 1999). Particle ingestion requires a second signal that can be provided by activation of protein kinase C (Blystone et al. 1994, 1999). α5β1 primarily mediates internalization. However, αvβ3 sometimes delivers an inhibitory signal that blocks internalization through α5β1 (Blystone et al. 1994, 1999). Inhibitory signaling may, in part, be due to the association of α5β1 with CD47 (also called integrin associated protein, IAP). Through its ligand SIRPα, CD47 inhibits both Fc- and complement receptor-mediated phagocytosis (Demeure et al. 2000; Oldenborg et al. 2001). Conversely, αvβ3 and another vitronectin-binding integrin, α5β1, can interact with CD36 (a class B scavenger receptor) to mediate phagocytosis of apoptotic cells, which is accompanied by anti-inflammatory signals (Albert et al. 1998; Fadok et al. 1998).

C-type lectin receptors (CTLRs) are expressed on phagocytes and mediate detection of self and foreign carbohydrates of microbes. Zymosan is a *Saccharomyces cerevisiae* yeast cell wall particle that is made up primarily of α-mannan and β-glucans (Di Carlo and Fiore 1958). The macrophage mannose receptor (CD206) binds to α-mannan and dectin-1 binds to β-glucan, which mediate phagocytosis of yeast and zymosan (Ezekowitz et al. 1990; Brown and Gordon 2001). The macrophage mannose receptor is a type I transmembrane protein with an extracellular domain consisting of eight C-type lectin carbohydrate recognition domains, a short amino terminal cysteine rich domain, and a fibronectin type II repeat (Stahl and Ezekowitz 1998; Fraser et al. 2000). There is no signaling motif in the short cytoplasmic tail and the exact signaling is yet to be determined. Dectin-1 was originally defined as a dendritic cell-specific receptor with a short extracellular domain consisting of a single C-type carbohydrate recognition domain and a short cytoplasmic tail containing an ITAM (Ariizumi et al. 2000a, b).

Subsequent analysis determined that dectin-1 is widely expressed on cells of myeloid lineage and may be the predominant β -glucan receptor for phagocytosis (Brown and Gordon 2001; Willment et al. 2001). Dectin-2 was recently isolated and classified as a different activation receptor via association with ITAM-bearing Fc γ R adaptor molecules (Dambuza and Brown 2015). Now dectin-1 comprise a clusters of many CTLRs: dectin-1 cluster (ITAM-1-containing Dectin-1, CLEC-2 and CLEC-9A, ITIM-1-containing MICL and MAH, and DDD/DDL motif-containing LOX-1 and CLEC-1) (Plato et al. 2013).

16.3.2 Scavenger Receptors for Exogenous and Endogenous Molecule Uptake

Scavenger receptors (SRs) comprise a diverse array of integral membrane proteins and soluble secreted extracellular domain isoforms. The first scavenger receptor was identified for the internalization of oxidized low-density lipoprotein into macrophages by Brown and Goldstein in the 1970s.

We have termed these proteins as belonging to the ‘SR super-group’ (Table 16.4), which is standardized according to the recent review by Prabhudas et al. (2014). Based on our current understanding of SR structure and biological function, these proteins were subgrouped into Classes A–J (Zani et al. 2015).

Class A proteins are Type II membrane proteins with an N-terminus comprising a short cytoplasmic domain followed by a single transmembrane region and a large extracellular domain that has a collagen-like domain and collagen-binding activity. Members include SR-A1, SR-A3, SR-A4, SR-A5 and SR-A6. Class A proteins form homotrimers at the cell surface (Gowen et al. 2001). SR-A1 binds to oxidized LDL, and activates JNK and p38 MAPK, leading to apoptosis or foam cell formation (Ricci et al. 2004).

Class B proteins comprise of SR-B1, SR-B2 and SR-B3. These molecules have two transmembrane regions located close to the N- and C-termini which straddle a central domain that is glycosylated and mediates ligand recognition. SR-B1 is originally cloned as HDL receptor, and SR-B2 binds to oxidized LDL, A β , and trigger immune signaling via Fyn/JNK/p38 MAPK for apoptosis, angiogenesis, and foam cell

Table 16.4 Adapted from Prabhudas et al. (2014)

Class	Receptors	Alternative protein names	Gene names
Class A	SR-A1	SR-A1, CD204, SCARA1, SR-AIII, MSR1	MSR1
	SR-A3	MSRL1, SCARA3	SCARA3
	SR-A4	SCARA4, SRCL, CL-P1, COLEC12	COLEC12
	SR-A5	TESR, NET33, SCARA5	SCARA5
	SR-A6	MARCO, SCARA2	MARCO
Class B	SR-B1	SR-BI, CD36L1, SCARB1	SCARB1
	SR-B1.1	LIMP2, CD36L2, LGP85, SCARB2	SCARB2
	SR-B2	CD36, SCARB3, FAT, GPIV, PAS	CD36
Class C	dSR-C1	dSR-C1	Sr-CI
Class D	SR-D1	CD68, gp110, SCARD1, LAMP4	CD68
Class E	SR-E1	LOX-1, SCARE1, CLEC8A, OLR1	OLR1
	SR-E2	Dectin 1, CLEC7A	Dectin1
Class F	SR-F1	SREC-1, SCARF1	SCARF1
	SR-F2	SREC-II, SR-F2, SCARF2	SCARF2
	SR-F3	MEGF10, EMARDD	MEGF1
Class G	SR-G	CXCL16, SR-PSOX	CXCL16
Class H	SR-H1	STAB1, FEEL-1	STAB1
	SR-H2	STAB2, FEEL-2	STAB2
Class I	SR-I1	CD163, M130	CD163
	SR-I2	CD163L1, M160, CD163B	CD163L1
Class J	SR-J1	RAGE, AGER (membrane form)	RAGE
	SR-J1.1	RAGE, AGER (secreted form)	RAGE
Unclassified	TBA	CD14	CD14
	TBA	Ly75, CD205	CD205
	TBA	MRC1, CD206	CD206
	TBA	Langerin, CD207	CD207
	TBA	DC-SIGN, CD209, CLEC4L	CD209/ DC-SIGN
	TBA	S4D-SRCRB	SRCRB4D
	TBA	unknown	SSC5D

formation, and Lyn/FAK/Vav for cell adhesion and migration (Silverstein et al. 2010; Liani et al. 2012).

Class C proteins are specific to insects, not present in mammals, and are involved in the innate immune response against pathogens such as bacteria (Ramet et al. 2001).

The **Class D** protein SR-D1 (CD68) is a Type I membrane protein and is heavily glycosylated. SR-D1 contains an N-proximal mucin-like domain, a proline-rich hinge region followed by a lysosome-associated membrane protein (LAMP) homology domain, a single transmembrane region and a short 12-residue cytoplasmic domain (Holness and Simmons 1993). SR-D1 can bind to oxidized LDL, lectins, selectins and also mediate phagocytosis and bone resorption (Ramprasad et al. 1996; da Silva and Gordon 1999).

Class E proteins are comprised of SR-E1 and SR-E2. SR-E1 is also known as lectin-like oxidized low-density lipoprotein receptor (LOX-1/OLR1). SR-E1 binds to oxidized LDL (oxLDL) and stimulates activation of p42/44 MAPK, p38MAPK and NF- κ B (Biocca et al. 2009; Khaidakov et al. 2011), triggering a key feature of pro-inflammatory response in immune and vascular cells. SR-E2 is dectin-1, which is discussed above as a C-type lectin receptor.

Class F proteins include SR-F1 (SREC1), SR-F2 (SREC2) and SR-F3 (MEGF10/EMARDD). Human SR-F1 and SR-F2 are Type 1 membrane proteins, an extracellular domain containing multiple EGF-like repeats, a single transmembrane region and a relatively large cytoplasmic domain. SR-F1 binds to carbamylated LDL (cLDL), AcLDL or OxLDL particles. However, SR-F2 lacks SR activity and suggested to suppresses the ligand-binding properties of SR-F1. SR-F1 not only recognizes a wide variety of modified lipid particles but also heat-shock protein HSP90 complexes (Murshid et al. 2010; Sano et al. 2012). SR-F3 is an ortholog of *Drosophila* Draper and *C. elegans* CED-1, and involved in the phagocytosis of apoptotic cells (Ziegenfuss et al. 2008; MacDonald et al. 2006; Zhou et al. 2001). This molecule will be described in more detail in the following section 16.3.3.

The **Class G** protein SR-G is also called chemokine 16 (CXCL16) or scavenger receptor for phosphatidylserine and oxidized lipoprotein (SR-PSOX). The SR-G extracellular domain mediates endocytosis of phosphatidylserine or OxLDL, and delivery to endosome-lysosome system. SR-G has important innate immunity functionality through recognition of bacteria and CpG-rich DNA found in other pathogens (Gursel et al. 2006; Sheikine and Sirsjo 2008). This molecule may play a vital role not only in recruiting but also promoting interaction of both T and natural killer cells to dendritic cells (Shimaoka et al. 2004).

Class H proteins include SR-H1 (stabilin 1/STAB1) and SR-H2 (stabilin 2/STAB2). The SR-H1 and SR-H2 membrane proteins are Fasciclin, EGF-like, laminin-type EGF-like and link (FEEL) domain-containing scavenger receptors. SR-H1 and SR-H2 bind to AcLDL, advanced glycation end-products (AGE) and bacteria. Macrophage SR-H1 mediates

recognition of matricellular secreted protein acidic and rich in cysteine (SPARC), a potent angiogenesis inhibitor, to promote endocytosis and lysosomal degradation (Kzhyshkowska et al. 2006). SR-H1 can also promote the clearance of apoptotic and necrotic bodies (Park et al. 2010; Kim et al. 2012). Macrophages also express SR-H2 to promote phagocytosis and clearance of aged cells, apoptotic bodies and heparin-linked proteins. These are also listed as phosphatidylserine receptors in the following section.

Class I proteins comprise of SR-I1 (CD163) and SR-I2 (CD163L). These receptors contains type B scavenger receptor cysteine-rich (SRCR) domain. SR-I1 is a single transmembrane Type 1 membrane glycoprotein and is primarily expressed in monocytes and macrophages. SR-I1 helps the removal of haptoglobin-hemoglobin (Hp-Hb) complexes via the heme oxygenase-1 (HO-1) pathway to reduce pro-inflammatory haem in the circulation (Thomsen et al. 2013). This indicates the role of SR-I1 in anti-inflammatory response by mediating the uptake of toxic haem in macrophages (Kristiansen et al. 2001). SR-I1 also has a role as a PAMP receptor: SR-I1 binds to both Gram⁻ and Gram⁺ bacteria (Fabriek et al. 2007). The expression of SR-I2 is uniquely restricted to tissue-resident macrophages with an anti-inflammatory or anergic phenotype (Gonzalez-Dominguez et al. 2015).

The **Class J** proteins SR-J1 and SR-J1.1 are membrane-bound or secreted form of the receptor for advanced glycation end-products (RAGE). SR-J1 is a 32 kDa multi-ligand transmembrane receptor that belongs to the immunoglobulin gene superfamily. SR-J1 is a PRP: SR-J1 can interact and be activated by a number of DAMPs such as A β (Yan et al. 1996), S100/calgranulin (Hofmann et al. 1999), phosphatidylserine (He et al. 2011) and HMGB1 (amphoterin) (Hori et al. 1995). Under physiological conditions, the expression of SR-J1 is low, but can be aggravated in response to chronic conditions. These pro-inflammatory endogenous molecules are involved in inflammation and physiological stress. AGE-bound SR-J1 is implicated in signal transduction mediating processes such as oxidative stress, apoptosis and inflammation (Xie et al. 2013).

Other unclassified proteins include CD14 (a co-receptor of TLR4 complex), CD205 (lymphocyte antigen), CD206 (macrophage mannose receptor), CD207 (Langerhans cell marker), CD209 (DC-SIGN), and S4D-SRCRB (scavenger receptor cysteine-rich superfamily, Group B 4 Domains) (Table 16.4).

16.3.3 Phosphatidylserine Receptors for the Phagocytosis of Apoptotic Cells

Apoptotic cells expose 'eat-me' signals so that phagocytes can recognize and distinguish them from their healthy counterparts. The most classic 'eat-me' signal is phosphatidylserine (PtdSer). PtdSer is normally sequestered on the inner

Table 16.5 Adapted from Penberthy and Ravichandran (2016)

Names	PtdSer recognition	Signaling pathway	Other ligand	SR classification
BAI1	Direct	ELMO-Dock180-Rac1	LPS	
TIM-1/HAVCR1/KIM1	Direct	p85/LC3	LDL, oxLDL, HAV	Proposed as Class J
TIM-4/SMUCKLER		Integrin signaling		
Tyro3	Via Gas6/Protein S	TK domain		
Axl	Via Gas6	TK domain		
MerTK	Via Gas6, Protein S, Tubby and Tubby-like protein	Integrin signaling		
STAB1/SR-H1	Direct	GULP/Rac1	oxLDL, AGE, Gram+ and Gram- bacteria	Class H
STAB2/SR-H2		Integrin/FAK/ELMO-Dock180-Rac1		
$\alpha\text{v}\beta 3$	Via MEGE8	CrkII/Dock180/Rac1, FAK/ELMO-Dock180-Rac1		
$\alpha\text{v}\beta 5$				
SREC-1/SR-F1	Via C1q		AcLDL, calreticulin	Class F
LRP1/CD91	CED-1 binds via TTR-52	GULP/Rac1	>40, such as HDL and HSPs	
MEGF10/SR-F3			A β	Class F

leaflet of the plasma membrane, and during apoptosis PtdSer is exposed on the outer leaflet of the plasma membrane, where it can be directly or indirectly detected by numerous PtdSer receptors on phagocytes. Ligation of PtdSer receptors on phagocytes triggers phagocytic cup formation, which then facilitates the engulfment of the dying cell. PtdSer receptors come in a variety of different molecular formats, with the only commonality being their ability to directly or indirectly recognize PtdSer. It should be noted that majority of PtdSer receptors recognize multiple ligands and play roles other than apoptotic cell clearance (Table 16.5). Several PtdSer receptors have already been categorized as scavenger receptors (Table 16.4). We will mainly discuss Brain angiogenesis inhibitor 1 (BAI1), T-cell immunoglobulin and mucin receptor 1 (TIM-1)/hepatitis C virus receptor (HAVCR1)/kidney injury molecule 1 (KIM1), TIM-4/spleen, mucin-containing, knockout of lymphotoxin (SMUCKLER), tyrosine-protein kinase receptor Tyro3, Tyrosine-protein kinase receptor UFO (Axl), Tyrosine-protein kinase Mer (MerTK), STAB2/SR-H2, multiple epidermal growth factor-like domains protein 10 (MEGF10)/SR-F3 and $\alpha\text{v}\beta 5$ integrins.

BAI1 is a type II adhesion G-protein coupled receptor (GPCR) family Das et al. (2011). Human BAI1 is a 1584 amino acid protein with a large extracellular region, and a seven transmembrane region, followed by a long intracellular tail. The cytoplasmic tail of BAI1 can directly interact with engulfment and cell motility protein (ELMO). ELMO form a complex with dedicator of cytokinesis protein 1 (Dock1/Dock180), a guanine nucleotide exchange factor (GEF), which activates Rac Rho small GTPases by exchanging bound GDP for free GTP. Activation of Rac leads to cytoskeletal reorganization and importation of phagosomes. BAI1 is essential for the engulfment of apoptotic cells Park et al. (2007). BAI1 also recognizes LPS Das (2011).

TIM-1/HAVCR1/KIM1 is a prototypical member of a novel class of scavenger receptors. It directly binds to PtdSer, oxLDL, and hepatitis A virus (HAV) Ichimura (2008); Kim et al. (2011); Feigelsstock et al. (1998); Curtiss (2011). Recent publication shows that binding to PtdSer to TIM-1 induces tyrosine phosphorylation of the cytoplasmic tail and its association with p85, which results in encapsulation of phagosomes by lipidated LC3 in multi-membrane organelles (Brooks et al. 2015).

TIM-4/SMUCKLER is structurally similar to TIM-1 and can directly bind to PtdSer Miyashita et al. 2007. TIM-4 uses integrin $\beta 1$ as a co-receptor in the process of apoptotic cell clearance Flannagan et al. 2014 and contributes to phagosome stabilization Mazaheri F et al. (2014).

Tyro3 is one of three TAM receptors (Tyro3, Axl, and MerTK) and has tyrosine kinase domain in the cytoplasmic tail Seitz et al. (2007). Tyro3 indirectly bind to PtdSer via Gas6 or protein S Lew et al. (2014); Lu et al. (1999), and signals through ELMO/Dock180/Rac1 pathway.

Axl also belongs to TAM receptor family and indirectly bind to PtdSer via Gas6 Abu-Thuraia et al. (2015); Seitz et al. (2007). ELMO2 is essential for Axl signaling Abu-Thuraia et al. (2015).

MerTK is another TAM receptor and binds to PtdSer via bridging molecules including Gas6, Protein S, Tubby, and Tubby-like protein 1 Wu et al. (2005); Caberoy et al. (2010); Burstyn-Cohen et al. (2012); Lew et al. (2014). Bridging elicits homo-dimerization of Mer and trans-phosphorylation of the cytoplasmic tyrosine kinase domain Tibrewal et al. (2008). This subsequently drives phosphorylation of phospholipase $\text{C}\gamma 2$ (PLC $\gamma 2$) Todt et al. (2004). PLC $\gamma 2$ can then recruit p130CAS to $\beta 5$ integrin and subsequently activate the CrkII-ELMO-Dock180 complex Wu et al. (2005).

STAB2/SR-H2 possesses multiple EGF-like repeat domains in its extracellular region that mediate direct

interaction with PtdSer Park et al. (2008a). Upon binding to PtdSer, STAB2/SR-H2 has been reported to promote corpse internalization via two independent, but parallel pathways. In the first signaling pathway, STAB2/SR-H2 binds to the PTB domain of the adapter protein GULP via an NPXY motif Park et al. (2008b). The *C. elegans* ortholog of GULP, CED-6, activates the Rac1 ortholog, CED-10 Kinchen et al. (2005). GULP can activate Rac1 as downstream signaling of SR-B1 Osada et al. (2009), it has not been shown in STAB2/SR-H2 signaling. Alternatively, STAB2/SR-H2 interacts with the $\alpha\beta 5$ integrin in the process of erythrocyte engulfment Kim et al. (2012). As discussed below, $\alpha\beta 5$ integrin can signal via the ELMO/Dock180 complex to activate Rac1, suggesting that STAB2/SR-H2 might utilize this downstream signaling module via cooperation with $\alpha\beta 5$ integrin Albert et al. (2000); Akakura et al. (2004).

MEGF10/SR-F3 and **LRP-1** are structurally similar to the *C. elegans* membrane receptor CED-1 Su et al. (2002); Suzuki et al. (2007). CED-1 clusters around apoptotic cells on the phagocyte membrane and is crucial for the clearance of apoptotic cells Ellis et al. (1991); Hedgecock et al. (1983); Zhou et al. (2001). CED-1 functions upstream of CED-6, the *C. elegans* ortholog of GULP Ellis et al. (1991); Su et al. (2002). Both MEGF10/SR-F3 and LRP-1 are capable of signaling via GULP, suggesting that this apoptotic cell recognition and signaling mechanism is evolutionarily conserved and utilized by a multitude of PtdSer receptors Su et al. (2002); Scheib et al. (2012).

$\alpha\beta 5$ integrin indirectly recognizes PtdSer through the soluble bridge, milk fat globule-EGF factor 8 (MFG-E8) or lactadherin Shi et al. (2004); Hanayama et al. (2002); Andersen et al. (1997); Andersen et al. (2000). Interestingly, $\alpha\beta 5$ integrin activates ELMO/Dock180 complex to elicit Rac activation and apoptotic cell engulfment Albert et al. (2000); Akakura et al. (2004). FAK is phosphorylated and recruits p130CAS that binds to CrkII Albert et al. (2000). CrkII then recruits Dock180 and triggers Rac1 activation Albert et al. (2000); Akakura et al. (2004). Overall, these molecules mediate phagocytic cytoskeletal changes via converging intracellular signaling.

16.3.4 TREM2/TYROBP Pathway for Sensing Apoptotic Cells and Diseases

Triggering receptor expressed on myeloid cells 2 (TREM2) belongs to the immunoglobulin superfamily based on shared structural features with immunoglobulin domain or fold. Members of the immunoglobulin superfamily include cell surface antigen receptors, co-receptors and co-stimulatory molecules of the immune system, molecules involved in antigen presentation to lymphocytes, cell adhesion mole-

cules, and certain cytokine receptors. TREM2 is thought to be involved in phagocytosis of damaged cells (Hsieh et al. 2009; Kawabori et al. 2015).

TREM2 is mostly expressed in myeloid cells (immature dendritic cells, microglia and osteoclasts). In the CNS, TREM2 is expressed at levels greater than 300-fold in microglia compared to astrocytes (Hickman and El Khoury 2014). Inflammation decreases TREM2 expression in microglia and their ability to phagocytize apoptotic neurons (Takahashi et al. 2007). TREM2 requires the interaction with the adaptor protein, TYRO protein tyrosine kinase binding protein (TYROBP; formerly DAP12) that facilitates the downstream signaling of phagocytosis (Paloneva et al. 2000). Thus, healthy coupling of TREM2 and TYROBP is essential for the phagocytic function of myeloid cells. No endogenous ligand has been confirmed for TREM2, but TREM2 is shown to bind to Gram⁺ and Gram⁻ bacteria and yeast in a charge-based method (Daws et al. 2003). Stimulation of TREM2 by a cross-linked TREM2 antibody is shown to activate TREM2. This activation causes actin polymerization and restructuring of the cytoskeleton (Takahashi et al. 2005).

TYROBP is a transmembrane adaptor protein that binds to TREM2 and signals through immunoreceptor tyrosine-based activation motifs (ITAMs). The ITAM signal is important for phagocytosis activation in microglia (Linnartz and Neumann 2013). Tyrosine phosphorylation of ITAM by Src family proteins activates TYROBP. The phosphorylated ITAM binds to protein spleen tyrosine kinase (Syk). Syk activates many downstream cascades linked to phagocytosis (Linnartz and Neumann 2013). The ITAM activation is negatively regulated by the immunoreceptor tyrosine-based inhibition motifs (ITIMs). When a regulatory ligand binds to receptor containing an ITIM, recruitment of SHP1/SHP2 occurs. SHP1/SHP2 can dephosphorylate and inhibit ITAM activation and other downstream cascades (Linnartz and Neumann 2013). TYROBP deficient mice show enhanced inflammatory phenotypes (Hamerman et al. 2005).

Mutations in TREM2 or TYROBP are thought to be the main cause of Nasu-Hakola (Paloneva et al. 2003). Nasu-Hakola diseases is polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOS), a rare autosomal recessive disease (Paloneva et al. 2003). Patients who are diagnosed with Nasu-Hakola disease suffer from bone cyst-like lesions and bone fractures often on their lower legs and also experience rapid pre-senile dementia (Paloneva et al. 2001, 2003). Around the age of 40, patients have severe neurodegeneration and suffer from severe dementia, gait disturbances, sensory agnosia, agraphia and in some cases develop tumors in the CNS (Hakola and Puranen 1993). The link between neurodegeneration and TREM2/TYROBP function provided by Nasu-Hakola disease indicates that the phagocytic and anti-inflammatory function of TREM2/TYROBP signaling is important for healthy brain function.

The missense R47H mutation in TREM2 is associated with LOAD, has an effect on both folding and ligand binding, and decreases the clearance of damaging debris (Abduljaleel et al. 2014). Similarly, FTDL-linked missense mutations in TREM2 (T66M and Y38C) also reduce phagocytic activity (Kleinberger et al. 2014). TYROBP is recently highlighted as one of the central genetic nodes and networks in LOAD (Paloneva et al. 2000, 2001; Zhang et al. 2013). Since both TREM2 and TYROBP are specifically expressed in myeloid cells and physically interact to transduce phagocytosis and anti-inflammatory signaling (Linnartz and Neumann 2013; N'Diaye et al. 2009; Paradowska-Gorycka and Jurkowska 2013), the TREM2/TYROBP pathway may play an important role for regulating peripheral or central inflammation.

16.4 Review Questions

1. Describe the difference in pattern recognition among Toll-like receptors (TLR) 2, TLR4 and TLR in terms of (1) ligand distinction, (2) intracellular signaling, and (3) co-activation signaling
2. Describe the representative danger-associated molecular pattern molecules (DAMPs) from nucleus, cytosol, and mitochondria in terms of (1) exact DAMPs, (2) corresponding receptors, and (3) co-activation signaling
3. Describe subclass of phagocytic receptors for microbes in terms of (1) receptor class, (2) representative receptors, (3) ligands
4. Describe the representative class of scavenger receptors for non-opsonized molecules in terms of (1) scavenger receptor classes, (2) representative receptors, (3) ligands
5. Describe the representative phosphatidylserine (PtdSer) receptors and their intracellular signaling

16.5 Answers

1. Listed in Table 16.1
2. Listed in Table 16.2
3. Listed in Table 16.3
4. Listed in Table 16.4
5. BAI1 is a type II adhesion G-protein coupled receptor (GPCR) family. The cytoplasmic tail of BAI1 can directly interact with engulfment and cell motility protein (ELMO). ELMO form a complex with dedicator of cytokinesis protein 1 (Dock1/Dock180), a guanine nucleotide exchange factor (GEF), which activates Rac Rho small GTPases by exchanging bound GDP for free GTP. Activation of Rac leads to cytoskeletal reorganization and importation of phagosomes. BAI1 is essential for the engulfment of apoptotic cells.

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Abstract

The role of cytokines and chemokines in neuroimmune regulation is highly broad and complex, due to the overwhelming number of molecules and their overlapping, synergizing and antagonizing effects of various factors. Classifying any individual factor or family of factors as beneficial or detrimental oversimplifies the interactions between various cell types and the signaling cascades initiated by them. Instead, cytokines need to be considered as a balanced network, where subtle modifications can shift cells towards different outcomes such as death, proliferation, migration, and induction of inflammation or inhibition of immune responses. Prolonged inflammation has a profound impact on the cytokine network and the cells they target, consequently altering the outcome of cell populations. The central nervous system is a unique environment that is exquisitely sensitive to cytokines, growth factors, and chemokines; the dysregulation that is rampant during neurodegenerative diseases permanently transforms brain function. The profile of cytokines, growth factors, and chemokines presented in the brain likely dictate the fate of neurons in the diseases. The understanding of cytokines, growth factor, and chemokine effects and how the expression or activity of those factors can be manipulated may provide the key to diagnosing or treating neuroimmunological diseases.

Keywords

Alzheimer's disease • Chemokine • Chemotaxis cytokine • Growth factor • Inflammation • Multiple sclerosis

17.1 Introduction

Communication between cells takes place either via direct cell-to-cell contact, extracellular vesicles, or soluble factors such as cytokines. Originally thought to be limited to the immune cells, cytokines are soluble protein messengers now known to be produced by a broad range of cells beyond the immune system, regulating key aspects of development,

homeostasis, inflammation, response to infection, and tumor. Among various families of cytokines, chemokines are a unique family in that all family members are primarily involved in cellular migration. Cytokines and chemokines are particularly relevant to neuroimmunology due to their critical role in communication between immune system and central nervous system (CNS). Aberrant regulation of cytokines and chemokines may facilitate the development of several CNS diseases. For example, HIV-1 associated neurocognitive disorders (HAND), Alzheimer's disease (AD), and multiple sclerosis (MS) share certain characteristics in their pathogenesis that include the production of high levels of proinflammatory cytokines. The elevation of proinflammatory cytokines within the CNS is commonly regarded

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as a perpetrator to the eventual impairment of neuronal function. Therefore, cytokines and chemokines are the subject of intense investigations for pharmacological intervention. This chapter provides an overview of cytokines, chemokines, and their roles in various pathological conditions. Understanding the involvement of chemokines and cytokines in the pathogenesis of CNS diseases may help identify novel therapeutic targets for the treatment of neurodegenerative disorders and neuronal injury.

17.1.1 Classification of Cytokines and Chemokines

Most cytokines are single polypeptide chains of various sizes, although they may form multimers in biological fluids. To learn about this exceedingly large group of proteins, it is helpful to classify them into families. The classification of cytokines and chemokines is typically based on the structure homology of their receptors (Vilcek 2003). It is important to note that while many of them share structural similarities or have functional homologues; most generalizations are riddled with exceptions. Similarly, the term “growth factors”, used in this chapter, also refers to a broad range of structurally diverse molecules. However, there are clearly some overlaps in term of terminology between cytokines and growth factors. These growth factors are introduced in individual cytokine families in this chapter.

17.1.2 Cytokine Families

When grouped into families based upon the structural homology, most cytokines and their receptors fall into the same family with few exceptions. Below is an effort to group cytokines and their families based on the structural homology of their receptors. This classification helps provide a foundation for thinking about these factors and their role in the brain.

17.1.2.1 Type I Cytokine Receptor Family

Also known as hemopoietic growth factor family, Type I cytokine family share a characteristic three-dimensional motif consisting of an extracellular region containing four α helices. Members of this family include receptors to interleukin-2 (IL-2), IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-12, granulocyte-colony stimulating factor (G-CSF) and granulocyte macrophage-colony stimulating factor (GM-CSF). Subfamilies of Type I cytokine receptor family exist. For example, IL-2 receptor subfamily includes IL-2, IL-7, IL-9, IL-15, and IL-21 receptors; IL-4 receptor subfamily includes IL-3, IL-4, IL-5, IL-13, and GM-CSF receptors; IL-12 receptor subfamily includes IL-12, IL-23, and IL-27 receptors. Among them IL-2, IL-3, IL-4 and IL-7 are T cell growth

factors; IL-6 and IL-12 are pro-inflammatory cytokines; and G-CSF and GM-CSF are important for survival and differentiation of hematopoietic lineages. It is important to note that Type I cytokine receptors are not to be confused with Th1 cytokine receptors. Classification of Type I cytokine receptors is based on structural biology, whereas classification of Th1 cytokine receptors is based on function. Type I cytokine receptors include both Th1 cytokine receptor (IL-2) and Th2 cytokine receptors (IL-4 and IL-5). In addition, members from the same subfamily do not necessarily exert similar biological effects.

17.1.2.2 Type II Cytokine Receptor Family

Also known as interferon family, Type II cytokine receptor family includes receptors to IL-10, IL-19, IL-20, IL-22, and interferons (IFN- α , - β , - ϵ , - κ , - ω , - δ , - τ and - γ). Subfamilies of Type II cytokine receptor family include IL-10 receptor subfamily, which comprises IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28A, IL-28B, and IL-29 receptors (Sabat 2010); IFN receptor subfamily, which comprises IFN- α/β receptor and IFN- γ receptor. Functions of this group include induction of cellular antiviral states, modulation of inflammatory responses, inhibition or stimulation of cell growth and production or inhibition of apoptosis, as well as affecting many immune mechanisms (Pestka et al. 2004; Renauld 2003). Notably, IL-10 is a potent anti-inflammatory, immunomodulatory cytokine. The IFN system is an important contributor to innate immunity with IFN- γ serving as a potent pro-inflammatory cytokine. The type II cytokine receptor family is also comprised of both Th1 and Th2 cytokine receptors; IFN- γ receptor is a Th1 cytokine receptor while IL-10 receptor is a Th2 cytokine receptor.

17.1.2.3 TNF Receptor Superfamily (TNFRSF)

TNF receptor subfamily is comprised of at least 29 type II transmembrane proteins that have partial homology in their extracellular domains. The ligands for these receptors include 19 members of TNF superfamily. Active TNF receptors typically form trimeric complexes on the plasma membrane. Members of this superfamily include TNF receptor 1 (TNFRSF1A), TNF receptor 2 (TNFRSF1B), Fas receptor (FasR, TNFRSF6), CD40 (CD40, TNFRSF5), and TNF-related apoptosis-inducing ligand receptors (TRAILRs, TNFRSF10A-D). Members of the TNF receptor superfamily are known for their apoptosis-inducing ability, though they have additional biological effects. TNF- α is an inflammatory Th1 cytokine released during infection; CD40 ligand (CD40L) is a strong activator of macrophages; FasL and TRAIL modulate immune responses and induce apoptosis in immune cells and tumor cells. In addition, TNF receptor superfamily also has been suggested to be involved in destructive effects toward tissue in many disease settings.

17.1.2.4 IL-1 Receptor Family

IL-1 receptor (IL-1R) family comprises ten members: IL-1R1, IL-1R2, IL-1R accessory protein (IL-1RAcP), IL-18R α , IL-18R β , IL-33R, IL-36R, single Ig IL-1R-related molecule (SIGIRR), three Ig domain-containing IL-1R related-2 (TIGGIR-2), and TIGGIR-1 (Palomo et al. 2015). Members of IL-1R family typically comprise three extracellular immunoglobulin domains. Therefore, the IL-1 receptor family belongs to the immunoglobulin superfamily. IL-1 β and IL-18, two prominent members of this cytokine family, are produced as biologically inactive pro-peptides that need to be cleaved by caspase-1 upon inflammasome activation in order to bind to their receptors. These IL-1 receptor ligands are potent pro-inflammatory cytokines that induce genes associated with inflammation and autoimmune disease. IL-1Ra, on the other hand, is the specific receptor antagonist for IL-1 α and IL-1 β but not for IL-18.

17.1.2.5 Transforming Growth Factor Beta (TGF- β) Receptor Superfamily

Based on structural and functional properties, TGF- β receptor family can be further divided into type I and type II receptor subfamilies (Herpin et al. 2004). In vertebrates, there are five type II receptors and seven type I receptors in the TGF- β receptor superfamily. However these receptors are paired up with the TGF- β superfamily members, including TGF- β , Activins, Nodals, Bone Morphogenetic Proteins (BMPs), and Growth and Differentiation Factors (GDFs) of a total of at least 30 secreted factors, remain unclear (Weiss and Attisano 2013). The receptors of TGF- β family have characteristic cysteine-rich extracellular domains and either a GlySer (GS) domain or a serine/threonine kinase domain. TGF- β has numerous functions including regulation of embryonic stem cell self-renewal, gastrulation, differentiation, organ morphogenesis, and adult tissue homeostasis. In immune system, TGF- β regulates both anti-inflammatory and proinflammatory T cell responses with the presence of other cytokines (Travis and Sheppard 2014).

17.1.3 Important Sub-families of Cytokines and Growth Factors

The five cytokines families based on their receptor structural homology are a broad term. Smaller groups of cytokines and growth factors may form more function-related sub-families and are discussed below.

17.1.3.1 IL-17 Family

IL-17 cytokine family includes IL-17A (previously referred to as IL-17), IL-17B, IL-17C, IL-17D, IL-17E (also known as IL-25) and IL-17F. All IL-17 family members bind to their receptors, which comprises five receptor subunits from

IL-17RA to IL17RE. These cytokines and their receptors have little homology to other cytokine and receptor families and therefore form a distinct group. The biological function and regulation of IL-17A, IL-17B, IL-17C, IL-17D, and IL-17F are best understood for their pro-inflammatory responses, whereas IL-17E plays distinct roles in immunity by regulating the Th2 response (Gaffen et al. 2014; Jin and Dong 2013).

17.1.3.2 Neurotrophin Family

The neurotrophin family includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), NT-4. All neurotrophins are neurotrophic factors capable of binding to a receptor called p75NTR (also known as TNFRSF16) and to one of the three tropomyosin-related kinase (TRK; also known as NTRK) receptors - NGF binds to TRKA, BDNF and NT4 bind to TRKB, and NT3 binds to TRKC (Park and Poo 2013). Neurotrophins secreted by cells protect neurons from apoptosis and are also important for differentiation and maintenance of nerve cells (Korsching 1993; Lewin and Barde 1996).

17.1.3.3 Growth Factor Families

Growth factor families include many each with unique functions. Members of vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) families are potent mitogenic and angiogenic factors with critical roles in embryonic development, wound healing, as well as the integrity of blood brain barrier (BBB). Epidermal growth factor (EGF) family members include EGF, neuregulins, amphiregulin, and betacellulin. These EGF family members mediate their growth and proliferate effects on cells of both mesodermal and ectodermal origin. Insulin-like growth factor (IGF)-I and IGF-II belong to the family of insulin-like growth factors that are structurally homologous to proinsulin. IGF-1 has a much higher growth-promoting activity than insulin and is highly expressed in all cell types and tissues. IGF-2 is expressed in embryonic tissues and is related to development. Fibroblast growth factors (FGF) constitute a large family of proteins involved in many aspects of development including cell proliferation, growth, and differentiation. They act on several cell types to regulate angiogenesis, cell growth, pattern formation, and embryonic development. Notably, EGF and basic FGF are commonly used for the culture of neural stem cells.

17.1.4 Structure and Classification of Chemokines and Their Receptors

First discovered in 1987 (Walz et al. 1987; Yoshimura et al. 1987), chemokines include a group of secreted proteins within the family of cytokines that by definition relate to the

induction of migration. These “**chemotactic cytokines**” are produced by and target a wide variety of cells, but primarily address leukocyte chemoattraction and trafficking of immune cells to locations throughout the body via a concentration gradient. There are two general categories of biological activity for chemokines, the maintenance of homeostasis and the induction of inflammation (Moser and Loetscher 2001; Balkwill 2012). Homeostatic chemokines are involved in roles such as immune surveillance and navigation of cells through hematopoiesis, and are typically expressed constitutively. Inflammatory chemokines are produced in states of infection or following an inflammatory stimulus. By targeting cells of the innate and adaptive immune system, inflammatory chemokines facilitate an immune response.

Chemokine activity is mediated by activating a family of seven transmembrane G-protein coupled receptors (GPCR). GPCRs interact with and signal through heterotrimeric guanine-nucleotide-binding regulatory proteins (G-proteins). Upon stimulation by a ligand, GPCRs undergo a conformational change that leads to activation of the G-protein by GDP-GTP exchange, followed by uncoupling of the G-protein from the receptor. Upon activation, G-proteins trigger a cascade of signaling events that regulate various cellular functions (Devi 2000).

Chemokine receptors are classified into four families based on the number and position of the N-terminal-conserved cysteine residues within the receptor-binding domain. These families include: α -chemokine receptors (such as CXCR2 and CXCR4); β -chemokine receptors (such as CCR5, CCR4, CCR3 and CCR2); γ -chemokine receptor (XCR1) and δ -chemokine receptors (CX3CR1) (Gabuzda et al. 1998; Hesselgesser and Horuk 1999; Klein et al. 1999; Miller and Meucci 1999; van der Meer et al. 2000; Cotter et al. 2002). Additionally, receptors that structurally resemble chemokine receptors but lack classical chemokine receptor signaling include Duffy antigen receptor for chemokines (DARC), D6 (also known as CCBP2), CC-chemokine receptor-like 1 (CCRL1; also known as CCXCKR and CCR11) and CXC-chemokine receptor 7 (CXCR7; also known as RDC1) (Nibbs and Graham 2013). These receptors are now grouped as the family of atypical chemokine receptors (ACKRs). DARC is known as ACKR1, D6 is known as ACKR2, CXCR7 is known as ACKR3 and CCRL1 is known as ACKR4 (Bachelier et al. 2014).

17.1.4.1 CXC Chemokines

These include CXCL1 to CXCL17 and CXC chemokine receptors include CXCR1 to CXCR7. CXC chemokines are further separated into two groups based upon the presence or absence of a specific three amino acid sequence found adjacent to the CXC. The Glu-Leu-Arg residues constitute the ELR motif, and if present, the CXC chemokine is considered to be ELR(+). The general function of ELR(+) chemokines

revolves around neutrophils inducing chemotaxis and promoting angiogenesis (Strieter et al. 1995). Chemokines in this group include CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8, and CXCL15; unlike ELR(-) members, these factors interact primarily with neutrophils via CXCR1 and CXCR2 receptors. In contrast, ELR(-) chemokines attract lymphocytes and monocytes with little affinity for neutrophils. This subgroup including CXCL4, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, CXCL14 and CXCL16, has a wider variety of activities, but generally have angiostatic properties and induce chemotaxis in mononuclear cells.

17.1.4.2 CC Chemokines

These include CCL1 to CCL28, CCL3L1 and CCL4L1, whereas CC chemokine receptors include CCR1 to CCR10. CC chemokines target primarily mononuclear cells and serve in both homeostatic and inflammatory capacities. The family can be divided into five functional groups including allergenic, pro-inflammatory, HCC, developmental and homeostatic groups. The developmental and homeostatic factors are, as expected, constitutively produced, whereas the other groups contain largely inducible signals. Allergenic CC chemokines target eosinophils, basophils and mast cells accounting for their name, and are both potent attractors and stimulants of histamine release. Both inflammatory and HCC subgroups are participants in inflammation but are separated because of phylogenetics.

17.1.4.3 CX3CL1 (Fractalkine, FKN)

CX3CL1 is the lone member of the CX3C subfamily with three intervening residues between the first two cysteines. FKN, the ligand for CX3CR1, is a 373-amino acid, multi-domain molecule found in a wide variety of tissues, including liver, intestine, kidney, and brain. Structural components of FKN include a 76-amino acid chemokine domain (CD) at the N-terminus, which is important in the binding, adhesion, and activation of its target cells (Harrison et al. 2001; Mizoue et al. 1999, 2001; Haskell et al. 2000; Goda et al. 2000). FKN also has an 18-amino acid stretch of hydrophobic residues that spans the cell membrane, and an extended carboxyl-terminus that anchors it to the cell surface (Hoover et al. 2000; Lucas et al. 2001; Cook et al. 2001), thus allowing a membrane form as well as a shed soluble form targeting monocytes and T cells (Bazan et al. 1997).

17.1.4.4 XCL1 (Lymphotactin)

XCL1 is the only representative of the C family. The chemokine targets CD4+ and CD8+ lymphocytes, but does not act on monocytes, and acts through a unique receptor, XCR1. Although having some homology to ligands CCL3 and CCL8, XCL1 lacks the first and third cysteines characteristic of the CC and CXC chemokines.

All of the above families are imperative for homeostatic functions as well as the orchestration of response to pathogenic insult by the immune system. As investigations carry on, this large family will likely see the emergence of additional cytokines and growth factors, and the family and sub-families of cytokine, growth factor, or chemokine receptors will continue to evolve and expand.

17.2 Cytokines and Growth Factors in the CNS

Cytokines and growth factors are an important element in the brain's immune function, serving to traffic leukocytes, maintain immune surveillance, and regulate inflammatory factors. Cytokines and their receptors that are detectable in brain tissues or neuronal and glial cultures under pathophysiological conditions are summarized in Table 17.1. The view of neuro-immunology has evolved from the early belief that regarded the CNS as an absolute immune privileged site to a view of significant CNS-immune system interactions. In the normal

physiological state, T cells, macrophages and dendritic cells readily cross the BBB and transverse specific parts of the healthy CNS (Hickey 1999; Louveau et al. 2015). As discussed in Sect. 17.3, CNS inflammation is unique due to the presence of the BBB. A restricted inflammatory process in the CNS is initiated upon encountering foreign antigen, inducing the production of recruitment factors (Irani 1998). Upon recruitment, monocytes and lymphocytes cross through the BBB to mount immune responses in the CNS. Recruitment is dependent upon the presence of chemotactic factors produced within the CNS that facilitate the crossing of the BBB. This inflammatory state is highly regulated, usually self-limited, and differs from the inflammation in peripheral tissues. The threshold to initiate immune responses against antigens in CNS is much higher than that in periphery (Matyszak 1998; Perry 1998). Avoidance of uncontrolled activation, release of toxic factors, edema and other effectors of robust inflammation is crucial to the maintenance of microenvironment in the CNS.

CNS-immune system interactions are regulated by cytokines, chemokines, and growth factors. Immune cells,

Table 17.1 Cytokine ligand and receptor expression in the CNS

		Cytokine expression	
Cytokines	Receptors	Cell types	References
Type I receptor family			
IL-6	IL-6 R	Macrophage/microglia	Perrella et al. (1992), Poluektova et al. (2004), and Griffin (1997)
		Astrocytes	
GM-CSF	GM-CSF R	Astrocytes	Perrella et al. (1992) and Perno et al. (1990)
IL-4	IL-4 R	T-cells	Wesselingh et al. (1993), Adorini and Sinigaglia (1997), and Kedzierska et al. (2003)
IL-2	IL-2 R	Macrophage/microglia	Wesselingh et al. (1993) and Adorini and Sinigaglia (1997)
BDNF	TrkB	Neuron, astrocyte, oligodendrocytes	Soontornniyomkij et al. (1998) and Boven et al. (1999)
Type II receptor family			
IL-10	IL-10 R	Macrophage/microglia	Gallo et al. (1994) and Poluektova et al. (2004)
IFN- α/β	IFN- α R	Macrophage/microglia	Perrella et al. (2001)
IFN- γ	IFN- γ R	T-cells macrophage/microglia	Griffin (1997)
M-CSF	M-CSF R	Macrophage/microglia	Gallo et al. (1994) and Griffin et al. (1995)
		Astrocyte	
TNF receptor superfamily			
TNF- α	TNF- α R	Macrophage/microglia	Shi et al. (1998) and Kast (2002)
TRAIL	TRAIL-R1, -R2, -R3, -R4	Macrophage/microglia	Ryan et al. (2004) and Dorr et al. (2002)
		T-cells	
NGF	TrkA	Neuron/astrocytes/microglia/macrophage/monocyte	Soontornniyomkij et al. (1998) and Boven et al. (1999)
IL-1 receptor family			
IL-1 α	IL-1 α R	Macrophage/microglia	Perrella et al. (1992)
IL-1 β	IL-1 β R	Astrocytes	Wesselingh et al. (1993), Zhao et al. (2001), and Poluektova et al. (2004)
TGF-beta family			
TGF- β	TGF- β R	Macrophage/microglia	Perrella et al. (2001) and Pratt and McPherson (1997)
		Astrocytes	

following interactions with pathogens or abnormal cells, release cytokines, which then interact with immunosensory tissues associated with the brain. Cytokines activate these tissues directly via specific receptors, or via diffusible mediators such as prostaglandins. Immunosensory structures respond to cytokines, then activate brain neuro-immune circuits responsible for the induction of the defensive response. Microglia are the resident macrophage of the CNS and present antigen, orchestrating the innate immune responses. Astrocytes have also occasionally been postulated to have a role in antigen-presentation to CD4⁺ T cells. Other resident cells, such as neurons or oligodendrocytes, are all capable of recruiting immune cells, modulating the immune response through cytokines, chemokines, or growth factors. In addition, neurons may also modulate the antigen-presenting function of microglia. For example, neuronal production of neurotrophins also suppresses IFN- γ induced MHC class II expression (Neumann et al. 1998).

Disease states within the CNS can lead to dysregulation of the inflammatory process. Continual activation and

recruitment of effector cells may establish a positive feedback loop that perpetuates inflammatory processes and can ultimately lead to neuronal injury and dropout. Similar yet distinct processes occur in multiple CNS disorders such as MS, HAD, and AD. One underlying similarity in each disease state is the cytokine driven inflammatory response (see Fig. 17.1). Numerous studies have demonstrated that microglia/macrophage and astrocytes are major sources of inflammatory mediators, including TNF- α and IL-1 β , IL-10, and nitric oxide (NO). Those mediators initiate or regulate inflammatory processes in the CNS (Aloisi 2001; Dong and Benveniste 2001; Hanisch 2002). In addition to the beneficial effects that glia have in initiating protective immune responses in the CNS, these cells have been implicated in contributing to tissue damage when chronically and/or pathologically activated. For example, many reports demonstrate that activated microglia may exacerbate AD and MS, as described later in this chapter, through secretion of a battery of inflammatory cytokines and cytotoxic agents (Benveniste 1997; Renno et al. 1995; Meda et al. 1995).

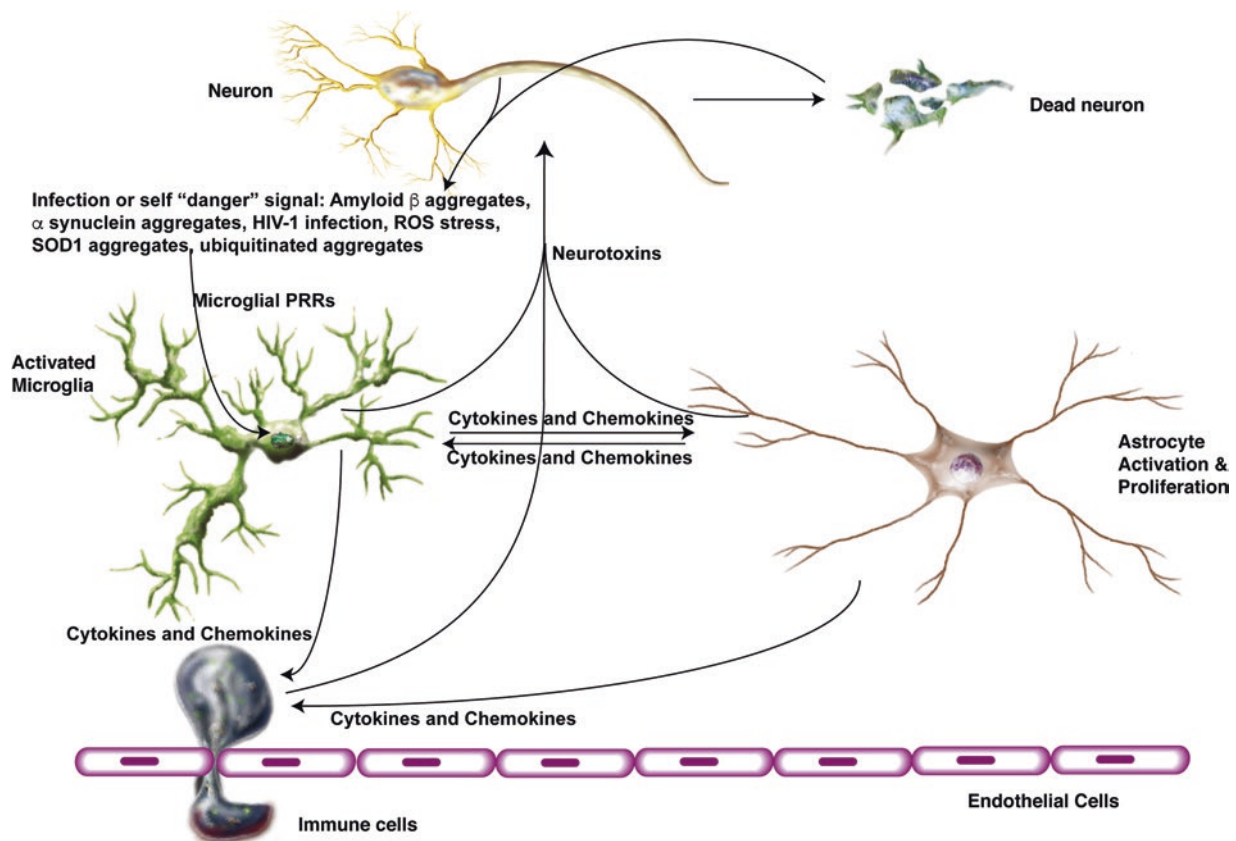


Fig. 17.1 Cytokine-driven inflammatory process in multiple disease states. Multiple disease states have similar cytokine-driven inflammatory processes. In HAND, HIV-1 infection of macrophages in the CNS leads to the activation of macrophages and microglia. Activated macrophage and microglia release cytokines and chemokines, which could be detrimental to neuron. Neuron injury further recruits macrophages.

This injury, recruitment, activation process forms a positive feedback loop and deteriorates the disease states. In AD and MS, either A β priming or T cell-mediated of macrophage/microglia activation may elevate similar inflammatory cytokines, chemokines to cause the pathogenic effects on the CNS

17.3 Molecular Mechanisms of Cytokine and Growth Factor Activity and Signaling

Multiple signaling pathways have been observed for various cytokines. We will first discuss the molecular signaling of neuronal survival and cell death to better understand the molecular mechanisms involved in cytokine-induced neurodegeneration.

The signaling events leading to apoptosis can be divided into two distinct pathways, involving either the mitochondria (*intrinsic*) or death receptors (*extrinsic*) [for reviews, see Green (2003) and Green and Reed (1998)]. The mitochondrial pathway is initiated through various stress signals that impair the mitochondrial membrane integrity. BCL-2 family proteins, including the anti-apoptotic members, i.e. BCL-2 and BCL-XL, and pro-apoptotic members, i.e. Bax, Bak, play a critical role in this pathway (Danial and Korsmeyer 2004; Gross et al. 1999; Wang 2001; Green 2003). The BH3-only BCL-2 family proteins, such as Bid, Bad, Bim, and PUMA, serve as sentinels to these stress signals. Once activated, BH3-only proteins translocate to the outer membrane of mitochondria, where they trigger the oligomerization and activation of both Bax and Bak. In turn, Bax and Bak cause the release of cytochrome c (cyto c) and other apoptogenic factors, including second mitochondrial-derived activator of caspase (SMAC), Htr2, apoptosis inducing factor (AIF), and endonuclease G (EndoG) (Green and Reed 1998). Cyto c then binds the cytosolic adaptor protein, Apaf-1, mediating the formation of the apoptosis complex “apoptosome”. Such complexes lead to the activation of caspase-9, which further processes and activates the effector caspases, pro-caspase-3, 6, or 7. Effector caspases then cleave death substrates and complete apoptosis. The death receptor pathway is initiated through interaction with death receptors and the recruitment of cytoplasmic proteins to specific regions in the intracellular domains of these receptors. The receptor adaptor protein complex then binds pro-caspase 8 and forms a death-inducing signaling complex (DISC) that subsequently releases active caspase 8. Active caspase 8 then activates a caspase cascade and eventually leads to apoptosis of the cell. Many cytokines influence the activation of apoptotic pathways. Members of TNF superfamily, such as TNF- α , TRAIL, or Fas ligand are able to interact directly with death receptors on neurons initiating neuronal apoptosis through activation of the caspase cascade.

In contrast to the apoptotic pathway, some cytokines and growth factors are able to induce survival and proliferation of neurons through survival pathways. The phosphatidylinositol-3-kinase (PI3K) pathway and Ras- or protein kinase C (PKC)-dependent mitogen-activated protein kinase (MAPK) pathway have been extensively studied as survival pathways. Activated PI3K leads to downstream Akt phosphorylation and activation. Notably, Akt activation con-

verges with death receptor mediated receptor interacting protein (RIP) activation on the phosphorylation of the IKK complex and the resulting IKB degradation. IKB degradation releases active nuclear factor κ B (NF- κ B) leading to transcription of genes necessary for cell survival. Similarly, activation of MAPK will lead to nuclear translocation of MAPK together with other transcription factors and co-activators and initiate the transcription of a variety of survival genes. Though not limited to those survival pathways, cytokines and growth factors such as NGF, GDNF, and BDNF play a role in protecting, repairing, or regenerating neurons. Neurons constantly receive survival and apoptotic signals from the extracellular environment, influencing the lifespan of each individual cell. Various cytokines such as BDNF or NGF increase during diseases and may be a compensatory mechanism to the neural injury of the CNS (Soontornniyomkij et al. 1998; Boven et al. 1999). IL-1 β from the IL-1 family or IL-6 from the Type I cytokine family can activate the NF κ B pathway, thus potentially promoting survival of neurons. Interestingly, IL-1, TNF and even TRAIL signaling pathways also converge on IKB degradation and NF κ B activation. IL-6 also activates the Ras-dependent MAPK pathway. Regarding IL-1 β , the activation of NF κ B and MAPK initiates transcription of a variety of genes for either survival or the inflammatory response, dependent upon cell type (Srinivasan et al. 2004).

In summary, various families of cytokine and growth factor may greatly influence the apoptotic or survival pathways of neurons and inflammatory state in the CNS. Extensive crosstalk occurs among signaling pathways activated by cytokines and growth factors. As a result, pathways regulate inflammatory process in neurodegeneration and neuroimmunologic diseases are often not exclusive.

17.4 Cytokines in Neurodegenerative and Neuroimmunologic Disorders

Many cytokines have been suggested to participate in neurodegeneration and neurotoxicity. Increases in factors such as TNF- α and IL-1 β have been observed before neuronal death (Esser et al. 1996; Guo et al. 1998; Little and O’Callaghan 2001; Matusevicius et al. 1996; Meda et al. 1999). However, a simple interpretation of such a pro-inflammatory cytokines effect is controversial. These same cytokines appear to present at least at part of the healthy CNS and has physiological functions. In experimental animals, forced IL-1 β or TNF- α expression does not universally lead to neuronal loss (Akassoglou et al. 1997; Shaftelet al. 2007), suggesting that the action of these cytokines in the CNS is more complex. However, prolonged expression of these cytokines is believed to compromise neuronal functions in disease states. The effect of cytokines in each individual disease will be discussed in the following sections.

17.4.1 Cytokines in HIV Associated Neurocognitive Disorders (HAND)

HIV-1 infection notoriously attacks the immune system in the periphery, but also often leads to neurocognitive impairment in the brain. HIV-1 enters the brain shortly after initial infection, crossing the BBB through peripherally infected monocytes (Koenig et al. 1986). Brain macrophages and microglia, unlike other cellular residents in the CNS, are able to sustain a productive infection within the brain (Eilbott et al. 1989). Although neurons are not infected by HIV-1, without antiretroviral therapy (ART), a dementia specific to HIV has been described as HIV associated dementia (HAD) (Navia et al. 1986; McArthur 1987). Application of antiretroviral therapy has dramatically lowered the rates of HIV-1 replication and HAD. However, substantial numbers of patients are still affected by mild or asymptomatic neurocognitive disorders after apparently successful ART. These disorders, along with HAD, were collected termed HIV associated neurocognitive disorders (HAND) (Sacktor et al. 2016; McArthur et al. 2010). The persistence of HAND indicates an ongoing impairment of neuronal function that cannot be fully restored by ART. The precise mechanism(s) of neuronal injury and dropout in HAND is still unclear. Notably, limited CNS penetration of antiretroviral drugs, presence of viral proteins, the neurotoxicity of ART drugs, co-morbidities with other infection and drugs of abuses may contribute to the neuronal injury associated with the cognitive impairment.

Although recent evidence suggests that HAND showed decreased association with immune activation (Sacktor et al. 2016; McArthur et al. 2010), mononuclear phagocytes (MP, perivascular and brain macrophages and microglia) are known to remain in a chronic state of inflammation (Gendelman and Folks 1999), particularly in the increasing HAND population of older individuals and drug abusers (Valcour et al. 2004; Bell et al. 1998). Pathological examination of patient tissues has revealed characteristic activated macrophage and microglia, as well as damage of neuronal dendrites, axons, and synaptic structure in the brain. Chronic inflammation has both detrimental and beneficial effects. On one hand, these responses are essential in limiting viral spread; yet on the other hand, excessive inflammation is detrimental to resident cells such as neurons. HAD was common in HIV-1-infected individuals who were naïve to ART. In the CNS, infected macrophages and microglia become the major cytokine producer (Meltzer et al. 1990; Wiley et al. 1986; Fischer-Smith et al. 2004; Griffin 1997; Kaul et al. 2001). Two distinct models have been proposed as mechanisms in HAD pathogenesis. The first is the direct neuronal injury model, where viral proteins (gp120, Tat and Vpr) directly interact with neurons causing neuronal injury through various mechanisms. The role of cytokines in this model has been controversial. On one hand, cytokines like

TNF- α have a synergistic apoptotic effect with viral proteins Tat (Shi et al. 1998) and gp120 (Kast 2002). However, other cytokines inhibit the effects of viral proteins on neurons. For example, TGF- β 1 prevents gp120-induced neuronal apoptosis by restoring calcium homeostasis (Scorziello et al. 1997); BDNF and IL-10 can inhibit gp-120 mediated cerebellar granule cell death by preventing gp120 internalization and caspase 3 activation in vitro (Bachis et al. 2001, 2003). The second model, referred to as the indirect neuronal injury model, proposes that neurons die as bystanders when excessive local concentrations of soluble pro-inflammatory and neurotoxic factors are released by infected MP and astrocytes. Studies have supported this notion including the observation that viral protein levels do not correlate with neuronal injury (Petito et al. 1994), while neuronal apoptosis correlates well with microglial activation (Adle-Biassette et al. 1995; Glass et al. 1993). Furthermore, studies have shown that cognitively impaired patients have elevated levels of inflammatory markers and activators in contrast to HIV patients without CNS impairment (Tyor et al. 1992; Sippy et al. 1995).

Although proinflammatory cytokines such as TNF- α and IL-1 β are key molecules in the pathogenesis of HAD, other cytokines may play their role as well. Elevated levels of IL-6, IL-2 and decreased levels of IL-4 have been reported in both CSF and brain sections of HAD patients (Perrella et al. 1992; Wesselingh et al. 1993; Griffin 1997). These factors are directly related to the inflammatory state of brain. The activation of a TH1 immune response by infected macrophages results in the synthesis of cytokines such as IL-2 and IL-6 that activate macrophage and coordinate the immune response towards HIV-1 infection. IL-2 induces T-cell proliferation and potentiates the release of other cytokines. IL-3 and GM-CSF stimulate the production of new macrophages by acting on hematopoietic stem cells in bone marrow. New macrophages are recruited to the site of infection by TNF- α and other cytokines on the vascular endothelium that signal macrophages to leave the bloodstream and enter the tissue. Moreover, many inflammatory signals have been shown to increase HIV replication. IL-2 and GM-CSF are both potent stimulators of HIV-1 replication in activated CD4+ T cells, and GM-CSF increases HIV-1 replication in macrophage cultures (Perno et al. 1990). The activity of type I cytokines during HIV-1 infection promotes the inflammatory response in an effort to eliminate virus, however in the CNS during HAD, type I cytokine mediated inflammation results in neuronal damage as well as facilitating the replication of HIV.

Type II cytokines are key to the balance of inflammation and regulation of response in HAD. Increased levels of IL-10 in the CSF have been reported in individuals with HIV-1 encephalitis (Gallo et al. 1994). In a study in an murine HIV-1 encephalitis model, mRNA levels of IL-10 were increased five-fold as compared to uninfected controls,

and this change is concurrent with down-regulation of proinflammatory cytokines (IL-1 β and IL-6) (Poluektova et al. 2004). Moreover, pretreatment with IL-10 attenuated the neurobehavioral damage induced by HIV-1 gp120 in an animal study (Barak et al. 2002). In addition to IL-10, IFN- α and IFN- β are induced by HIV-1 infection and suppress HIV replication at multiple steps of the viral life cycle in macrophage (Gessani et al. 1994; Gendelman et al. 1992). Levels of IFN- γ have been shown to be elevated in HAD patients (Shapshak et al. 2004). IFN- γ enhances HIV-1 replication in CD4 $^{+}$ T cells in an autocrine manner, while in macrophage culture IFN- γ enhances HIV-1 replication when added prior to infection but inhibits replication when added post-infection. The active effects of these immunomodulatory and antiviral cytokines suggest that their increase may be an attempt to control inflammation and infection, and to restore tissue to homeostasis.

Among the pro-inflammatory cytokines, TNF- α is the most studied. Elevated levels of TNF- α mRNA is seen in brain tissue collected from HAD patients (Wesselingh et al. 1993). Studies have shown that TNF- α levels in vulnerable brain regions correlate with neurological disease severity in HIV patients (Gelbard 1999). During HAD, microglia, macrophages and monocytes show increased expression of TNF- α and TNF receptors (Tyor et al. 1992), an effect promoted by both IFN- γ and IL-1. Under ART, HAD patients have a marked decrease in soluble TNF- α levels in the cerebrospinal fluid; this drop in TNF- α coincides with decreased viral load and marked improvement in the neurological function of patients (Gendelman et al. 1998). TNF- α may promote neuronal demise through various mechanisms. Increased BBB permeability and recruitment of activated immune cells facilitates viral invasion of the CNS. Synergy of TNF- α , viral proteins and excitotoxic glutamate activates glia to produce neurotoxins or leads to neuronal apoptosis directly [see review Saha and Pahan (2003)]. The effects of TNF- α are complex; differing experimental systems or approaches have also shown TNF- α to assume a neuroprotective role. TRAIL, another member of TNF family, is not normally expressed in the CNS. However, upon HIV-1 infection of CNS, TRAIL was upregulated primarily by infiltrated macrophages. Neurons express TRAIL-receptors and have been indicated as a target of TRAIL (Cantarella et al. 2003; Aktas et al. 2005; Ryan et al. 2004). We have demonstrated that TRAIL is upregulated on the cell surface of HIV-infected or immune-activated macrophages (Ryan et al. 2001, 2004). Also, TRAIL has been reported to be expressed on the cell membrane of MP, Natural killer cells, and CD4 $^{+}$ T lymphocytes and can be cleaved into a soluble, secreted form (Ehrlich et al. 2003). The plasma level of TRAIL is increased to ng/ml ranges in HIV-1-infected patients, particularly those with high viral loads (Herbeuval et al. 2005). Thus TRAIL may be one of the neurotoxic mediators in the pathogenesis of HAD.

Another proinflammatory cytokine IL-1 β is rapidly produced upon HIV-1 infection within the CNS. The induction of IL-1 β has been shown to be associated with HAD (Zhao et al. 2001). IL-1 β in this case serves as an upstream signal for multiple proinflammatory cytokines, notably TNF- α and IL-6, initiating and amplifying inflammation in the brain, which is responsible for the global activation of macrophage and microglia (Chung and Benveniste 1990; Aloisi et al. 1992; Lee et al. 1993). IL-1 β directly activates HIV-1 replication in a monocytic cell line by transcriptional and post-transcriptional mechanisms independent of NF- κ B. IL-1 also synergistically enhances HIV-1 replication with multiple cytokines including IL-4 and IL-6 (Kedzierska et al. 2003).

TGF- β is expressed in the brain by astrocytes, MP and oligodendrocytes, and has been shown to exert multiple effects on neurons and glial cells both in vitro and in vivo. Effects of TGF- β include cell cycle control, differentiation, extracellular matrix formation, hematopoiesis, and chemotaxis. Importantly in the CNS, TGF- β has key a role in regulating neuron survival and repair processes. TGF- β has also been shown to play a role in several varieties of CNS pathology including ischemia, excitotoxicity and neurodegenerative diseases such as multiple sclerosis. In mild HAD, the cerebral levels of TGF- β has an inverse correlation to IFN- α and HIV RNA; while in the severe form of HAD, TGF- β is undetectable (Perrella et al. 2001). In specific culture conditions, TGF- β has neurotrophic effects similar to BDNF and NGF, but a change in conditions can shift the effect of TGF- β towards neurotoxicity (Prehn and Miller 1996).

In summary, various cytokines are upregulated and strongly associated with brain inflammation and neurological symptom in HAD patients. However, introduction of ART has reduced such cytokine dysregulation in HAND patients. This trend of cytokine regulation is consistent with the clinical and pathological manifestation of HAND.

17.4.2 Cytokines in MS

MS is a chronic neurological disease with the hallmark pathological findings of perivascular inflammation and demyelination. Histological sections from MS patients show demyelinating plaques are distributed within the white matter of the CNS, but the most frequently affected sites are the optic nerves, the brainstem, the cerebellum and the spinal cord. Although the pathological hallmark of the MS lesion is focal demyelination, axonal damage and neuronal dysfunction are also found in the pathology of MS (Trapp et al. 1999; De Stefano et al. 2001; Bjartmar et al. 2003). The selective loss of axons and myelin sheaths has been postulated to occur via a variety of different mechanisms. Traditionally, MS has been considered an autoimmune disorder consisting of myelin auto-reactive T-cells that drive an inflammatory process, leading to

secondary macrophage recruitment and subsequent myelin and oligodendrocyte destruction. However, recent detailed studies on a large collection of MS lesions have indicated that structural features of the plaques are extremely variable and the events involved in the immunopathogenesis of MS may be more complicated (Carson et al. 2005).

Cytokines are extensively involved in the pathogenesis of MS. Generally, Th1 of activated CD4⁺ cells produce IL-1 β , IL-2, IFN- γ , and TNF- α to mediate inflammatory pathological processes in immune-mediated tissue damage seen in MS and experimental autoimmune encephalomyelitis (EAE), a mouse model of MS (Adorini and Sinigaglia 1997). In contrast, Th2 cells produce IL-4, IL-6, and IL-10 to mediate antibody production and downregulation of Th1 cellular responses (Adorini and Sinigaglia 1997). The inflammatory reaction in MS and EAE is associated with the upregulation of a variety of Th1 cytokines, including IL-2, IFN- γ , and TNF- α . Adaptive transfer of IFN- γ -producing Th1 cells but not Th2 cells induce EAE, suggesting the pathogenic role of Th1 cells in EAE. However, cells other than the classical Th1 T-cells may contribute to the inflammatory process in autoimmune disease. Studies in the last decade led to the identification of a functionally distinct T cell subset called T helper type 17 (Th17) cells that produce a signature cytokine, interleukin-17A (IL-17A), which play a crucial role in EAE pathogenesis (Korn et al. 2009). Furthermore, IL-23, a cytokine from IL-12 subfamily, has been identified to be a pivotal factor in inducing Th17 cells into a more pathogenic phenotype (Gaffen et al. 2014). This is also evidence suggesting that IL-9-producing Th9 cells can also induce EAE upon adaptive transfer, albeit with a different phenotype (Jager et al. 2009). These data suggest that the heterogeneity of multiple sclerosis lesions may relate to different disease-causing T cells and their associated effector cytokines.

Many inflammatory cytokines from TNF superfamily, such as fas ligand and TNF- α , are important effectors for neuronal death in EAE. Overexpression of Bcl2, an anti-apoptotic protein that blocks death receptor-initiated signaling events, protects mice primed to develop EAE (Offen et al. 2000). Further, elevated death ligand TRAIL expression level in the blood MP of MS patients had been reported (Huang et al. 2000). Oligodendrocytes and neurons have recently been shown to be one of TRAIL's targets (Matysiak et al. 2002; Wosik et al. 2003; Aktas et al. 2005). Although inflammatory mechanisms seem to be an important aspect contributing to tissue injury in MS, whether the degree to which demyelination and axonal injury are a direct consequence of inflammation, is still not clear.

Although pro-inflammatory cytokines associated with type I, IL-1 β , and TNF receptor families are thought to play an important role, characterization of MS and EAE animal model generally support the pathogenic role of IL-17A in the pathogenesis of MS (Korn et al. 2009; Glass et al. 2010).

Th17 cells and IL-17A are frequently detected in lesions from patients with multiple sclerosis and mice with EAE. The differentiation and activation of Th17 cells requires not just IL-17A and IL-17F but cytokines including IL-1 β , IL-6, TGF- β as well as IL-23 and other cytokines. Therefore, targeting IL-17A with neutralizing antibodies has become a new therapeutic approach in treating multiple sclerosis (Gaffen et al. 2014). An ongoing clinical trial with the IL-17A-specific antibody secukinumab in active relapsing-remitting multiple sclerosis has now provided preliminary results that support the role of IL-17A in multiple sclerosis in humans (Baeten and Kuchroo 2013).

In addition to cytokines, T cells, macrophage, and microglia also produce neurotrophic factors and other neuroprotective factors during inflammation, which indicates an important role for inflammation in the repair of MS lesions (Stadelmann et al. 2002; Hohlfeld et al. 2000; Gielen et al. 2003). FGF2 and PDGF have been demonstrated to be the factors regulating remyelination (Frost et al. 2003). CNTF family factors, such as CNTF, leukemia inhibitory factor, cardiotrophin-1, and oncostatin M have been reported to induce a strong promyelinating effect in a myelination model (Stankoff et al. 2002). NGF enhances neural growth and also has the ability to switch the T-cell phenotype from Th1 to Th2, which reduces CNS tissue damage (Villoslada et al. 2000). Interestingly, IL-1 β is crucial to the remyelination of the CNS and this action is suggested to the induction of astrocyte and microglia-macrophage-derived IGF-1 (Mason et al. 2001). IGF-1 may, in addition, have antiapoptotic properties for oligodendrocytes (Mason et al. 2000).

Cytokines has a long history to be used for MS therapy. IFN- β has been in clinical use as an immunomodulatory drug for the treatment of MS for more than 10 years. Administration of IFN- β decreases the relapse rates and new MRI lesions in patients with relapsing/remitting MS (The IFN- β Multiple Sclerosis Study Group 1993) or delay disease progression in patients with secondary progressive MS (Jacobs et al. 1996; George C Ebers et al. 1998). However, the exact mechanisms of action of IFN- β in MS are unknown. IFN- β is effective in preventing the IFN- γ -induced upregulation of MHC-II on antigen-presenting cells (Yong et al. 1998). In an earlier study, patients treated with IFN- γ exhibited an alarmingly high relapse rate during the initial month of treatment (Panitch et al. 1987). IFN- β can also downregulate the expression of costimulatory molecules and inhibit the activation and proliferation of T cells. Another effect of IFN- β is the alteration of cytokine levels. IFN- β decreases the level of Th1 cytokine such as IFN- γ , IL-12 and TNF- α (Yong et al. 1998). IFN- β treatment of astrocytes has been reported to stimulate production of NGF in-vitro (Boutros et al. 1997) and may thus promote remyelination in the CNS.

In summary, cytokines and growth factors are very important mediators in the pathogenesis of MS. More effort should

be brought to explore the complex interactions among immune cells, cytokines, growth factors and CNS resident cells to identify the main perpetrator(s).

17.4.3 Cytokines in AD

AD is the most common chronic neurodegenerative disorder characterized by plaque deposits comprised of highly insoluble β -amyloid peptides ($A\beta$), cleaved products of the amyloid precursor protein (APP), intracellular neurofibrillary tangles (NFTs) generated by hyperphosphorylated forms of the microtubule-binding protein tau, and inflammatory proteins in the cerebral cortex and hippocampus (Ringheim and Conant 2004; Selkoe 2001; Glass et al. 2010). A number of factors have been thought to contribute redundantly to pathogenesis of AD, including unbalanced calcium homeostasis, cell-cycle protein dysregulation, excitatory amino acids, as well as DNA damage. Deposition of $A\beta$ and hyperphosphorylated tau in the brain parenchyma appears to be the crucial factor for the onset of AD, although mechanisms underlying $A\beta$ effects are far from understood. $A\beta$ at high concentrations (generally $>10 \mu\text{M}$) has been found to act as a potent neurotoxic agent to both neuronal and non-neuronal cells. Furthermore, $A\beta$ formation and hyperphosphorylated tauopathy appear to be sufficient to cause stress response in neurons. The stress response in neurons could further activate microglia and astrocytes and to local inflammation that may further amplify neuronal injury and loss.

Inflammation has been suggested to contribute to the $A\beta$ deposition and neuropathology associated with AD. For example, many clinical studies have indicated that anti-inflammatory drugs have a protective effect upon the development of AD (Stewart et al. 1997; Broe et al. 2000). AD is associated with the increase of pro-inflammatory cytokines including IL-6, TNF- α , M-CSF, IL-1 β , TGF- β , PDGF (Griffin et al. 1989, 1995; Akiyama et al. 2000; van der Wal et al. 1993; Tarkowski et al. 1999). These cytokines are produced by activated glia and some (IL-1 β and TNF- α) are capable of perpetuating glial activation, leading to a cycle of overproduction of these potentially neurotoxic molecules. Microglia isolated post-mortem from AD patients have been shown to either constitutively express cytokines, including IL-1 β , IL-6, and TNF- α , or produce these cytokines in response to $A\beta$ (Lue et al. 2001). It should be noted that a complex relationship seems to exist between $A\beta$ deposition and the $A\beta$ -induced inflammatory response.

IL-1 β regulates the synthesis of the APP from which the pathologic and diagnostic amyloid plaques of AD originate (Goldgaber et al. 1989). The functions of IL-1 β and its association with AD pathology suggest that this cytokine plays a key role in the pathogenesis of AD. Brain tissue levels of IL-1 β are markedly elevated in AD, and the numbers of IL-1-

immunoreactive microglia are markedly increased in tissue sections of Alzheimer brain, mirroring the distribution of microglia within cerebral cortical layers in brains of normal controls (Sheng et al. 1998), suggesting that the distribution of microglia within the brain determines in part the distribution of $A\beta$ plaques in AD.

In summary, cytokines and growth factors discussed in this section are implicated in the pathophysiological processes of AD, likely amplifying inflammatory response that ultimately causes neuronal injury. Although the extent of involvement by cytokines, chemokines, and growth factors in AD is still up to debate, many clinical studies will be directed to address this question.

17.5 Chemokine in the CNS

While produced most often by immune cells, chemokines are also expressed by cells within the brain including endothelial cells, neurons, astrocytes, microglia and oligodendrocytes. They regulate a number of brain functions including the migration, recruitment, accumulation, and activation of leukocytes in the brain (Wu et al. 2000). For a summary of chemokines in the CNS, see Table 17.2. Some chemokines such as stromal derived factor-1 alpha (SDF-1 α , CXCL12) and FKN (CX3CL1) are constitutively produced in the brain and likely play an important role in CNS homeostasis and development. Others, such as macrophage inhibitory proteins - one alpha and one beta (MIP-1 α , CCL3 and MIP-1 β , CCL4), monocyte chemotactic protein-1 (MCP-1, CCL2), and the regulated upon activation normal T-cell expressed and secreted (RANTES, CCL5), are induced by inflammatory stimuli. These chemokines are likely involved in the pathogenesis of a variety of neurodegenerative diseases, where inflammation plays a role in pathogenesis, such as MS, AD, and HAND.

The inflammatory response manifested in the brain during HIV-1 infection leads to the development of a chemo-attractant gradient to facilitate the formation of multinucleated giant cells in HIV encephalitis (Williams et al. 2002). This enables inflammatory monocytes to enter the brain, become activated and expand the sources of neurotoxic secretory factors that lead to the pathological and clinical aspects of disease (Gendelman et al. 1997). Moreover, chemokine receptors are critical for infection in perivascular macrophages and microglia. Studies have shown chemokines and their receptors play a more direct role in the neuropathogenesis of HIV-1 infection. It is now clear that neurons, glia and neural stem cells express chemokine receptors and the interactions of HIV-1 gp120 with neuronal chemokine receptors leads to apoptosis of neurons (Cotter et al. 2002; Ryan et al. 2002; Peng et al. 2004; Tran and Miller 2003; Kaul et al. 2001). These effects may be manipulated by chemokines

Table 17.2 Chemokine ligand and receptor expression in CNS

Cell type	Ligands chemokine (systematic name)	Receptors	
Monocytes/macrophages	MCP-1 (CCL2)	CCR2	Bernasconi et al. (1996), Cinque et al. (1998), Kelder et al. (1998), Collman et al. (2000), and Gabuzda et al. (2002)
	MIP-1 α/β (CCL3/CCL4)	CCR3, CCR5	
	RANTES (CCL5)	CCR3, CCR5	
	IL-8 (CXCL8)	CXCR3	
	IP-10 (CXCL10)	CXCR4, CCR4	
Microglia	MIP-1 α/β (CCL3/CCL4)	CCR3, CCR5	Bernasconi et al. (1996), Cinque et al. (1998), Kelder et al. (1998), and Harrison et al. (1998)
	RANTES (CCL5)	CCR3, CCR5	
	MCP-1,3 (CCL2,CCL7)	CCR2	
	IL-8 (CXCL8)	CXCR3	
	IP-10 (CXCL10)	CCR4, CXCR4, CX3CR1	
Astrocytes	MCP-1 (CCL2)	CCR2	Conant et al. (1998) and Zheng et al. (1999b)
	MIP-1 α/β (CCL3/CCL4)	CCR3, CCR5	
	RANTES (CCL5)	CCR3, CCR5	
	SDF-1 α (CXCL12)	CXCR4	
	IL-8 (CXCL8)	CCR4	
	IP-10 (CXCL10), FKN (CX3CL1)		
Neurons	FKN (CX3CL1)	CCR2	Coughlan et al. (2000), Erichsen et al. (2003), and Cook et al. (2001)
	MCP-1 (CCL2)	CCR5, CXCR4	
	GRO- α (CXCL2)	CXCR2	
		CXCR3	
Endothelium	IL-8 (CXCL8)	CXCR4, CXCR7	Gabuzda et al. (2002) and Stumm et al. (2002)
	IP-10 (CXCL10)	CCR5	
	MGSA- α , - β , - γ (CXCL1, 2, 3)		
	MIG (CXCL9)		
	MCP-1,3,4 (CCL2, 7, 13)		
	SDF-1 α (CXCL12)		

that act on the same receptors. The presence of chemokine receptors on neural cells also supports the notion that chemokines modulate neuronal physiological functions. Thus, chemokine receptors might have a crucial role in the balance between neuronal protection and injury.

17.6 Chemokines and Their Receptors in Neurodegenerative and Neuroimmunologic Disorders

17.6.1 Chemokines and Their Receptors in HAND

17.6.1.1 HIV-1, Chemokines and HIV-1 Co-receptors

As previously discussed, chemokines are important players in immune homeostasis and inflammatory response. Their essential roles in immune system have made them targets of invading pathogens. Studies have shown multiple viruses including herpesvirus, poxvirus, retrovirus, and lentivirus take advantage of the chemokine system, posing as analogs, to presumably gain a survival advantage by avoiding or altering immune detection and elimination (Murphy 2001).

Another manipulation of chemokine immune defense first described in 1996, is the use of a chemokine co-receptor in HIV infection (Feng et al. 1996). Initially, HIV was assumed to rely solely on the CD4 surface protein found on T-cells and macrophages for entry into host cells [CD4 as an HIV receptor is reviewed in Sattentau and Weiss (1988)]. However, CD4 alone did not accurately predict the cell interactions of HIV. This eventually led to the breakthrough revealing that chemokine receptor-mediated viral membrane fusion with human host cells. Each HIV strain has different specificities and interactions with various chemokine receptors, but the two primary HIV coreceptors are CCR5 and CXCR4. Macrophage or M-tropic viral strains utilize CCR5 for infection; T-cell or T tropic viral strains rely upon CXCR4. There is another viral subset, dual tropic or R5X4 strain that employs both coreceptors. Further, additional receptors have been shown to have more limited viral interactions including CCR2, CCR3, CCR8, CX3CR1 and others, but the pathophysiological relevance has yet to be determined (Gabuzda and Wang 2000).

The co-receptor requirement is a result of receptor ligand interactions between the chemokine receptors and the HIV coat protein gp120. Virus-cell interactions characteristically begin with gp120 binding CD4, inducing a conformational

change in gp120. This change alters the affinity of gp120 for a coreceptor, either CCR5 or CXCR4, resulting in a trimolecular interaction between gp120, CD4 and the coreceptor (Berson and Doms 1998; Dimitrov et al. 1998; Berger et al. 1999). The multi-molecular interaction then permits fusion of HIV viral membrane to the host cell, permitting entry and consequent integration into the host DNA. Chemokine receptors play a critical role, particularly in the early stages of HIV cell entry both in protective and liable capacities. CCR5 and CXCR4 are the major co-receptors for viral entry into CD4+ cells (Feng et al. 1996; Cocchi et al. 1995; Dragic et al. 1996; Deng et al. 1996), whereas the presence of their ligands can sometimes help prevent infection. In contrast, a deletion within the CCR5 gene confers resistance to HIV-1 infection (Samson et al. 1996). Furthermore, transplantation with stem cells from homozygous CCR5 deletion has produced a first function cure HIV-1 patient, demonstrating the critical role CCR5 plays in maintaining HIV-1 infection (Hutter et al. 2009). These observations have elicited intense interest in using CCR5 as a drug target for the treatment of HIV-1 infection.

Neurons express both chemokines and chemokine receptors, and although not infected by HIV, neurons do express the coreceptors CXCR4 (Zhang et al. 1998) and CCR5 (Rottman et al. 1997). Similar to cells infected by HIV, the neuron coreceptors have affinity for HIV envelope protein gp120, regardless of CD4. Many groups have since shown neuronal toxicity mediated by viral proteins, particularly gp120 (Hesselgesser et al. 1998; Ohagen et al. 1999; Kaul and Lipton 1999; Zheng et al. 1999b; Chen et al. 2002; Garden et al. 2004). Upon interaction with coreceptors, gp120 induces signaling cascades that may play a role in promoting apoptosis. Blocking of these cascades can block neuronal death in some cases. Interestingly, different viral strains induce varying levels of neuronal toxicity (Gabuzda and Wang 1999; Zheng et al. 1999a).

17.6.1.2 Neuroprotective and Neurotoxic Effects of Chemokines and Their Receptors in HAND

In contrast to HIV-1 coreceptors, some chemokine ligands have the ability to reduce or ablate neuron toxicity. High levels of chemokines RANTES, MIP-1 α , and others have been shown to reduce neuron death (Meucci et al. 1998; Kaul and Lipton 1999), while SDF-1, at higher concentrations may actually promote neuronal death (Kaul and Lipton 1999; Hesselgesser et al. 1998; Zheng et al. 1999b). The mechanism is not yet completely understood, but may rely upon simple competitive inhibition, receptor expression changes on the cell surface, or other unknown mechanisms.

FKN (CX3CL1): In the CNS, FKN is constitutively produced by neurons, and its receptor (CX3CR1) is predominantly expressed in the microglia. FKN appears to have

physiological functions since mice lacking the chemokine receptor Cx3cr1 exhibit a transient reduction of microglia during the early postnatal period and a consequent deficit in synaptic pruning (Zhan et al. 2014). FKN levels are higher in the CSF of cognitively impaired HIV patients than in infected subjects without cognitive impairment. Moreover, FKN can affect the chemotaxis of primary monocytes across an artificial blood brain barrier, and is neuroprotective to cultured neurons (Meucci et al. 2000; Tong et al. 2000). Thus, this neuronal chemokine may serve as a damage signal to recruit macrophages and microglia to the site of injury (Jung et al. 2000; Tong et al. 2000; Zujovic et al. 2000; Erichsen et al. 2003). Subsequent chemokine-MP interactions can initiate inflammatory responses through the production of chemokines/cytokines or protective responses through the production of neurotrophins (Xiao and Link 1998; Cotter et al. 2002; Kaul et al. 2001).

MCP-1 (CCL2): Despite some association of chemokines and neuroprotection, there are also detrimental effects of chemokine function during HAD pathogenesis. Shown to be expressed in the brains of HAD patients (Conant et al. 1998), MCP-1 (CCL2) is a potent chemoattractant for monocytes and may help fuel the positive feedback loop of inflammation in the HAD brain. MCP-1 recruits monocytic phagocytes to sites of inflammation as was evidenced by a study using a mouse system with elevated levels of MCP-1 resulting in increased phagocytic cells at lesion sites (Fuentes et al. 1995). A high level of MCP-1 in CSF versus plasma was shown to be predictive of dementia development in monkeys (Zink et al. 2001). While clearly possessing chemotactic properties, the ability of MCP-1 to recruit phagocytes through the BBB was further elucidated with a study showing changes in BBB permeability in the presence of MCP-1 (Song and Pachter 2004). Increased levels of MCP-1 were shown to result in initial protection from infection, however, upon successful HIV-1 infection, increased MCP-1 was shown to lead to increased susceptibility to the development of HAD (Gonzalez et al. 2002).

Interferon gamma inducible protein 10, (IP10, CXCL10): IP-10 is a CXC chemokine. As indicated by its name, IP-10 is highly induced by interferon as well as other factors, yet is also produced constitutively throughout the body. IP-10 targets multiple subtypes of activated T-cells and macrophages for migration. IP-10 has been found in very high levels in CSF as well as shown to recruit cells into the CNS in the setting of HAD. While clearly a player in recruitment and inflammation, IP-10's role has also been shown to include cytotoxic effects towards neurons (van Marle et al. 2004) and may stimulate HIV-1 replication in macrophages (Lane et al. 2003).

IL-8 (CXCL8): An endogenous ligand for CXCR2, IL-8 is secreted in high levels by HIV-1 infected lymphocytes and macrophages. Although expressed constitutively, immune activation potentiates IL-8 production from infected or uninfected

macrophages by agents such as LPS or CD40L (Zheng et al. 2008). IL-8 levels are increased in the CSF of HAD patients, more so than those lacking cognitive symptoms, supporting the role IL-8 in HAD (Zheng et al. 2008).

SDF-1 (CXCL12): Chemokines have also been shown to have a neuromodulatory capacity, in some cases decreasing excitation and avoiding toxicity. A complicated example is the effect of SDF-1 on glutamate toxicity and uptake, specifically as regulated through astrocytes. SDF-1 is a member of the C-X-C chemokine subfamily and is the only known physiological ligand for CXCR4 (Rossi and Zlotnik 2000). SDF-1 is a potent chemoattractant for resting lymphocytes, monocytes, and CD34-positive hematopoietic progenitor cells (Kim and Broxmeyer 1999). CXCR4 is upregulated in HIV and SIV encephalitis, experimental allergic encephalitis (EAE), and brain tumors (Jiang et al. 1998; Sanders et al. 1998; Vallat et al. 1998; Westmoreland et al. 1998). SDF-1 transcripts are predominantly expressed by oligodendrocytes, astrocytes and neurons in the cortex, hippocampus and cerebellum (Gleichmann et al. 2000; Stumm et al. 2003). SDF-1 has been shown to be upregulated in the brain of patients with HIV encephalitis (Langford et al. 2002; Rostasy et al. 2003). Studies conducted in different settings have shown SDF-1 to promote neuronal survival, reducing glutamate toxicity (Meucci et al. 1998), while another study has shown SDF-1 to increase neuronal death by increasing the release of glutamate and TNF- α from glial cells (Bezzi et al. 2001). This may be due to experimental variation or a concentration dependent effect of SDF-1 and glutamate regulation. Recently, it was suggested that SDF-1 could be cleaved to SDF-1 (5-67) and mediate direct neurotoxicity (Zhang et al. 2003).

17.6.1.3 Therapeutic Avenues Directed Toward Chemokines and Their Receptors

Because HIV requires coreceptors for the induction of productive viral infection, and naturally occurring alleles have been shown to effectively limit HIV entry [reviewed in Tang and Kaslow (2003)], potential therapies may rely upon exploitation of CXCR4 and CCR5. However, the ability of HIV to mutate presents a great hurdle in the development of effective therapeutic receptor antagonists. Another layer of complexity is the potential side effects of blocking one or multiple chemokine receptors that have homeostatic and inflammatory roles. Despite the inherent difficulties, multiple approaches have been studied that led to the development of a new class of HIV drugs called CCR5 antagonists (Flexner 2007). Among these new drug maraviroc was approved in 2007 for the treatment of HIV-1 infection. Since then, maraviroc has become one of the two chemokine receptor-targeting drugs that are approved for clinical use. New approaches are also begin developed to target CCR5 for

anti-HIV treatment, which include siRNAs, engineered zinc finger nuclease, and CRISPR/Cas9 (Zhou et al. 2004; Gu and Chen 2014; Saayman et al. 2015).

Some chemokine ligands inherently disrupt viral pathogenesis or provide protection against cell death during the disease process. Ligands of HIV co-receptors, such as SDF-1 and RANTES, have been shown to block infection of cells in different systems (Bleul et al. 1996; Lederman et al. 2004). Similarly, ligands to co-receptors have been shown to block HIV envelope protein induced toxicity to neurons (Alkhatib et al. 1996; Meucci and Miller 1996). The mechanism may be simple blocking or internalization of receptors, or may rely upon the signaling downstream of chemokine interaction.

17.6.2 Chemokines and Their Receptors in MS

Similar to cytokines, the chemokine expression pattern is also a Th1-mediated response in MS. Pro-inflammatory cytokines activate resident macrophages and microglia within the CNS. Recruitment and attraction of these cells occurs via integrins and chemokines and is believed to contribute to tissue injury and demyelination. Selective expression of individual chemokines may influence the cellular composition of inflammatory lesions because chemokine receptors are associated with either Th1 or Th2 responses. Th1 proinflammatory cells may express CCR5 (receptor for chemokines RANTES, MIP-1 α and MIP-1 β) and CXCR3 (receptor for IP-10 and MIG), whereas Th2 inflammatory cells may shift toward the display of CCR3 (receptor for MCP-3, MCP-4, and RANTES) and CCR8. MCP-1, MCP-2, and MCP-3 were found to abundantly express within the lesion center, with more intense staining apparent for MCP-1 and MCP-2 (McManus et al. 1998; Van Der Voorn 1999; #5468).

In MS autopsy brain sections, CCR5 and CXCR3 are overexpressed in peripheral and lesion associated T-lymphocytes (Balashov et al. 1999; Simpson et al. 1998). The ligands for CCR5 and CXCR3, RANTES and IP-10, also increase in the CSF (Lucchinetti et al. 2003). For the cell source of chemokines, IP-10 was associated with astrocytes and perivascular astrocytic processes within MS lesions (Sorensen et al. 1999; Balashov et al. 1999; Simpson et al. 1998). These results suggested that interaction between IP-10 and CXCR3 might be the mechanism that traffic T-cells into MS lesions. Similarly, the elevated CCR5 in activated macrophages was found to be in MS lesions, these results indicated RANTES might mediate the recruitment and activation of monocytes and macrophages in MS (Sorensen et al. 1999).

Chemokines play an important role in remyelination of MS. Chemokine CXCL12 regulates remyelination through oligodendrocyte progenitor cells (OPCs) (Patel et al. 2010).

Blockade of CXCL12 and its receptor CXCR4 signaling limits OPC maturation during remyelination (Carbajal et al. 2010). Interestingly, antagonism of CXCR7, an atypical chemokine receptor that binds CXCL12, augments OPC proliferation, leading to increased numbers of mature oligodendrocytes within demyelinated lesions (Williams et al. 2014). These results suggest that CXCR7 could serve as a new therapeutic target to promote remyelination in MS.

Taken together, existing literature suggests extensive chemotactic interactions between the glia cells in MS lesion and the infiltrating cells. Whether these interactions ultimately promote destructive inflammation or recruitment of protective regulatory cells that enhance tissue repair is still not clear. This is a crucial question to answer before potential pharmacological intervention based on chemokines and their receptors could be used.

17.6.3 Chemokines and Their Receptors in AD

Immunohistochemical analysis of tissue from human brains with AD have revealed that in AD, there is increased expression of MIP-1 β and IP-10 by activated astrocytes, and of the chemokine receptors CCR3 and CCR5 on activated microglia, adjacent to A β deposits in AD (Xia et al. 1998, 2000). Dystrophic neuritis has been shown to express the IL-8 receptor CXCR2 within the A β plaques of AD (Xia et al. 1997). A β is capable of modulating the inflammatory processes involved in AD through chemokine production. MCP-1 is found in activated microglia and within neuritic A β plaques, but not in early plaque forms (Ishizuka et al. 1997). A β promotes production of MCP-1, MIP-1 α , MIP-1 β , or IL-8 by monocytes and microglia (Meda et al. 1999; Fiala et al. 1998); A β also promotes expression of MCP-1 and RANTES by astrocytes (Johnstone et al. 1999). These observations collectively suggest that chemokines may play a role in the pathogenesis of AD (Xia and Hyman 1999). Indeed, Ccr2-deficient mice are viable and in mouse model of AD, Ccr2-deficiency blocks microglial accumulation in the brain, suggesting that CCL2 and CCR2 are pathogenic in Alzheimer's disease. Production of this chemokine promotes the recruitment and accumulation of astrocytes and microglia in senile plaques (Cartier et al. 2005).

Although the exact role of chemokines in the AD is still not yet well defined, neuroprotective effects of chemokine have also been noted thus make chemokines double edged roles in the AD. Fractalkine/CX3CL1 is suggested to have a protective role in AD. It has been reported that Fractalkine/CX3CL1 inhibits the production of IL-6, TNF- α and NO with A β -primed microglia, thereby improving the neuronal survival rate (Mizuno et al. 2003; Zujovic et al. 2000). GRO- α (CXCL2), the ligand for CXCR2 has been suggested to trigger ERK1/2 and PI-3K pathway in a way similar to

BDNF, thus indicates GRO- α , or perhaps other CXCR2 ligands, could have neurotrophic effect on neurons and other cell types (Xia and Hyman 2002). Neuroprotective effects of other chemokines, such as RANTES, SDF-1 α , IP-10, and MIP have also been documented (Meucci et al. 1998).

With the current understanding of the dual roles of chemokines and their related regulations, it is not likely that the pharmaceutical intervention will soon be used to treat the AD. New therapeutic modalities targeting chemokines and their receptors may become available with a more detailed understanding of these agents and their receptors in the inflammatory changes responsible for the CNS degeneration.

17.7 Review Questions

1. What are the difference between type I cytokines and Th1 cytokines?
2. How does IL-1 β contributes to the development of HIV-1 associated dementia, Alzheimer's disease, and multiple sclerosis?
3. Why does signaling of TNF family of cytokine need to be tightly regulated during CNS inflammation.
4. Why are there more chemokines than chemokine receptors.
5. How do different stimuli lead to CNS inflammation in the pathogenesis of HIV-1 associated dementia, Alzheimer's disease, and multiple sclerosis?
6. Briefly discuss the approach to classify chemokines and provide an example for each type.
7. Which chemokine receptors have been identified as coreceptors for HIV?
8. Many cytokines and chemokines are upregulated during the pathogenic processes in MS and AD, what is the best approach to identify them as therapeutic targets?
9. Which of the following statements about chemokines is correct?
 - (a) Chemokines and chemokine receptors play a significant role in pathogenesis of inflammatory disorders.
 - (b) Stromal derived factor-1 α (SDF-1 α , CXCL12) and fractalkine (FKN, CX3CL1) are constitutively produced in the brain.
 - (c) Macrophage inhibitory proteins-one alpha and one beta (MIP-1 α , CCL3 and MIP-1 β , CCL4), and monocyte chemotactic protein-1 (MCP-1, CCL2) are induced by inflammatory stimuli in the CNS.
 - (d) All of the above are correct.
10. Propose a potential therapeutic approach using a cytokine or chemokine in HIV-1 associated neurocognitive disorders.

17.8 Answers

1. Classification of Type I cytokine receptors is based on structural biology, whereas classification of Th1 cytokine receptors is based on function. Type I cytokine receptors include both Th1 cytokine receptor (IL-2) and Th2 cytokine receptors (IL-4 and IL-5).
2. IL-1 β is a potent proinflammatory cytokine. HIV-1 infection, A β deposition, and the infiltration of activated T cell into the CNS will lead to IL-1 β production in microglia and macrophage. IL-1 β induces genes associated with inflammation and amplifies inflammation in the CNS particularly in the chronic state.
3. This inflammatory state is highly regulated, usually self-limited, and differs from the inflammation in peripheral tissues. The threshold to initiate immune responses against antigens in CNS is much higher than that in periphery.
4. There are few chemokine receptors that bind to a single ligand, and several chemokines can bind to multiple receptor
5. In HAD, HIV-1 infection of macrophage in the CNS leads to the activation of macrophage and microglia. Activated macrophage and microglia release cytokines, and chemokines that can be detrimental to neurons. Neuronal injury recruits additional macrophages. This process of injury, recruitment, and activation forms a positive feedback loop and advancing the disease state. In AD and MS, either A β priming or T cell-mediated of macrophage/microglia activation may utilize similar inflammatory cytokines and chemokines to cause the pathogenic effects on the CNS.
6. Chemokines are classified based upon the arrangement of two N-terminal cysteine pairs. Chemokines with two cysteines separated by one amino acid are called CXC or α with examples being SDF-1 (CXCL4) and IL-8 (CXCL8). Chemokines with two cysteines immediately next to each other are called CC or β chemokines, with examples including MCP-1 (CCL2), MIP-1 α/β (CCL3,4), and RANTES (CCL5). The two remaining groups are called C or δ chemokines and CX3C or γ chemokines and are represented by Lymphotactin (XCL1) and Fractalkine (CX3CL1).
7. CCR5, CXCR4, and CX3CR1.
8. Targeting IL-17A with neutralizing antibodies has become a new therapeutic approach in treating multiple sclerosis.
9. Because of the great diversity among cytokines, there are nearly limitless potential therapeutic avenues through their manipulation. The use of anti-inflammatory cytokines such as TGF- β and IL-10 may be useful for preventing or limiting excessive inflammation in some neurodegenerative disorders. On the other hand, inflammatory cytokines such as IFN- γ or TNF- α may potentially be used to enhance the immune response, perhaps

facilitating the removal of immune insults such as HIV or A β protein aggregates. One final approach may be through the manipulation of survival pathways; for instance, pro-apoptotic factor TRAIL may have therapeutic uses for clearance of infected cells, whereas BDNF may be a useful agent to enhance neuron viability. It is important to keep in mind a major pitfall is the means of delivery for this type of treatment and ensuring the specificity of response to the area of insult.

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Growth and Neurotrophic Factors in HIV-Associated Neurocognitive Disorders

18

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Abstract

The advent of combination antiretroviral therapy (cART) has successfully controlled replication of the human immunodeficiency virus (HIV) leading to reduced morbidity and increased longevity of infected people. Paradoxically however, as patients live longer lives, the prevalence of HIV-associated cognitive impairments has risen due to drug toxicity and its limited cART central nervous system (CNS) penetrance. Additionally, the presence of HIV proteins, which are not impacted by antiretrovirals, are a source of neuroinflammation. As a result, almost 50 % of infected individuals on effective cART exhibit various forms of HIV-associated neurocognitive disorders (HAND) whose mechanism(s) include deficits in calcium flux, excitotoxicity, cell signaling, oxidative stress and autophagy. Additionally, defects in the processing and functioning of neurotrophic factors such as fibroblast growth factors, brain-derived growth factor, insulin-like growth factor, platelet-derived growth factor, and glial cell derived neurotrophic factor have also been implicated in disruption of neuronal function. Importantly, these factors have been shown to interfere with the fundamental mechanism of apoptotic cell death, the underlying feature of HAND and other neurodegenerative diseases. Additionally, neurotrophic factors are also capable of promoting neuronal differentiation, neurogenesis and angiogenesis, thereby facilitating repair. Currently, there are no effective adjunctive treatments for disease. Thus, identifying novel neuroprotective molecules for treating such diseases is highly warranted in this field. This chapter discusses the beneficial roles of those neurotrophic and growth factors in the context of potential HIV therapies.

Keywords

Brain-derived growth factor • Fibroblast growth factors • Glial cell derived neurotrophic factor • HIV-associated neurocognitive disorders • Human immunodeficiency virus infection • Insulin-like growth factor • Platelet-derived growth factor • Neurodegeneration • Neuroinflammation

18.1 Introduction

Currently over 40 million people are living with HIV-1 worldwide. The advent of combination antiretroviral therapy (cART) has successfully controlled replication of the virus leading to increased longevity of those infected. As a result, disease morbidities and mortality rates for patients with HIV has improved dramatically. Paradoxically however, increased survival rates resulting from broad cART availability and usage have also resulted in an undesirable increase in the

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prevalence of mild neurocognitive impairments collectively referred to as HIV-associated neurocognitive disorders (HAND). Clinically, HAND is associated with reduced quality of life, memory loss, and social and occupational disabilities. Pathologically, in the most severe forms, patients can exhibit protracted forms of HIV-encephalitis (HIVE) and in the most mild subtle evidence gliosis. Pathological findings, however, do not always correlate with disease signs and symptoms and patients manifest with common and subtle cognitive deficits rather than overt dementia. When neurodegenerative pathology is seen it is characterized by involvement of cortical and subcortical regions (milder phenotypes) and simplification of the synaptodendritic structure of neuronal populations in the neocortex and hippocampus.

Central nervous system (CNS) homeostasis is a fine balance between the presence of neurotrophic and neurotoxic factors. While there is abundant information on the neurotoxic factors, the neurotrophic factors have been rather understudied and include brain derived neurotrophic factor (BDNF), fibroblast growth factor (FGF), nerve growth factor (NGF) and glial cell derived neurotrophic factor (GDNF) have been implicated in the protection of neurons against various neurotoxins (Alzheimer and Werner 2002; Deierborg et al. 2008; Almeida et al. 2005; Colafrancesco and Villoslada 2011). Downregulation of BDNF follows HIV infection leading to imbalance of anti- and pro-apoptotic neurotrophins (Avdoshina et al. 2011; Bachis et al. 2012; Mocchetti et al. 2007; Nosheny et al. 2007; Bachis and Mocchetti 2005). Findings from our lab have identified yet another factor, platelet-derived growth factor (PDGF) that plays a crucial role in reversing neuronal toxicity mediated by HIV proteins, Trans-activator of transcription (Tat) and HIV-1 gp120 (Yao et al. 2009). Neurotrophic factors exert their effects by preventing synaptic and axonal degeneration that follows neurotoxin exposures. These factors interfere with the fundamental mechanism of apoptotic cell death; the underlying feature of many neurodegenerative diseases. Additionally, neurotrophic factors are also capable of promoting neuronal differentiation, neurogenesis and angiogenesis, thereby facilitating repair (Avdoshina et al. 2011; Bachis et al. 2012; Mocchetti et al. 2007; Nosheny et al. 2007; Bachis and Mocchetti 2005). Neurotrophic factors can promote axonal growth and maintain normal neuronal morphology. For HAND, a loss of expression of neurotrophic factors and/or their receptors can impair neural signaling and affect disease. Although cART can inhibit systemic virus replication, HIV proteins (not impacted by cART) such as Tat or HIV-1 gp120 present in CNS and the lymph node tissues can induce neurotoxicity. Viral proteins interfere with neuronal survival by a number of mechanisms including the production of free radicals, nitric oxide, and the release of glutamate, inflammatory cytokines or other excitotoxins (Kaul et al. 2001). Additionally, neurotrophic factors are also capable of promoting neuronal

differentiation, neurogenesis and angiogenesis, thereby facilitating repair. Currently, there are no effective treatments for most neurodegenerative diseases. Thus, identifying novel neuroprotective molecules for treating such diseases is highly warranted. This chapter discusses the beneficial roles of several neurotrophic factors such as FGF, BDNF, IGF, PDGF, and GDNF in the context of HIV infection.

18.2 Fibroblast Growth Factor (FGF)

The superfamily of FGF encompasses 18 family members that are grouped into several subfamilies based on their sequence homology and phylogeny. Originally this superfamily included four additional ligands, now termed FGF homologous factors (previously FGF11-14; now FHF1-4), that have since been removed (Goldfarb et al. 2007) as they are not capable of activating FGF receptors although they share structural similarity with other member ligands. The 18 ligands bind to four distinct membrane-bound FGF receptor subtypes with differing affinities (Reuss and von Bohlen und Halbach 2003). Following binding to the ligand, FGF receptors undergo dimerization and phosphorylation followed by activation of three primary downstream signaling pathways: RAS/mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)/AKT and phospholipase gamma (Fig. 18.1).

Ten of eighteen FGFs are expressed in the brain (Goldfarb et al. 2007) with the highest abundance for FGF1 and FGF2 in the adult brain (Turner et al. 2006; Wilcox and Unnerstall 1991; Gomez-Pinilla et al. 1992). FGF2, also known as basic fibroblast growth factor, was the first FGF to be cloned in the rat (Kurokawa et al. 1988). Among the various brain regions, hippocampus and the cortical regions exhibit the highest expression of FGF2 (Gomez-Pinilla et al. 1994). At the cellular level, FGF2 is expressed in both neurons and glial cells with astrocytes representing increased expression (Gonzalez et al. 1995). FGF1, known as acidic fibroblast growth factor, is predominantly expressed in neurons and relatively little outside of the nervous system (Goodrich et al. 1989). FGFs have been well characterized for their multifarious roles in neuronal differentiation, survival as well as angiogenesis in the CNS. FGFs have also been shown to promote gliogenesis and neurogenesis when added to cultures of precursor cells from various brain areas (Vescovi et al. 1993; Qian et al. 1997) or when expressed in developing (Raballo et al. 2000) or adult brains in vivo (Shihabuddin et al. 1997; Yoshimura et al. 2001). Additionally, the neuroprotective roles of FGFs have also been demonstrated in neurons exposed to toxic insults (Frim et al. 1993; Zechel et al. 2010). Specifically, FGF2 has been demonstrated to inhibit excitotoxicity associated with excessive glutamatergic stimulation (Fernandez-Sanchez and Novelli 1993; Kirschner et al. 1995; Brandoli

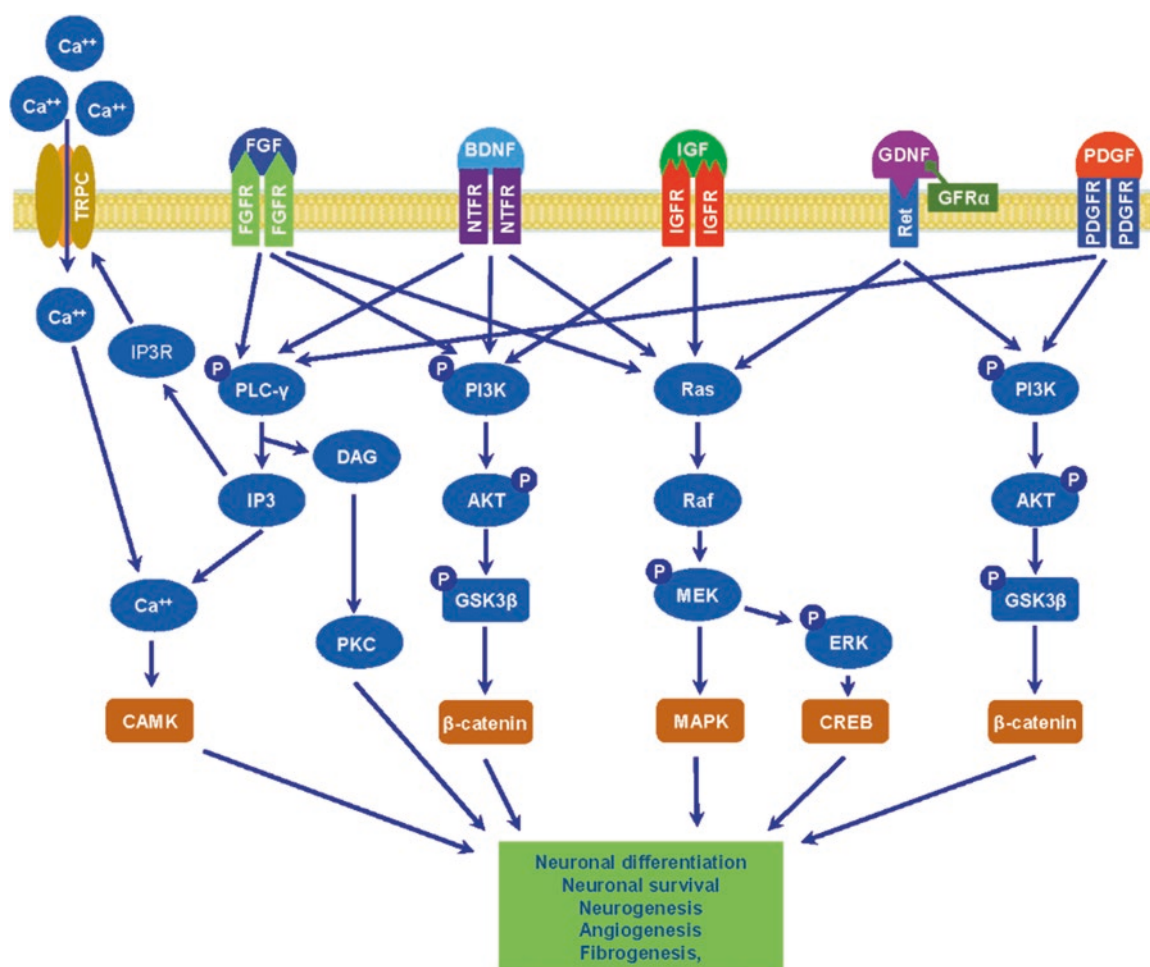


Fig. 18.1 Schematic representation of growth and neurotrophic factors signaling in HAND. After HIV infection, the HIV proteins and other cellular neurotoxins involved in the activation of various signaling

through its receptor interactions thereby affecting/modulating the downstream signaling processes as an outcome these pathways intensify the neurotoxic effects of HIV proteins

et al. 1998) while also reducing inflammatory responses and improving recovery of neuronal function following mechanical injury or stroke (Peterson et al. 1996; Kawamata et al. 1997; Teng et al. 1999).

Based on the ability of FGFs in exerting protective effects on mature neurons exposed to toxic stimuli, it has been proposed that enhanced expression of these neurotrophic factors could be of therapeutic value as a mechanism to protect the CNS against toxicity mediated by HIV proteins such as Tat and gp120 (Everall et al. 2001; Langford et al. 2002). In fact, FGF1 levels have been shown to inversely correlate with neurotoxic effects observed in HIV patients (Everall et al. 2001). For example, in archived brain tissues from HIV positive encephalitic patients with no obvious signs of neurodegeneration, FGF1 levels were significantly elevated compared with the levels of the neurotrophic factor in HIV positive individuals exhibiting both encephalitis and signs of neurodegeneration (Crews et al. 2009; Everall et al. 2001). In vitro studies using primary human neuronal cultures have

also demonstrated that FGF1 protected the neurons against gp120 toxicity in a dose-dependent manner (Everall et al. 2001) and that FGF1 overexpression ameliorated neurodegeneration in gp120 expressing transgenic mice in vivo (Crews et al. 2009). In addition to the damage to neurons, gp120 also mediates injury to endothelial cells lining the blood-brain barrier (BBB). This in turn leads to breach of the BBB integrity resulting ultimately into enhanced migration of HIV-infected cells from the periphery into the brain (Nakamuta et al. 2008; Yang et al. 2009a).

FGF2 that is produced primarily by astrocytes has the ability to promote endothelial cell fitness and angiogenesis. For example, pretreatment of human umbilical vein endothelial cells with FGF2 protected cells from gp120-induced angiotoxicity. It was demonstrated that ERK signaling pathways was responsible for these protective effects of FGF2 (Langford et al. 2005). Other possible protective roles for FGFs in the CNS include the dampening of the pathology and symptomatology resulting from excessive glutamate and

the ensuing neuroinflammation in HIV patients. In fact, both FGF1 and 2 have been shown to reduce the expression of CXCR4, a chemokine receptor that mediates the entry of T-tropic HIV (Dittmar et al. 1997). Furthermore, it was also shown that FGFs levels inversely correlated with CXCR4 expression in postmortem AIDS brains (Sanders et al. 2000). This could explain in part, why reduced expression of FGFs, in turn, lead to enhanced neuroinflammation and increased virus replication, thereby contributing to exacerbated progression to HAND.

HIV patient brains exhibit not only morphological and structural alterations in mature neurons, such as decreased synapse numbers, reduced spine density and neuronal loss, but also significant defects in adult hippocampal neurogenesis (Avraham et al. 2014; Lee et al. 2013). Similar defects in neurogenesis that are observed in HIV patient brains have been also recapitulated in a rodent model of HAND—the gp120 transgenic mice (Okamoto et al. 2007). Interestingly, over-expression of FGF1 (Crews et al. 2009) diminishes these neurogenesis defects as indicated by increased numbers of doublecortin expressing neurons (Langford et al. 2005). Based on these findings, it has been suggested that the mechanism(s) responsible for impaired neurogenesis during HIV infection involve neurotoxic effects of HIV proteins such as gp120, vpr, and Tat on neuronal progenitor cells through dysregulation of the neurotrophic factor signaling pathways. In the setting of HIV-mediated encephalitis, alterations in GSK3 β (Maggirwar et al. 1999; Nguyen et al. 2009; Kehn-Hall et al. 2011) and abnormal activation of CDK5 (Patrick et al. 2011; Lee et al. 2013) have also been suggested to play important roles in the culmination of neurodegeneration and ultimately HAND. It was shown that over-activation of CDK5 resulted in hyperphosphorylation of collapsing-response mediator protein-2 (CRPM2) and decreased neurite outgrowth. Reciprocally, inhibition of CDK5, or expression of a non-phosphorylatable (S522A) CRPM2 construct in adult hippocampal neurons reversed neurite outgrowth deficits (Lee et al. 2013). Likewise, CDK5-mediated CRPM2 phosphorylation was significantly increased in the hippocampus of HIV positive subjects and in gp120 transgenic mice, and these effects were rescued by genetic down-modulation of CDK5 in the mouse model (Crews et al. 2011). These results thus underscore that abnormal CDK5 activation and CRPM2 hyperphosphorylation could contribute to defective neurogenesis in neurodegenerative disorders such as HIVE (Crews et al. 2011).

Taken together, these reports highlight the fact that FGFs, especially FGF1 and FGF2 play critical roles in the CNS, not only by supporting the survival of mature neurons exposed to toxic stimuli, but also by promoting neurogenesis and angiogenesis to ensure normal brain functioning and homeostasis. FGFs are also beneficial in enhancing neuronal survival and protection against toxic insults in the HIV infected brain.

Development of FGFs as therapeutic agents could thus serve as alternative approaches for ameliorating functional deficits associated with HAND.

18.3 Brain-Derived Neurotrophic Factor (BDNF)

BDNF is a member of the neurotrophin family that includes NGF and the neurotrophins NT-3 and NT-4. In general, BDNF supports the survival of mature neurons and promotes the neurogenesis and differentiation of new neurons in the CNS and the peripheral nervous system. In the brain, BDNF is expressed in the hippocampus, cortex, and basal forebrain—areas vital for learning and memory. BDNF primarily binds to two types of receptors: TrkB and the LINGFR (low-affinity nerve growth factor receptor, also known as p75). Pro-BDNF and mature BDNF have different binding affinities for TrkB and p75. Pro-BDNF binds with a higher affinity to p75 while mature BDNF has a preference for TrkB. Such distinct binding patterns could likely explain the differences in cellular fates induced by pro- or mature BDNF exposure; with pro-BDNF binding to p75 resulting in pro-apoptotic signaling while mature BDNF predominantly supporting neuronal survival. Mechanistically, BDNF executes its functions by enhancing glutamatergic excitatory signaling (elevations of NMDA and AMPA Receptors activity) and preventing the GABAergic inhibitory signaling strength. BDNF play critical roles in neuronal survival and growth. Impaired functioning of BDNF has been implicated in many neurodegenerative disorders including Alzheimer's disease (AD), drug addiction and schizophrenia. For example, in the brains of AD patients, there is alteration in BDNF transport resulting in degeneration of selected neuronal populations located in the basal nucleus of Meynert and hippocampus (Mufson et al. 1989; Mufson and Kordower 1992; Poon et al. 2013). Furthermore, reduced BDNF release/levels have also been shown to closely correlate with depression (Duman 2004) and Huntington's disease (Zuccato and Cattaneo 2009).

Recent evidence has also shown that BDNF may play a neuroprotective role in the context of HIV-1. BDNF has been shown to protect neurons against gp120-mediated toxicity both in vivo and in vitro (Bachis et al. 2003) by regulating neuronal expression of the HIV co-receptor, CXCR4. This receptor is abundant in neurons and areas of the CNS such as the cortex, hippocampus and striatum (Ahmed et al. 2008). Increasing evidence shows that neuronal CXCR4 is a target of BDNF. Indeed, previous studies have shown that BDNF decreases the expression of CXCR4 (Nosheny et al. 2007; Bachis et al. 2003). Conversely, heterozygous BDNF knockout animals exhibited increased levels of CXCR4 mRNA in the cortex, hippocampus and striatum compared with the wild type controls (Ahmed et al. 2008). Moreover, in these mice,

increased CXCR4 expression correlated with a more robust neurotoxic effect of gp120 (Nosheny et al. 2004). Thus, BDNF is particularly important as a neuroprotective factor against HIV envelope gp120 mediated neurotoxicity, which is primarily transmitted via the CXCR4 receptors. Furthermore, BDNF has also been shown to reduce glial expression of CXCR4, leading in turn, to effective blocking of virus infection in microglia and astrocytes in the CNS (Nosheny et al. 2004). Mechanistically, BDNF initially binds to the tyrosine kinase receptor, TrkB, followed by induction of receptor dimerization (Fig. 18.1). This in turn, leads to increased activation of ERK signaling which culminates in decreased expression of CXCR4, blocking of caspase-3 and decreased gp120-mediated neuronal apoptosis (Bachis et al. 2003). CXCR4 internalization is important for BDNF-mediated down-regulation of CXCR4 levels (Bachis et al. 2003; Mocchetti and Bachis 2004; Mocchetti et al. 2007, 2008). Intriguingly, another study validating these findings demonstrated that BDNF was not able to prevent gp120 neurotoxicity in neurons that did not express TrkB (Ahmed et al. 2008).

There are other mechanisms as well, by which BDNF exerts its beneficial effects in the context of HIV. BDNF supports the survival of T cells (Maroder et al. 1996; De Santi et al. 2009), which are the key cells that are depleted in AIDS patients. BDNF thus exerts its effects by maintaining the pool of CD4⁺T cells thereby delaying the progression to AIDS. Preservation of CD4⁺T cells can in turn, can have broader implications for CNS functioning. It has been demonstrated that onset of severe immunodeficiency precedes progressive neurological deficits. For example, it has been shown that during a state of chronic inflammation induced in a non-human primate following infection with simian immunodeficiency virus, the brain is positive for peripheral circulating monocytes trafficking from the bone marrow (Burdo et al. 2010). Inflammation in this case correlated with the severity of encephalitis. It has been shown that uncontrolled activation of cytokine and chemokine receptors in the context of HIV infection leads to dendritic beading and loss of dendritic spines (Suzumura et al. 2006). These changes are accompanied by failure of long-term potentiation (LTP), which in turn, underlies learning and memory deficits. BDNF rescues neuronal deficits by promoting dendritic branching and spine morphology (Horch and Katz 2002; Tanaka et al. 2008), which are crucial for BDNF-mediated LTP (Figurov et al. 1996). This in part, explains how BDNF maintains neuronal structural integrity and morphology while abrogating memory and learning deficits.

HIV proteins have been shown to regulate BDNF levels in the brain. For example in the HIV brain, gp120 and TNF α antagonized BDNF functions by inhibiting the anterograde transport of BDNF, reducing the intracellular stores and stimulating the NMDA receptor activity (excitotoxicity), leading in turn, to reduced neuronal survival (Bachis et al. 2003;

Nosheny et al. 2004). HIV also inhibits BDNF activity via an indirect mechanism via stimulation of TNF α . TNF α has been shown to prevent glucocorticoid receptor (GR) activity, leading to reduction of hippocampal neurogenesis. HIV-induced neuroinflammation thus indirectly leads to neurodegeneration by silencing GR activity (Suri and Vaidya 2013). Additionally, BDNF pro-survival effect is also reduced as HIV downregulates its levels in lymphocytes, which in turn, leads to T-cell apoptosis and neurodegeneration (Avdoshina et al. 2011). The molecular mechanism(s) underlying down-regulation of BDNF by HIV remain unclear. Recent data however, suggests that gp120 reduced pro-BDNF processing in neurons by decreasing furin levels and by effectively altering the balance of anti- and pro-apoptotic neurotrophins (Bachis et al. 2012). Another study also showed that in the older patients with HIV or with HIVE exhibited decreased levels of BDNF and increase levels of pro-BDNF protein.

Several cellular mechanisms are involved in the processing of pro-BDNF into mature BDNF. ProBDNF is cleaved within the endoplasmic reticulum by furin and in regulated secretory vesicles by proconvertase enzymes. If proBDNF reaches the extracellular milieu, it is processed by plasmin, and the mature BDNF produced then activate cell surface TrkB receptors (Pang et al. 2004). On the other hand, extracellular proBDNF binds with p75NTR and become endocytosed and then cleaved to produce mature BDNF that either activates TrkB within endosomes (Nykjaer et al. 2004) or is recycled to the cell surface (Pang et al. 2004). The site of BDNF translation within the neuron determines the form of BDNF released. BDNF mRNA with a short 3' UTR accumulates in the neuronal soma, whereas BDNF mRNA with a long 3' UTR is trafficked to dendrites. The soma supports BDNF cleavage within the Golgi, but the majority of dendrites lack Golgi elements necessary for processing of proBDNF, and therefore proBDNF is the predominant form released (Barker 2009; Yang et al. 2009b). Conversion of pro-BDNF to mature BDNF is an important process for synaptic plasticity. In fact, pro-BDNF can bind with high affinity to p75NTR (Greenberg et al. 2009) and promote neuronal loss (Teng et al. 2005). The altered mBDNF/proBDNF ratio in HAND could thus compromise synaptic connections and neuronal survival. These findings were recently corroborated by a report demonstrating that reduced neurite out growth and impaired neurogenesis was reversed by exercise via the production of BDNF in a gp120 transgenic mouse model (Lee et al. 2013).

Taken together, it is reasonable to suggest that HIV promotes neuronal degeneration by lowering BDNF levels and that BDNF treatment could be considered a beneficial therapeutic approach for ameliorating complications of the CNS in HIV-infected patients. Corroborating this was the observation that both brain (Bachis et al. 2012) and serum (Avdoshina et al. 2011) BDNF levels were significantly lower in HIV positive individuals compared with the HIV

negative subjects. Furthermore, low levels of BDNF have been shown to impair the innervation of the cortex by serotonergic fibers (Lyons et al. 1999). Similarly, gp120-mediated reduction of BDNF levels appears to cause neuronal degeneration even in the absence of inflammation (Nosheny et al. 2004). In summary, these findings suggest that low levels and activity of BDNF could be a risk factor for the development of HAND and that delivery of BDNF to neurons could be an effective means of treating HIV-associated neurodegeneration.

18.4 Insulin-Like Growth Factors

Insulin-like growth factors (IGFs) are signaling protein molecules that regulate key cellular activities, specifically cell proliferation, differentiation, and apoptosis. Recent reports have shown that IGFs are potential neurotherapeutic agents that modulate brain development and plasticity (Bondy and Cheng 2002; Ciucci et al. 2007; Tropea et al. 2006). IGF signaling is an intricate system, comprises of two ligands, IGF-1 and IGF-2, two-cell surface receptors (IGF1R and IGF2R), and a family of six high-affinity IGF-binding proteins (IGFBPs) and four low-affinity IGFBPs. The sequence homology of IGF-1 and IGF-2 are similar to each other as well as with proinsulin (Daughaday and Rotwein 1989; Rotwein 1991). IGF-1 and IGF-2 normally bind to the insulin/IGF family of cell surface receptors and activate their intrinsic tyrosine kinase activities. Since IGF-1 has a higher binding affinity to IGF1R than that of insulin receptor, IGF1R is commonly referred to as a physiological receptor. IGF2R on the other hand is commonly referred as a clearance receptor, because IGF2R can only bind with IGF-2 thereby sequestering the IGF-2 and preventing IGF-2 mediated downstream signaling (Nakae et al. 2001). IGF signaling elicits pleiotropic activities such as proliferation, maturation, survival, and/or growth of most neural cells including neural stem cells, lineage restricted neural precursor cells, post-mitotic neurons, oligodendrocytes and astrocytes in the developing brain. The biological activities of IGFs however, seem to vary based on the specific cell types, the local microenvironment, and the stage of development (O'Kusky and Ye 2012).

IGF-1, a small polypeptide trophic hormone, is mainly synthesized in the liver and is transported to the brain simply by IGFBPs, found in the plasma and cerebrospinal fluid (Jones and Clemmons 1995). IGF1 is also produced by all the cell types in the brain, and its expression is highest perinatally in regions such as the brainstem, cerebellum, cortex, hippocampus, hypothalamus and the spinal cord. The primary functions of IGF-1 include regulation of development, cell differentiation, plasticity, and survival of the nervous system (Benarroch 2012). In addition, IGF-1 is involved in regulating brain development and promoting neuroprotection following cellular

insult, neurogenesis, myelination and synaptogenesis. IGF-1 binds to the cell surface IGF1R, which is expressed specifically during the development of the brain or in response to injury, thereby producing growth-promoting signals. IGF-1 mediated canonical signaling through the IGF-1 receptor comprises of phosphoinositide-3-kinase-AKT-FOXO, and RAS-MAPK signaling pathways (Fig. 18.1). These signaling pathways modulate active gene transcription and activate numerous downstream kinases and phosphatases, that eventually perturb vital cellular functions including protein synthesis, autophagy, apoptosis and protection from oxidative stress associated with immunity and inflammation (Fernandez and Torres-Aleman 2012). In addition, IGF-1 has an essential role in the promotion of dorsal root ganglion neuronal development and peripheral axonal regeneration as well as neuritogenesis (Kimpinski et al. 1997; Jones et al. 2003). Essentially, the effects of IGF-1 are more prominent in the adult brain than in the developing brain (Bondy and Lee 1993).

The association between IGF axis and HIV is very complex. HIV infection is frequently interconnected with a notable decline in the IGF-1, IGF-2, and IGFBP-3 levels with a significant elevation in IGFBP-1 and IGFBP-2 concentrations. In recent years, IGF-1 has gained significant attention for its modulatory role in adult neurogenesis (Aberg et al. 2000, 2003; Lichtenwalner et al. 2001). In addition, circulating IGF-1 plays an essential role in the mediation of exercise-induced augmentation of hippocampal neurogenesis in the adult brain (Trejo et al. 2001). IGF-1 also acts as a neuroprotective agent against neuronal death induced by supernatants from HIV-1-infected macrophages in vitro (Ying Wang et al. 2003). Low levels of IGF-1 in conjunction with elevated levels of TNF- α are known to exacerbate HIV-1 mediated neurodegeneration (Kumar et al. 2003). In addition, low levels of peripheral IGF-1 have been reported in HIV-patients with wasting syndrome and in children with failure to thrive. Since IGF-1 acts as a primary mediator for growth hormone action, its role in anabolic effects has encouraged studies on IGF-1 levels in HIV-infected patients and the use of IGF-1 and growth hormone in the treatment of cachectic patients (Wilk et al. 2011; Mynarcik et al. 1999). However, few studies suggest limited resistance to IGF-1 and growth hormone therapies in the setting of HIV-1 wasting syndrome (Jain et al. 1998). Therefore, diminished levels of IGF-I in the CNS or alternatively development of insulin resistance may compromise neuronal survival during HIV infection.

18.5 Glial-Derived Neurotrophic Factor

Glial-derived neurotrophic factor (GDNF) was originally isolated from the supernatant fluids of cultured rat glioma cells and was shown to have profound effects on the survival and

differentiation of motor, and dopaminergic neurons of the midbrain and striatum (Henderson et al. 1994; Yan et al. 1995). GDNF exerts its neurotrophic effects on variety of cells such as the sensory and autonomic ganglia (Buj-Bello et al. 1995; Ebendal et al. 1995), Purkinje cells of the cerebellum (Mount et al. 1995), hippocampal neurons (Martin et al. 1995), as well as noradrenergic (Arenas et al. 1995), serotonergic (Ohta et al. 2002), and cholinergic neurons (Williams et al. 1996). GDNF belongs to a family of neurotrophic factors, which are comprised of four proteins—artemin, GDNF, neurturin, and persephin. These neurotrophic factors belong to the transforming growth factor- β (TGF- β) superfamily. GDNF however, is distinct and demonstrates limited amino acid sequence homology with other members of the superfamily. The conformational similarity of GDNF is significantly conserved with other members of the superfamily. All the members of this family function as homodimers to elicit both protective and restorative signaling in the developing and adult CNS (Allen et al. 2013; Airaksinen and Saarma 2002). GDNF and its associated family members mediate their signaling through the canonical signaling receptor Ret and a glycosyl-phosphatidylinositol-anchored co-receptor, GDNF family receptor α (GFR α) (Fig. 18.1). There are four isoforms of GFR α such as, GFR α 1, GFR α 2, GFR α 3 and GFR α 4 and that have been shown to bind to the ligands artemin, GDNF, neurturin and persephin, respectively (Naveilhan et al. 1998).

This family of neurotrophic factors has been shown to play key roles in diverse biological processes including cell survival, differentiation and migration, as well as neurite outgrowth (Lin et al. 1993; Tomac et al. 1995). GDNF has been demonstrated to promote exclusively the survival of dopaminergic neuronal cells in a mouse model of Parkinson's disease (Hegarty et al. 2014; Zhao et al. 2014). These studies implicate GDNF as a promising therapeutic agent for the treatment of Parkinson's disease (Yasuda and Mochizuki 2010; Hegarty et al. 2014). GDNF has also been shown to protect neonatal and adult facial motor neurons from axotomy-mediated cell death (Yan et al. 1995). Similar to GDNF, neurturin has also been shown to protect non-human primate dopaminergic neurons from 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced neurotoxicity (Grondin et al. 2008).

The neurotrophic potential of GDNF is specifically important for HAND, especially in the late phase of HIV-associated dementia (HAD). During this late stage, the manifested clinical features of HAD resemble to those observed in Parkinson's disease, such as bradykinesia, loss of fine motor skills, involuntary movements and postural instability (Berger and Nath 1997; Mocchetti et al. 2014). It can be envisioned that GDNF can be considered a potential therapeutic agent for HAD. Along these lines and similar to the findings for PDGF

(described above), intrastriatal microinjections of HIV-1 envelope protein gp120 significantly reduces levels of GDNF in the rat substantia nigra thereby causing retrograde degeneration of nigrostriatal neurons (Nossheny et al. 2006). Using the same model the authors have demonstrated chronic and progressive neurodegeneration mediated by gp120 (Nossheny et al. 2006). Interestingly similar to the experimental model, GDNF expression was notably diminished in the substantia nigra in patients with as compared to those without dementia. Altogether, the neurotrophic potential of GDNF is vital for protecting the survival of substantia nigra neurons in HIV-1 patients (Mocchetti et al. 2014).

18.6 Platelet-Derived Growth Factor

Platelet-derived growth factor (PDGF) is a family of mesenchymal cysteine-knot-type mitogens and is synthesized in several tissues including brain cells such as neuronal progenitors, neurons, astrocytes, oligodendrocytes, and Schwann cells in the peripheral nervous system. This growth factor acts in an autocrine and paracrine manner and plays a key role from gastrulation period to adult neuronal maintenance. This growth factor family consists of four chains (five isoforms), which are disulfide-linked to form homo- or heterodimers of A-, B-, C-, and D-polypeptide chains, i.e. PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC, and PDGF-DD (Heldin and Westermark 1999). These growth factors elicit their biological functions on cells through binding with their cognate receptors, namely PDGF receptor (PDGFR)- α , and PDGFR- β by their receptor tyrosine kinase activity. Binding of PDGF stimulates PDGFR dimerization, which further initiates downstream signaling. Based on PDGF isoforms and the pattern of receptor expression, specific receptor dimers play key roles in eliciting signaling specific to a particular cell. Typically, PDGFR- α binds to the A-, B-, and C-chains of PDGF, while PDGFR- β binds to the B- and D-chains of PDGF (Andrae et al. 2008; Chen et al. 2013).

Though the mitogenic activity of PDGFs in most of the cell types has been well studied, their key roles in various disorders of the CNS are a subject of extensive exploration. PDGF-A is also shown as a potent mitogen for oligodendrocyte progenitors, whereas postnatally surviving PDGF-A null mice are developed tremor owing to severe deficiency of myelination in the central nervous system (Betsholtz 2004; Fruttiger et al. 1999). In addition, PDGF-BB has been shown to protect primary hippocampal neurons from glutamate-induced neuronal injury by up-regulation of PDGFR- β signaling, especially targeting NR2B receptors (Beazely et al. 2009). Several studies have demonstrated the neuroprotective potential of PDGFs via their role in guarding neuronal/brain cells from direct in vitro glutamate-induced

excitotoxicity and hypoxic-ischemic injury (Egawa-Tsuzuki et al. 2004; Tseng and Dichter 2005; Ishii et al. 2006). In addition, PDGFs and PDGFR activation is essential for neuronal progenitor cells differentiation into neurons (Erlandsson et al. 2001; Williams et al. 1997) and is shown to protect hippocampal neurons from oxidative insults and energy deprivation (Cheng and Mattson 1995). Additionally, PDGFR activation has also been shown to be protective in the context of Alzheimer's disease pathology, possibly by inhibiting NMDA receptors. Taken together these studies suggest a neuroprotective effect of PDGF produced by neurons themselves.

HIV infection primarily targets the basal ganglia region of the brain leading to dopaminergic neuron degeneration (Nath et al. 2000) with the development of HIV-associated dementia and other neurological complications at the end stage of disease (Mocchetti et al. 2007). Recent investigations have also revealed the novel neuroprotective role of PDGFs that function by reversing neuronal toxicity induced by HIV-1 proteins Tat and gp120 in the primary midbrain neurons (Yao et al. 2009, 2014) as well as in the SHSY-5Y neuroblastoma cells (Peng et al. 2008a, b; Zhu et al. 2009). Furthermore, it was also shown that the neuroprotective potential of PDGF was mediated through the transient receptor potential canonical channels (TRPC), which are Ca^{2+} -permeable, non-selective cationic channels that play key roles in various physiological functions including neuronal survival (Fig. 18.1). In keeping with the cell culture findings, it was further validated that PDGF-mediated neuroprotection was attenuated in mice pretreated with the TRPC blocker. Additional molecular mechanism(s) underlying PDGF-mediated neuroprotection against HIV-1 protein toxicity include activation of PI3K/AKT and ERK/CREB pathways and concomitant inactivation of the mediators of the proapoptotic pathway. In addition to its role in neuroprotection, PDGF-BB also promotes differentiation of rat hippocampal neural progenitor cells through activation of TRPC channels, influx of calcium, and activation of ERK signaling and the downstream transcription factor, CREB thereby reversing the impairment mediated by HIV-1 Tat protein (Yang et al. 2015). PDGF exposure of neurons has also been shown to decrease the release of extracellular glutamate and induce upregulation of the synaptic plasticity proteins (Peng et al. 2008a, b; Tseng and Dichter 2005; Yao et al. 2009, 2014). Arc/Arg3.1 is one such synaptic plasticity protein, whose expression in the neurite has been implicated as an index of synaptic activation. Interestingly, it has been demonstrated that PDGF-BB regulates the expression of *Arc/Arg3.1* gene in the rat hippocampal neurons (Peng et al. 2010). Furthermore, it has been shown PDGF-CC also protects the rat cortical neurons against HIV-1 Tat protein-mediated neurotoxicity through activation of the TRPC and AKT signaling (Peng et al. 2012).

18.6.1 PDGF Paradox in HAND

While the PDGF family members play protective roles on neuronal fitness and survival, in the endothelial cells (Mermis et al. 2011; Yao et al. 2011) and astrocytes (Bethel-Brown et al. 2011, 2012) their effects are more cytotoxic. For example in the context of pulmonary hypertension associated with HIV-infection, it has been shown that exposure of HIV-1 proteins to pulmonary endothelial cells induced oxidative stress with subsequent activation of hypoxia inducible factor-1 α leading in turn, to increased expression of PDGF, that functions as a mitogen for the neighboring smooth muscle cells (Mermis et al. 2011). In addition, HIV-1 infection of human primary astrocytes resulted in increased expression of PDGF, which in turn, led to increased expression of the chemokine MCP-1 through the ERK1/2, JNK MAPK and NF κ B activation pathway. Increased MCP-1 expression, ultimately resulting in increased monocyte transmigration and disruption of the endothelial barrier permeability in the brains of individuals infected with HIV-1 (Bethel-Brown et al. 2012). Upregulated expression of PDGF in astrocytes is thus deleterious for endothelial cells.

Among all the cells of the CNS, the pericytes are known to express high constitutive levels of PDGF receptor. However, their role in the context of HIV infection is rather understudied (Nakagawa et al. 2012). It has been shown that pericytes potentiate and enhance the transcytosis of HIV-1 across brain microvascular endothelial cells via lipopolysaccharide-mediated release of inflammatory cytokines (Dohgu and Banks 2013). New findings have also shed light on the role of PDGF-BB in pericyte functioning under both physiological as well as pathological conditions (Lindahl et al. 1997; McCarty et al. 2007). For example, PDGF has been shown to increase the migration of retinal microvascular pericytes and plays an essential role in pericyte maintenance (Nadal et al. 2002). On the other hand, exposure of pericytes to HIV-1 Tat protein was shown to induce the expression and release of PDGF-B, which in turn, via the autocrine loop resulted in increased loss of pericytes from the endothelial barrier resulting in increased neuroinflammation (Niu et al. 2014). The mechanism of HIV-1 Tat-mediated expression of PDGF-BB in pericytes involved activation of the ERK and JNK MAPK pathways along with the downstream activation of NF- κ B (Niu et al. 2014).

18.7 Future Perspectives

The key challenge in the field of growth factor therapy is drug delivery to the CNS. Though these factors cross the BBB minimally via peripheral administration, direct administration of these factors into the CNS is essential in order to reach neurons of the brain or spinal cord. Furthermore, when these

growth and neurotrophic factors ‘flood’ the nervous system after direct administration into the CNS, they cause adverse effects that are intolerable over extended time-periods. Thus, the ideal system for administration of growth factors to the nervous system must achieve effective concentrations at precise sites of degenerating neurons, while limiting the spread to distant sites, which could generate adverse effects. In addition, delivery must be sustained over time. The absence of a safe and reliable delivery system for growth factor treatment in humans has limited the ability to investigate whether these proteins can effectively treat neurological diseases. Potential approaches that could solve the delivery problem of growth factors to the nervous system include intraparenchymal protein infusion, gene delivery using viral vectors, drug-induced increases in growth factors, and the use of peptide mimetics. Ongoing research in the field is aimed at addressing these challenges by improving gene therapy vectors to achieve persistent, safe and regulatable growth factor expression in the CNS; by enhancing the accuracy of vector and growth factor targeting to the brain using real-time monitoring during treatment; and by developing peptide mimetics of growth factors with specificity for degenerating cell systems. This could replace the need for invasive central administration and avoid adverse effects of non-targeted growth factor distribution.

18.8 Conclusions

Taken together the neurotrophic factors play critical role in the rescuing neurons from HIV protein mediated toxicity. So an effective therapy for HAND will require both cART and adjunct therapies that include neuro-protective and neuro-regenerative agents. Neurotrophic factors are well known for their pro-survival and pro-neurogenesis of neurons that make them perfect candidates as alternative agents for individuals living with HIV. Further effective treatment for HIV-mediated CNS diseases will definitely entail both cART and adjunctive therapies with neurotrophic factors, because these factors are considered as potent modulators of synaptic plasticity and neurogenesis in the adult CNS. Additionally, the biological effects mediated by these factors definitely play significant role in limiting the HIV-mediated neuronal degeneration following with stimulation of neuronal function. Though the effects of these agents are only examined in the cell lines/animal models of HAND, the therapeutic properties of neurotrophic factors provide incentive for further research and raise hope for the therapeutic possibilities in the near future. However, more research on this field is still needed for their clinical use in the future. Additionally, based on the paradoxical nature of most of these factors, caution needs to be exerted in the development and delivery of these agents in the CNS to avoid off-target effects.

18.9 Review Questions

1. Loss of expression of neurotrophic factors and/or their receptors in HIV-associated neurocognitive disorders results in:
 - (a) *Impaired neuronal signaling*
 - (b) Axonal growth
 - (c) Maintenance of normal neuronal morphology
 - (d) Neuronal differentiation
2. Which of the following downstream signaling pathways are activated following fibroblast growth factor binding to its receptors?
 - (a) RAS/mitogen-activated protein kinase signaling
 - (b) Phosphatidylinositol 3-kinase/AKT signaling
 - (c) Phospholipase gamma signaling
 - (d) *All the above*
3. Brain-derived neurotrophic factor primarily binds to:
 - (a) TrkB receptor
 - (b) Low-affinity nerve growth factor receptor
 - (c) Dopamine receptor
 - (d) *a and b*
4. In the context of HIV-1 infection, brain-derived neurotrophic factor elicits neuroprotective effects through:
 - (a) Superactivation of ERK signaling
 - (b) Blocking of caspase-3 expression
 - (c) Decreased expression of CXCR4
 - (d) *All the above*
5. HIV-1 mediated neurodegeneration is exacerbated by:
 - (a) *Low levels of IGF-1 with elevated levels of TNF- α*
 - (b) High levels of IGF-1 with low levels of TNF- α
 - (c) Low levels of TNF- α
 - (d) None of the above
6. GDNF belongs to a family of neurotrophic factors:
 - (a) Belong to the transforming growth factor- β superfamily
 - (b) Mediate their signaling through the canonical signaling receptor, Ret
 - (c) Promote exclusively the survival of dopaminergic neuronal cells
 - (d) *All the above*
7. Platelet-derived growth factors are produced by:
 - (a) Neuronal progenitor cells
 - (b) Neurons
 - (c) Oligodendrocytes
 - (d) *All the above*
8. Exposure of HIV-1 proteins to pulmonary endothelial cells induces oxidative stress with subsequent activation of hypoxia inducible factor-1 α leading to:
 - (a) *Increased expression of PDGF*
 - (b) Increased expression of BDNF
 - (c) Decreased expression of GDNF
 - (d) None of the above

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Tara E. Vanderweyde and Benjamin Wolozin

Abstract

Studies of the molecular genetics for Amyotrophic lateral sclerosis (ALS) and other motor neuron disorders have thrust a class of proteins, termed RNA binding proteins (RBPs), into the limelight. A striking number of mutations linked to motor neuron diseases occur in RBPs. The reason appears to derive from the unique structures and biology of RBP, as well as the unique abundance of these proteins in the extra-nuclear domains of neurons. The material discussed in this chapter will explicate the biology of RBPs, provide insight into how this contributes to the pathophysiology of neurodegenerative diseases, and also speculate on potential roles of RNA binding proteins in the inflammatory response.

Keywords

Aggregation • Amyotrophic lateral sclerosis • Prion • Protein synthesis • RNA binding proteins • RNA translation • Stress granules • Stress response

19.1 Overview

Regulating mRNA translation and protein synthesis allows a cell to rapidly alter the proteome in response to various signals. The discovery of RBPs, RNA granules, and their critical role in determining the fate and activity of mRNA transcripts has brought the importance of translational control into sharp focus. The interaction between RBPs and the

types of granules described here controls the stability and translational activity of mRNAs and plays a critical role in fine-tuning protein expression under both normal conditions and under conditions of stress (Adeli 2011).

The RBP family is comprised of about 800 proteins that share conserved domains structures and related functions. These RBPs generally contain two types of conserved domains: glycine rich domains and RNA recognition motifs (RRM). The glycine rich domain is hydrophobic and mediates the reversible aggregation of these proteins. The RRM have broad specificity, but differ in the spectrum of transcripts bound, and this specificity can also be dependent on the type of stress presented to the cell (Wolozin 2012; Paz et al. 2014). RBPs and the granules they form regulate all aspects of RNA biogenesis including RNA maturation, surveillance, transport, subcellular localization, translation, and RNA degradation. RBPs form dynamic interactions with coding, untranslated, and non-protein-coding RNAs in functional units called ribonucleoprotein (RNP) complexes. The RBPs within RNP complexes can remain stably bound to the RNA throughout its journey from synthesis to degradation, or associate with the RNAs selectively in a temporal and spatial manner (Lukong et al. 2008).

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The functions of RBPs can generally be divided into nuclear and cytoplasmic activities. In the nucleus, RBPs regulate mRNA maturation, including splicing, RNA helicase activity, RNA polymerase elongation, and nuclear export. In the cytoplasm, RBPs regulate RNA transport, silencing, translation, and degradation. These cytoplasmic RBPs regulate transcript activity and distribution by forming RNA granules that are macromolecular complexes containing RBPs, translational machinery, and mRNA transcripts consolidated to form granules through protein/protein interactions mediated by the glycine rich domains and protein/mRNA interactions mediated by RRM. RNA granules vary by molecular composition and function. RNA degradation is mediated by a type of RNA granule, termed Processing-bodies or P-bodies (PBs). Transport granules play important roles in neurons, where they move transcripts from the soma into the dendritic, and possibly axonal, arbors. Stress granules (SGs) are important for the mammalian stress response, sequestering mRNAs and allowing for dynamic sorting of mRNAs for translation, storage, or degradation to allow for cell survival (Wolozin 2012) (Fig. 19.1).

19.2 RBPs Exhibit Reversible Liquid to Solid Phase Transitions

The ability of RBPs to form granules represents a fundamental aspect of their biology. RBPs have an innate tendency to self-aggregate (Alberti et al. 2009). This ability is mostly determined by the low complexity glycine rich domains. For many RBPs, such as TIA1 cytotoxic granule associated protein (TIA1), these low complexity domains resemble the low complexity region of the yeast prion protein, Sup35, which is also a RBP (Phillips et al. 2004). The prion-like nature of these proteins provides insight into how the low complexity domains impact on RBP biology. Mathematical modeling shows that the low complexity domains function on the cusp of aggregation, so that small changes in their structure or environment can cause them to aggregate (Alberti et al. 2009).

Work by the McKnight group highlights the propensity of these proteins to aggregate (Han et al. 2012; Kato et al. 2012). The group generated recombinant forms of the low complexity domains from many different RBPs, but particularly focused on fused in sarcoma (FUS), which is linked to ALS. They showed that the low complexity recombinant protein could reversibly aggregate in a manner that was controlled by temperature and concentration (Han et al. 2012; Kato et al. 2012). The biological function of this aggregation propensity translates to an ability to serve as a scaffold for binding and sequestering transcripts. The McKnight group used this RNA binding ability to concentrate transcripts selectively bound by specific RBPs, such as FUS. This concept of reversible aggregation directly translates to disease. Several teams analyzed FUS and hnRNPA1, and showed that

disease-linked mutations shift the aggregation characteristics such that the liquid to solid phase transition occurs more quickly and becomes persistent after several transitions (Patel et al. 2015; Nott et al. 2015; Molliex et al. 2015; Lin et al. 2015). The persistence of the FUS granules suggests a mechanism through which mutations might cause disease by inducing formation of persistent FUS granules in the brain; the abnormal stability of these granules would lead to persistent sequestration of transcripts bound by FUS and proteins associated with FUS into the pathological granules (Patel et al. 2015). Thus, a gain of FUS aggregation would cause a loss of function by sequestering FUS, FUS binding proteins and FUS binding transcripts into pathologically persistent RNA granules (Patel et al. 2015). Multiple proteins that accumulate in neurodegenerative diseases contain prion-like and poly-glycine rich domains, and their aggregation mirrors that of proteins linked to neurodegenerative diseases (Gilks et al. 2004). Neurodegenerative-linked proteins Huntingtin, Prion Protein (PrP) and TAR DNA-binding protein 43 (TDP-43) have all been shown to associate with SGs, and mutations or malfunctions in some of these RBPs can directly cause neurodegeneration (Lukong et al. 2008).

19.3 RNA Granules

RBPs regulate all aspects of RNA biogenesis including RNA maturation, surveillance, transport, subcellular localization, translation, and RNA degradation. RBPs are able to interact with mRNA and other protein components and consolidate to form discrete granules in the cytoplasm, and based upon their composition and function can be identified as transport RNPs, SGs, or P-bodies. These RNA granules share common protein components, but each kind of RNA granule contains a distinct population of proteins and performs separate functions: (i) RNA transport granules deliver transcripts to dendrites while inhibiting RNA translational activity. RBPs such as fragile X mental retardation protein (FMRP) and Pumilio (PUM1) participate in dendritic transcript transport and function as translational repressors. (ii) Stress granules (SGs), which will be the main focus of this review, form transiently to reprogram RNA translation under stressful conditions. The primary nucleating SG proteins include TIA-1, G3BP, TIA-1 related protein (TIAR), FMRP, and survival of motor neuron protein (SMN), but a number of disease-linked proteins also associate with SGs as they expand; these proteins include fused in sarcoma (FUS), TDP-43, and ataxin-2 (ATXN2). (iii) Processing-bodies (PBs) are the sites for mRNA degradation, often integrating with miRNA machinery. Dcp1a (decapping enzyme 1a) is a RBP that is classically used to identify PBs. These granules are dynamic and are able to interact with each other and with active polysomes; this regulated trafficking can allow the cell to tailor translation to changes in its environment (Adeli 2011).

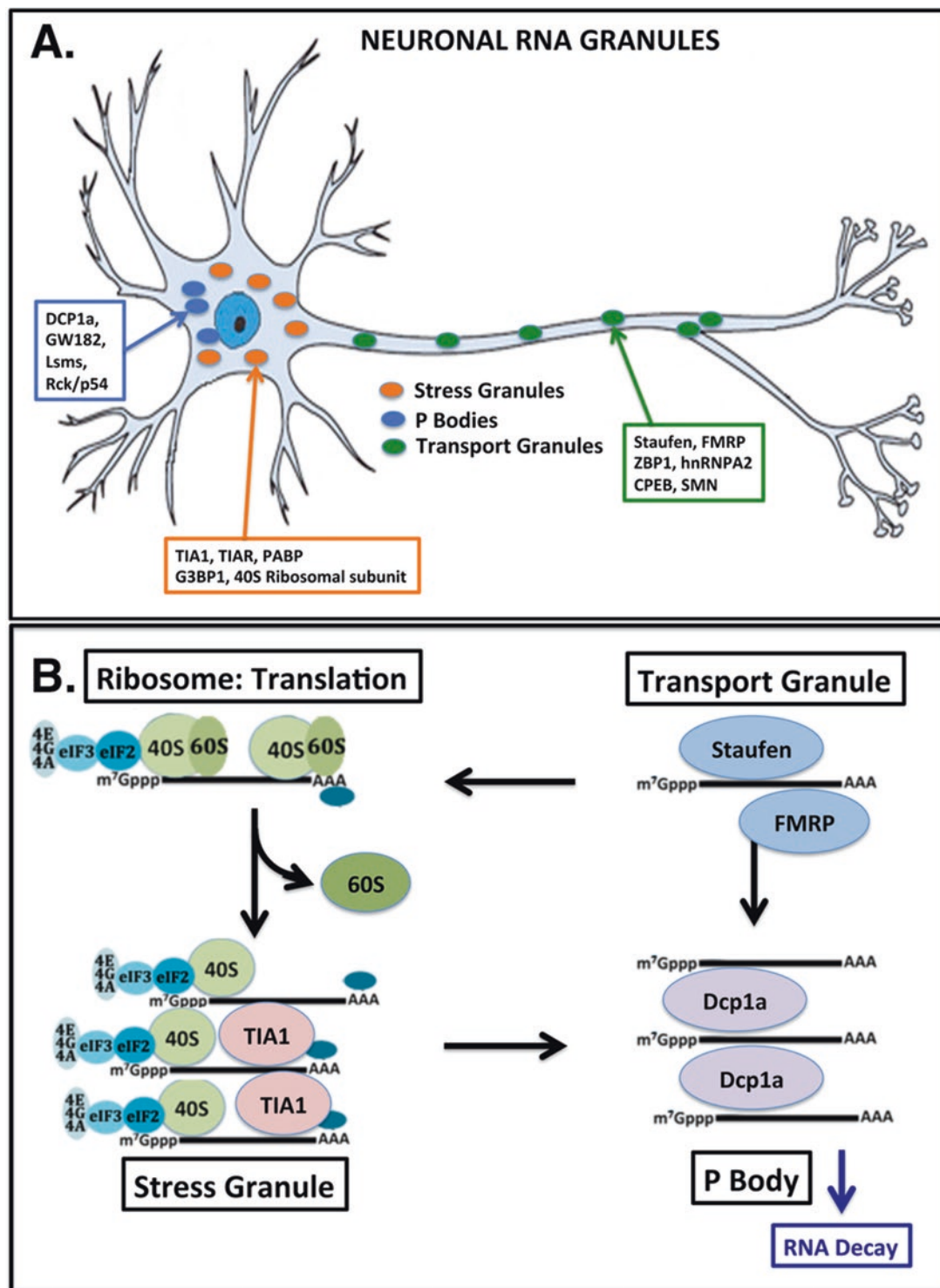


Fig. 19.1 Relationship between SGs, PBs and transport granules in neurons. (a) Types and locations of RNA granules in neurons. Neurons are atypical for having abundant cytoplasmic granules. They have a strong need for neuronal transport granules, in addition to having P-bodies and stress granules. (b) Relationship between the differing

types of RNA granules. Transcripts translocate to the cytoplasm where they are transported to polysomes for translation (protein synthesis). Transcript levels can be regulated by degradation in P-bodies. Stress leads to stalling of translation and formation of stress granules, which lack many (but not all) ribosomal components

19.3.1 Neuronal Transport Granules

Many mRNAs have localization elements within their 3' or 5' untranslated regions (UTRs); RBPs are able to recognize these elements and direct message localization while suppressing translation until the message is delivered to its destination. These mRNAs are delivered by transport granules composed of RBPs and silenced transcripts. Transcripts may also be stored in the cytoplasm in a repressed state by similar RNA storage granules, which act as a pool of mRNA that, depending on metabolic state and environmental changes, can be directed for activation, repression, or decay (Adeli 2011).

Normal neuron physiology largely depends on the presence of these types of granules, which are the functional units for transport and translation regulation of mRNAs from the soma out into the dendrites to synapses. These RBPs also mediate the process of activity dependent protein synthesis, which is critical in all aspects of biology, but has attracted particularly strong attention at the synapse where it controls synaptic plasticity, habituation and memory. For example, the synaptic function of FMRP has been studied extensively and is known to regulate dendritic sprouting.

Neuronal RNA transport granules are distinct from other RNA granules, although they can share some components and get in close contact with other RNA granules likely allowing a flow of mRNAs and proteins. There is also an apparent relationship between neuronal RNA granules and SGs, as both contain polyadenylated mRNAs, ribosomal subunits and a number of common RBPs, including SMN, Staufen, FMRP, Pumilio, TDP-43, and FUS, among others (Thomas et al. 2011).

19.3.2 Stress Granules

The stress response in eukaryotic cells involves the activation of defense mechanisms that either promotes survival or the initiation of apoptosis. The cellular response depends on the type of stress presented. Type I stress, including hypoxia, heat-shock, and oxidative stress, inhibits translation initiation and induces the formation of SGs as a defense mechanism promoting cell survival (Arimoto et al. 2008). SGs contain non-translating mRNAs, translation initiation components, and many additional proteins effecting mRNA function (Buchan and Parker 2009).

In metazoans, five eukaryotic initiation factor 2 alpha (eIF2 α) kinases monitor environmental stress and directly modulate the translation machinery. These include: (i) PKR (protein kinase R), a double-stranded RNA-dependent kinase that is activated by viral infection, heat and UV irradiation; (ii) PERK (PKR-like endoplasmic reticulum kinase, also known as PEK, or pancreatic eIF2 α kinase), a resident endoplasmic reticulum (ER) protein that is activated when unfolded proteins accumulate in the ER lumen; (iii) GCN2

(general control nonderepressible 2), a protein that monitors amino acid levels in the cell and responds to amino acid deprivation; (iv) HRI (heme-regulated initiation factor 2 α kinase), a protein that ensures the balanced synthesis of globin chains and heme during erythrocyte maturation and senses oxidative stress produced by arsenite; and (v) Z-DNA kinase, an enzyme involved in the host antiviral response. Stress-induced phosphorylation of eIF2 α on Ser51 inhibits global protein translation through depletion of the eIF2–GTP–tRNA-met ternary complex, thus permitting the RBP TIA-1 to bind the 48S complex instead of the ternary complex. This promotes polysome disassembly and the consequent recruitment of mRNAs to SGs (McDonald et al. 2011; Kedersha et al. 1999).

These non-translating mRNAs are necessary for SG formation and lead to primary nucleation of SGs involving RBPs including TIA-1, TTP, and G3BP, with specific mRNA transcripts (Fig. 19.4, stage 2). This family of proteins contains prion domains and poly-glutamine rich domains, which confer the ability to reversibly aggregate (Gilks et al. 2004). The primary aggregation of mRNA binding proteins with stalled mRNA transcripts induces protein-protein interactions and cross-linking by proteins such as poly-A binding protein (PABP-1) that seed an increase in aggregation with non-mRNA binding proteins (Fig. 19.2, stage 3). This results in the secondary aggregation of proteins with diverse physiological roles that can be modulated by SGs. The composition of SGs includes translation initiation components, small ribosomal subunit (40S), PABP, other RBPs, and mRNAs coding for most cellular proteins except those involved in the stress response. There is also piggy-back recruitment of many important signaling proteins into these granules that may affect cell signaling and survival (Fig. 19.2, stage 4) (Anderson and Kedersha 2008). However, SG composition may be subtly different according to the nature of the stress stimulus, and it may also change progressively during the response (Thomas et al. 2011).

Once formed, SGs serve as centers of mRNA triage by dynamically sorting sequestered mRNAs for re-initiation, storage, or degradation, and may be required to allow optimal translation of stress-responsive anti-apoptotic mRNAs and thus appear to be protective. SGs have been shown to associate with PBs, with TTP proposed to play an integral role in the shuttling of mRNAs destined for decay from SGs to PBs. Once the stress is removed there is rapid translational recovery in concert with SG disassembly. The mechanism of SG disassembly is poorly understood, but may be dependent upon interactions with heat shock proteins, and active transport by dynein and kinesin motors (Kedersha and Anderson 2007). SGs can also be disassembled chemically via treatment with emetine and cycloheximide. These drugs inhibit translational elongation and block the disassembly of polysomes, thereby preventing the translocation of mRNA into SGs and PBs. Additionally, the drug puromycin promotes premature translation termination and promotes SG and PB assembly (Kedersha et al. 2005).

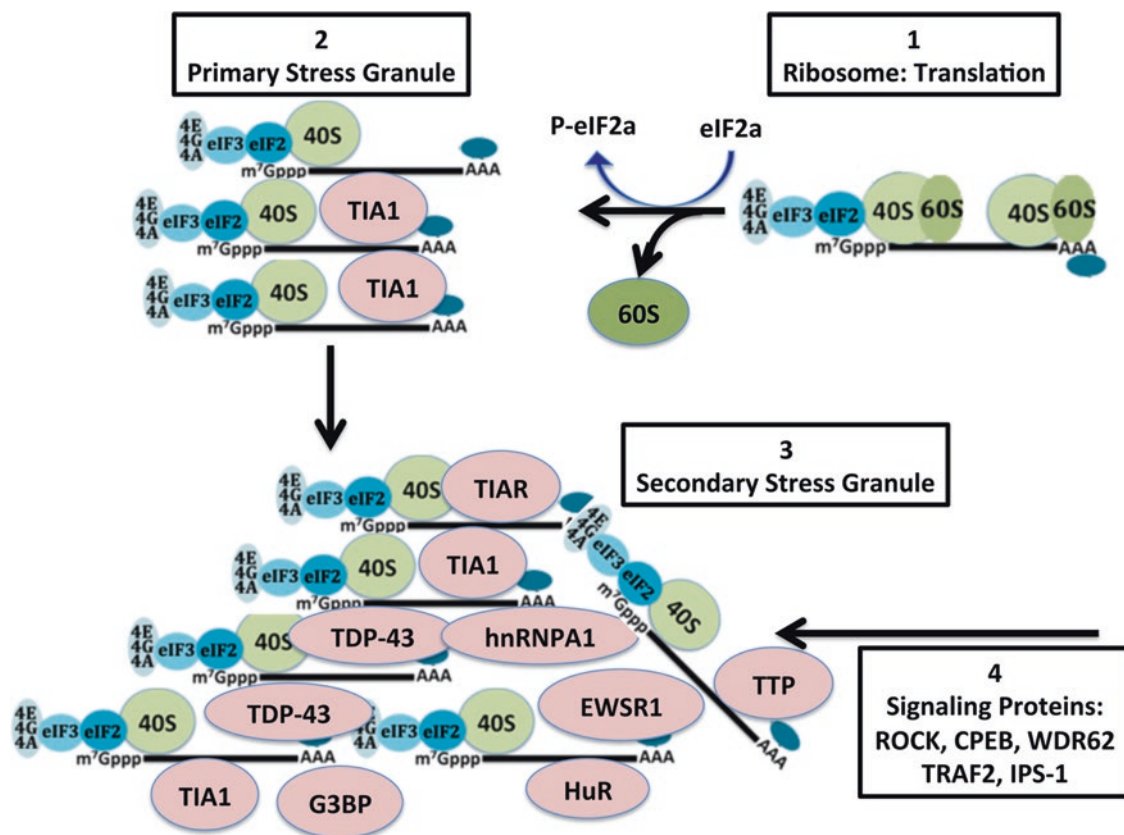


Fig. 19.2 A model of stress granule assembly. The process of SG assembly can be divided into discrete stages that are marked by the specific composition and localization of mRNPs subject to translational arrest. Adapted from Anderson and Kedersha (2008)

SGs have been suggested to suppress apoptosis by suppressing the stress-activated mitogen activated protein kinase (MAPK) pathway (Dewey et al. 2012). Additional cell survival mechanisms linked with SG formation are likely to occur, and an emerging example is the sequestration of proapoptotic molecules. The TNF receptor associated factor 2 (TRAF2) was the first case reported. TRAF2 facilitates apoptosis by two independent pathways: TNFR activation and caspase activation upon ER-stress induction. TRAF2 interacts with eukaryotic initiation factor 4G (eIF4G) and is therefore retained in SGs, thus avoiding apoptosis. More recently, two key molecules that activate the p38/JNK apoptotic pathway, namely RACK1 and ROCK1 were shown to be localized in SGs, thus favoring cell survival (Thomas et al. 2011).

An interesting possibility is that formation of SGs is required to allow optimal translation of stress responsive mRNAs. The translational arrest that accompanies environmental stress is potentially selective: one study shows that the translation of ~25 % of mRNAs is significantly reduced, whereas the translation of another 25 % of mRNAs (including transcripts encoding heat-shock proteins) is significantly enhanced. Stress-induced reprogramming of protein expression also entails stabilizing or destabilizing selected groups

of mRNAs. Thus, post-transcriptional reprogramming of mRNA translation and decay reconfigures the proteome during adverse environmental conditions (Anderson and Kedersha 2008).

The importance of SGs for cytoprotection is highlighted by the effects of knockout of SG proteins, such as TIA-1, or inhibition of eIF2 α phosphorylation, which render cells more vulnerable to acute stresses. Conversely, inhibiting eIF2 α dephosphorylation protects against some forms of stress. However, the actual mechanisms by which SGs mediate protection are poorly understood (Wolozin 2012).

19.3.3 Processing Bodies

P-bodies are another dynamic structure that contains mRNA decay machinery components. Core components of PBs are translationally inactive mRNA and the decapping factor, which induces mRNA decay and blocks translation by decapping and induction of 5'-3' mRNA decay. Key to these complexes are Dcp1/2 (decapping enzymes), the Lsm1-7 complex (activators of decapping), Xrn1 (5'-3' exonuclease), and nonsense mediated decay machinery. P-body

assembly factors recruit mRNA into P-bodies through multimerization domains (Adeli 2011).

P-bodies are often observed juxtaposed to SGs but are also present in cells not under stress. Several observations demonstrate that SGs interact with PBs and are likely to exchange mRNAs between them: (i) mammalian PBs and SGs transiently dock with one another during arsenite treatment and can show prolonged docking when TTP is overexpressed and (ii) PBs and SGs share many protein components and the same mRNA species (Buchan and Parker 2009). In neurons, FRAP analysis indicates that the turnover of DCP1a in PBs is dramatically enhanced by synaptic stimulation, indicating that PB dynamics and the release of mRNAs to allow their translation are controlled by neuronal activity, likely playing a role in regulating local protein synthesis at the post-synapse (Thomas et al. 2011).

19.3.4 Microtubule Dependence

The formation and persistence of RNA granules depends on the movement of RNA and proteins in the cytoplasm and their accumulation in particular cytoplasmic foci. It has been shown that SG formation is microtubule-dependent process and likely is facilitated by the motor protein-driven movement of individual SG components along microtubules. Disruption of MT array with nocodazole treatment abolished the formation of SGs in response to a stressor (Ivanov et al. 2003). Thus it is possible that the distribution of SGs in cells might correlate with the distribution of MTs, and any dysfunction in the MT network would prevent normal SG formation and dynamics.

19.4 RNA Granule Markers

19.4.1 TIA-1

19.4.1.1 Gene and Protein Structure

T-cell intracellular antigen-1 (TIA-1) is a RBP of the RNA recognition motif (RRM)/ ribonucleoprotein (RNP) family that regulates pre-mRNA splicing, mRNA translation, stress-induced translational arrest, and has been implicated as an

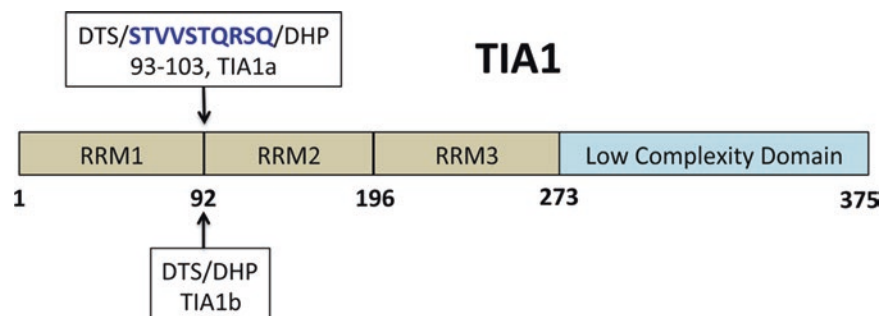
effector of apoptotic cell death (Beck et al. 1996; Gilks et al. 2004). The gene encoding the 43 kDa TIA-1 protein locates at chromosome 2p13.3 (Kawakami et al. 1994). TIA-1 has 3 RRM domains at its N-terminus that confer high-affinity binding to AU-rich motifs, and a glycine-rich, low complexity, carboxyl-terminal domain that allows it to reversibly aggregate, as shown in Fig. 19.3 (Wolozin 2012). The low complexity domain share homology to yeast prion proteins, such as Sup35 (Phillips et al. 2004).

TIA-1 presents in two major isoforms (TIA-1a and TIA-1b) generated by alternative splicing of exon 5 that differ by 11 amino acids as shown in Fig. 19.3. Exon 5 inclusion generates TIA-1 isoform a, whereas exon skipping produces the shorter b isoform. The 11-amino acid peptide encoded by exon 5 is located between RRM1 and 2. The relative expression of TIA-1 isoforms varies in different human tissues and cell lines, suggesting distinct functional properties and regulated isoform expression. Both TIA-1 isoforms show similar subcellular distribution and RNA binding, but TIA-1b displays enhanced splicing stimulatory activity compared with TIA-1a (Izquierdo and Valcarcel 2007).

19.4.1.2 General Biological Functions

TIA-1 is known to regulate pre-mRNA splicing, mRNA translation, stress-induced translational arrest, and has been implicated as an effector of apoptotic cell death (Beck et al. 1996; Gilks et al. 2004). TIA-1 regulates the alternative pre-mRNA splicing of various human and *Drosophila* genes (FGFR-2, msl-2, TIAR, cystic fibrosis transmembrane conductance regulator, and Fas) through binding to U-rich stretches, facilitating atypical 5'-splice site recognition by U1 small nuclear ribonucleoprotein (snRNP) (Izquierdo and Valcarcel 2007). TIA-1 has also been well characterized as a translational regulator. This protein has been implicated in stress-induced translational arrest, colocalizing after stress with poly(A)⁺ RNA in the cytoplasmic foci known as stress granules. TIA-1 is able to bind to the 3'-untranslated regions (UTRs) of the translational regulatory AU-rich elements of tumor necrosis factor α (TNF α), human matrix metalloproteinases-13 (MMP13), cyclooxygenase-2 (COX2), β 2-adrenergic receptor, mitochondrial cytochrome c, GADD45 α , and β -F1-ATPase mRNAs (Izquierdo and

Fig. 19.3 The structure of human TIA-1 gene and protein. Domain structure of human TIA-1 isoforms (Kawakami et al. 1994). The 11-amino acid peptide encoded by exon 5 is shown in *bold*. TIA-1 proteins include three RRM domains and a carboxyl-terminal glutamine-rich domain. Numbers indicate amino acid residues corresponding to each RRM and carboxyl-terminal domain of the TIA-1b isoform. Adapted from Izquierdo and Valcarcel (2007)



Valcarcel 2007). As a translational silencer, TIA-1 binds to these transcripts and suppresses their translation. Thus, mutant mice lacking TIA-1 have a hyperinflammatory phenotype because of the lack of suppression of TNF α and COX2 that results in increased cytokine levels (Yamasaki et al. 2007; Piecyk et al. 2000; Anderson and Kedersha 2008).

Interestingly, the aggregation of TIA-1 is regulated by molecular chaperones (Gilks et al. 2004) and is blocked by HSP70 overexpression. Minimal constitutive levels of HSP70 are continuously required to prevent TIA-1 aggregation. Stress-induced denaturation of other cytoplasmic proteins recruits HSP70 for protein renaturation, thus diverting HSP70 away from TIA-1, promoting TIA-1 aggregation and consequent SG nucleation. The successful refolding of denatured proteins releases HSP70 and the free HSP70 then solubilizes TIA-1, the subsequent TIA-1 disaggregation promotes SG disassembly (Anderson and Kedersha 2008).

19.4.1.3 Mutations Linked to Disease

Recently, a single missense mutation in TIA-1 (p.E384K) was found to cause Welander Distal Myopathy (WDM). WDM is a dominant late-onset muscular dystrophy mainly found in Finland and Sweden. Onset is usually at 40–60 years of age, presenting with weakness and atrophy in the fingers, progressing to the hand muscles and lower legs. Rare homozygotes exhibit earlier onset, faster progression, and proximal muscle involvement. These homozygous patients become wheelchair-bound by age 50 (Hackman et al. 2013).

Pathologically there are myopathic changes and prominent rimmed vacuoles in distal muscles. TIA-1 increased cytoplasmically and granules or bulky aggregates appeared in some atrophic and rimmed-vacuolated fibers. These rimmed vacuoles consistently showed strong LC3b, ubiquitin, and p62 reactivity indicating a potential deficit in protein clearance mechanisms. The mutation in TIA-1 is present in the glutamine-rich C-terminal domain, this domain is both required for enhanced interaction with the U1 splicing complex as well as the self-assembly of TIA-1, thus an assessment of both of these functions was necessary. Assessments of splicing patterns in known TIA-1 splice targets (FAS, PLOD2, and TIAR) suggested that there was no splicing abnormality associated with the mutation. In vitro assessments of mutant E384K TIA-1 indicates that the mutation is able to increase SG formation slightly, and that these stress granules exhibit altered trafficking (Hackman et al. 2013).

19.4.2 TTP

19.4.2.1 Gene and Protein Structure

Tristetraprolin (TTP) is an mRNA-binding protein containing tandem CCCH zinc fingers, involved in post-transcriptional regulation of AU-rich element (ARE)-containing mRNAs.

The gene encoding the 34 kDa protein is located on chromosome 19q13.2. TTP protein has two tandem CCCH zinc finger motifs that allows it to bind mRNAs (Kawakami et al. 1994).

19.4.2.2 General Biological Functions

TTP binds mRNAs through two tandem CCCH zinc finger motifs and promotes their decay. TTP target mRNAs typically contain the AU-rich sequence, where TTP can bind and destabilize its targets including TNF- α , granulocyte macrophage colony stimulating factor (GM-CSF), COX-2, Interleukin 3 (IL-3), IL-10, and interferon- γ (IFN- γ). Mice that lack TTP spontaneously develop erosive arthritis, cachexia, alopecia, dermatitis, autoantibodies, and myeloid hyperplasia indicating its importance in regulating the inflammatory response. TTP is a protein associated with PBs, and may receive transcripts from TIA-1 positive SGs in a hand-off mechanism (Rigby et al. 2005).

Although TTP is a phosphoprotein reported to be a substrate for multiple kinases including ERK, JNK, p38, and MAPKAP kinase 2 (MK2), the role of phosphorylation in regulating TTP function is unclear (Mahtani et al. 2001). While extensive TTP phosphorylation has been mapped, only the serine–threonine phosphatase PP2A has been established as promoting TTP dephosphorylation. TTP can be phosphorylated by MK2, a downstream target of p38, at serines 52 and 178 in mouse, 60 and 186 in humans. mRNA binding was not altered in response to phosphorylation, but it did prevent mRNA decay by preventing TTPs association with SGs or PBs, instead promoting its association with 14-3-3 proteins (Stoecklin et al. 2004; Rigby et al. 2005). Thus, exclusion of TTP from SGs correlates with inhibition of TTP activity, which suggests that TTP induces the degradation of translationally stalled mRNAs via its association with PBs.

19.4.3 G3BP

19.4.3.1 Gene and Protein Structure

G3BP was initially characterized through its interaction with a Ras-GTPase-activating protein (RasGAP p120), and is known to both activate and repress mRNA transcripts in a transcript dependent manner. There are three isoforms of G3BP: 1, 2a, 2b, which are products of two distinct genes. G3BP1 and 2 are encoded by distinct genes on human chromosomes 5 and 4. There is a 74 % similarity between G3BP1 and G3BP2a protein. G3BP2b is a splice isoform of G3BP2a, lacking 33 amino acids in the central region.

G3BP's C-terminus is comprised of two motifs traditionally associated with RNA binding. These are a RRM, and a loosely conserved RGG (arginine-glycine rich) box (Kennedy et al. 2001; Irvine et al. 2004). The N-terminus of G3BP is a nuclear transport factor-2 (NTF-2)-like domain, which influences the cellular localization of proteins and its

oligomerization with itself or with other partners. G3BP's central regions comprise varying numbers of proline-rich (PxxP) motifs and an acid-rich domain (Rigby et al. 2005; Irvine et al. 2004).

19.4.3.2 General Biological Function

The majority of publications concerning G3BP1 support a function in cell proliferation and/or survival downstream of Ras, and this is dependent on regulation of phosphorylation/dephosphorylation at S149. Thus, G3BP is very important in embryonic development and knockout is embryonically lethal (Tourriere et al. 2003). Both G3BP1 and G3BP2 are dramatically overexpressed in human cancers, in particular breast cancers. Over-expression of G3BP1 has been demonstrated in a range of human tumors, including breast, head, neck, colon, and thyroid (Guitard et al. 2001; Irvine et al. 2004).

G3BPs are also found to be components of mRNPs and are necessary to form SG complexes. In response to stress, G3BP becomes dephosphorylated at S149 allowing it to aggregate. The RRM domain mediates the binding of G3BP to specific mRNA sequences so that G3BP can exert its function as a dinucleotide-specific single-strand-specific endoribonuclease. G3BP can bind to the 3' untranslated region (3'UTR) of human c-Myc mRNA in a phosphorylation-dependent manner to increase its degradation in vitro (Cohen et al. 2003). *Supporting this*, several studies implicate G3BP1 in cell cycle regulation, with G3BP1 over-expression increasing S-phase entry and this was dependent on an intact RNA-binding domain (Guitard et al. 2001; Irvine et al. 2004).

There is also an interaction between G3BP1 and the de-ubiquitinating enzyme, USP10. Two substrates for the USP10/G3BP1 de-ubiquitinating complex have been discovered so far: Sec23, a component of the COPII complex involved in anterograde protein export from the endoplasmic reticulum (ER) to the Golgi network and β -COP, a component of the COPI complex required for retrograde protein transport from the Golgi to the ER. De-ubiquitination of Sec23 and β -COP rescues them from degradation by the proteasome, thereby maintaining the activity of the retrograde and anterograde protein secretion pathways (Irvine et al. 2004; Cohen et al. 2003).

19.5 RNA Binding Proteins and Inflammation

Very little is known about the role of RNA granules in inflammation, however it is very clear that RNA granules occur in inflammatory cells, and that they regulate the process. Proteins such as PARP12 localize to SGs in inflammatory cells and stimulate their formation (Welsby et al. 2014).

SG formation is stimulated by cytokines, such as TNF α . Formation in monocytes appears to suppress HSP70 synthesis and promotes apoptosis (Qi et al. 2011). In contrast, formation of a different kind of RNA granule, termed the anti-viral granule (AVG) plays an important role in virtually every viral infection. This field is vast and best addressed by reading current reviews that specifically address this important topic (Onomoto et al. 2014).

19.6 RNA Binding Proteins and Neurodegeneration

Stress granules are also important in the context of unfolded protein diseases. Many aspects of SGs resemble aggresomes and unfolded protein aggregates present in neurodegenerative pathologies (Thomas et al. 2011). For example, both are mediated by specific prion-like and poly-glutamine containing protein-aggregation domains, the dissolution requires molecular chaperones; they contain ubiquitinated proteins and are enhanced by inhibitors of protein degradation machineries. These commonalities make it tempting to speculate that SGs and intracellular protein aggregates may interact. However, the aggregation processes characterizing the biology of RBPs differ from the conventional models of protein aggregation in that they serve distinct biological functions and are reversible. In addition, aggresomes tend to form a single large inclusion, originating at the microtubule-organizing center, whereas SGs occur as multiple complexes scattered throughout the cytoplasm.

19.6.1 RNA Binding Proteins in Neurological Diseases

Perhaps most convincing of the increased recognition of RBPs in neurodegeneration is that mutations or malfunctions in some of these proteins can directly cause neurological disorders. For instance, impaired expression of FMRP, due to trinucleotide repeat expansions is the cause of fragile X mental retardation syndrome (FXS), which is the most common cause of inherited mental retardation, and with aging also leads to a related neurodegenerative condition. Expanded trinucleotide repeats in several different ataxin genes are the cause of spinocerebellar ataxia. Mutations in survival of motor neuron (SMN1) are linked to spinal muscular atrophy (SMA), and mutations in TDP-43, FUS, ATX2, optineurin (OPT) and angiogenin (ANG) all cause motor neuron diseases including amyotrophic lateral sclerosis (ALS). These disease processes have now been linked to dysfunctional or dysregulation of neuronal RNA granules and SGs (Wolozin 2012; Liu-Yesucevitz et al. 2011).

19.6.2 Regulated Protein Aggregation

The potential importance of SGs for neurodegenerative disease becomes apparent because the process of SG formation presents a biological pathway that could be vulnerable to the protein aggregates that accumulate in disease. RBPs are a group of proteins that naturally form insoluble aggregates, yet the aggregated material can disperse and resolubilize. Most, if not all, RBPs linked to neurodegenerative diseases associate with SGs in cell culture. TDP-43, FUS, ATX2, SMN, OPT, and ANG have all been shown to co-localize with classic SG markers (TIA-1, TTP, and/or G3BP) in cells undergoing stress. SG proteins such as TIA-1, eIF3, and PABP also co-localize with neuropathology in brain tissue of subjects with FTDP-17, FTLDP, and ALS, or animal models of these diseases. In addition, as mentioned above with respect to ALS, SMA, and FFR the dysfunctions of the disease may actually be linked to dysregulation of RNA granules (Wolozin 2012).

Recently our lab (Wolozin 2012), proposed that the aggregation of many pathological, intracellular proteins, including TDP-43 and FUS, proceeds through the SG pathway. Mutations in genes coding for SG associated proteins or prolonged physiological stress, lead to enhanced SG formation, which accelerates the pathophysiology of protein aggregation in neurodegenerative diseases. Alternatively, the formation of long-lived stable insoluble protein aggregates seen in disease may lead to accelerated, long-lived SG formation. These highly insoluble aggregates could also serve as a nidus for further aggregation of SGs, by binding with other RBPs and also binding RNA as part of the process of SG maturation. The result would be an overactive SG pathway. Over-active stress granule formation could act to sequester functional RBPs and/or interfere with mRNA transport and synthesis, each of which might potentiate neurodegeneration (Fig. 19.4a—normal, Fig. 19.4b—stress/normal SG formation, Fig. 19.4c—chronic stress/pathologic SG formation). The reversibility of the SG pathway also offers novel opportunities to stimulate endogenous biochemical pathways to reverse these pathological stress granules and also perhaps delays the progression of disease (Wolozin 2012; Vanderweyde et al. 2012).

19.6.3 Potential for Pharmacological Intervention

The RBPs addressed in this section (TIA-1, G3BP, TTP, SMN, and TDP-43) are essential players in RNA metabolism, with the RNPs forming a dynamic regulatory system for all aspects of the life of an mRNA, including nuclear processing,

transport, translation, and decay. These proteins are essential in the coordination of gene expression of many proteins, and their disruption could impair cell function and interfere with appropriate distribution and translation of mRNA in response to signaling (Vanderweyde et al. 2012). Protein levels of RBPs are known to change with aging perhaps making cells more susceptible to dysfunction and disease processes.

Whether hyperactive SG formation is good or bad remains to be determined. Acutely SG formation is known to be protective and anti-apoptotic, until the stress is resolved. In neurodegeneration and other aging-associated diseases, there is no resolution and the stress is chronic. At this point SG formation and recruitment of RBPs from their normal functions act as a loss of function situation where RNPs become dysfunctional, and RNA metabolism is no longer in check. Neurons require SGs for an effective stress response, but overactive, overly stable SG complexes could easily interfere with neuronal function by silencing transcripts and sequestering important proteins. Mutations associated with disease-linked RBPs increase the aggregation propensity or cause dysfunctional RNPs, which provides a direct mechanism for overactive SG formation. Chronic stressful diseases or environmental conditions might also stimulate overactive SG formation. For instance, the oxidative stress associated with aging, the trophic stress associated with diabetes, or the cellular stress associated with cancer all enhance SG formation creating the conditions for overactive SG aggregation (Wolozin 2012).

The effect of modulating the protein synthesis/SG pathway was recently evaluated in an animal model of Creutzfeldt Jacob disease, where pathological misfolding of PrP precipitates neurodegeneration. Mallucci and colleagues forced expression of GADD34, a phosphatase to reduce eIF2 α phosphorylation, inhibit SG formation and stimulate protein synthesis (Moreno et al. 2012). This intervention reduced PrP-induced neurodegeneration. In contrast, salubrinal, which increases and prolongs eIF2 α phosphorylation, increased SG formation, inhibited protein synthesis, and accelerated neurodegeneration (Moreno et al. 2012). These results suggest that inhibiting the SG pathway and stimulating protein synthesis can inhibit PrP-mediated neurodegeneration. The discovery of over-active SG formation in other diseases raises the possibility that these pathways are over-active in multiple neurodegenerative diseases and other aging processes, and pharmacotherapy that targets SG formation might be protective (Wolozin 2012). A major challenge in the future will be to define the components of each RNP complex and pinpoint the defects associated with RBP dysfunction. An important issue to resolve is why the absence of certain RBPs or accessory proteins leads to cell-specific defects (Lukong et al. 2008).

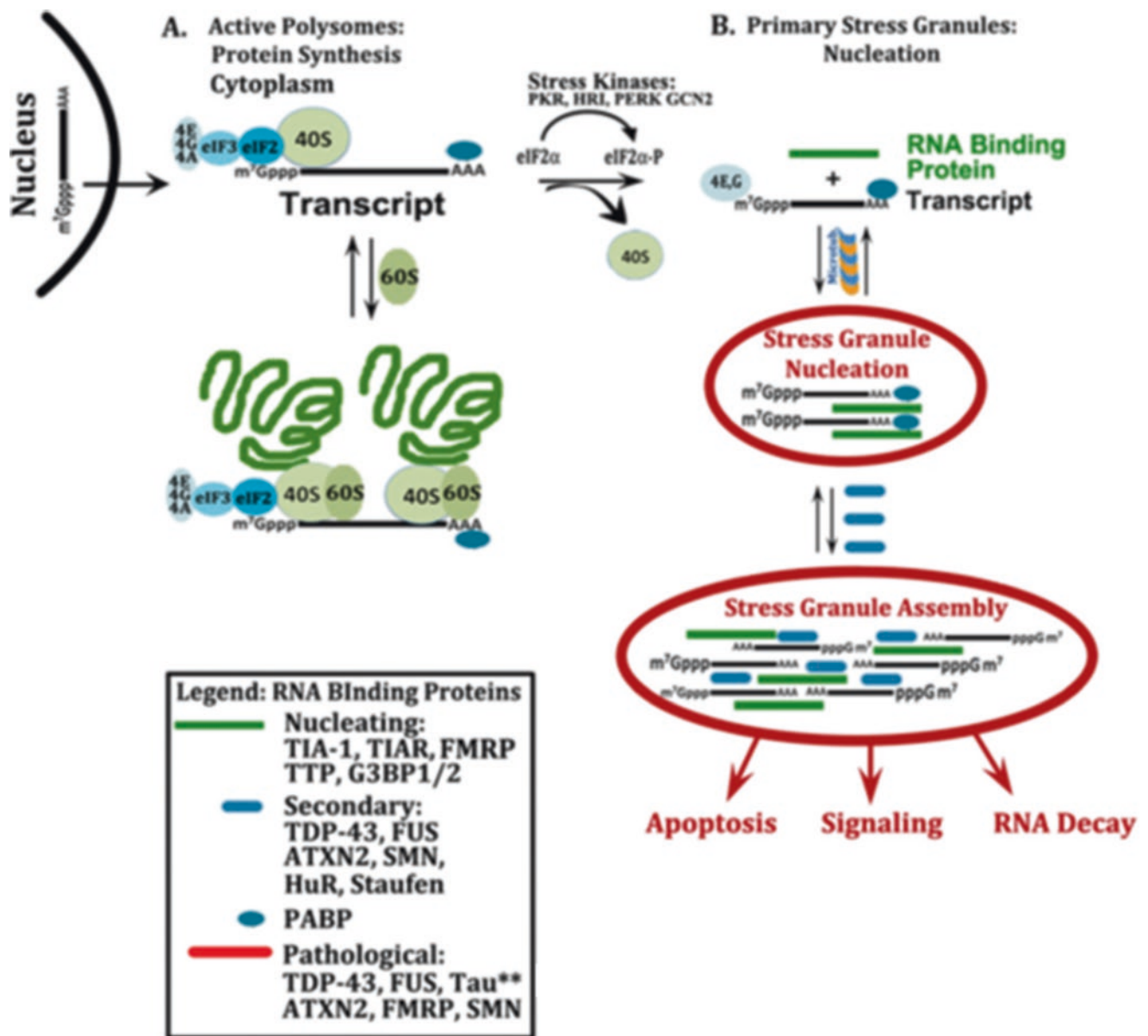


Fig. 19.4 Mechanism of normal and pathological stress granule formation. (a) Under normal, physiological conditions, neurons synthesize specialized proteins from capped transcripts. The proteins eIF4A, E and G complex to form the eIF4F pre-initiation complex, which interacts with the ribosome (40S) as well as other translational regulators to synthesize proteins. Association with the 60S ribosome complex allows protein synthesis to begin. (b) Stress leads to phosphorylation of eIF2 α , dissociation of ribosomes and many of the translation initiation factors, leaving mRNA bound eIF4G and poly-A binding protein. Nucleating RBPs bind the free RNA and also form protein/protein complexes, which initiate SG formation. Once initiated, other RBPs bind to the

mRNA and to the nucleating RBPs to increase the size and complexity of SGs. These SGs are rapidly reversible upon removal of the stress; however prolonged SG formation affects cell biology by interacting with biological systems regulating apoptosis, signaling and RNA decay. (c) Pathological proteins, such as TDP-43, FUS and tau, have a strong tendency to form oligomers, and then fibrils. The consolidation of RBPs during SG formation might promote oligomerization by creating cellular domains with higher concentrations of these proteins. Conversely, the increased stability of oligomers and fibrils might serve as a nidus for SG formation, leading to over-active SG formation. Adapted from (Wolozin 2012)

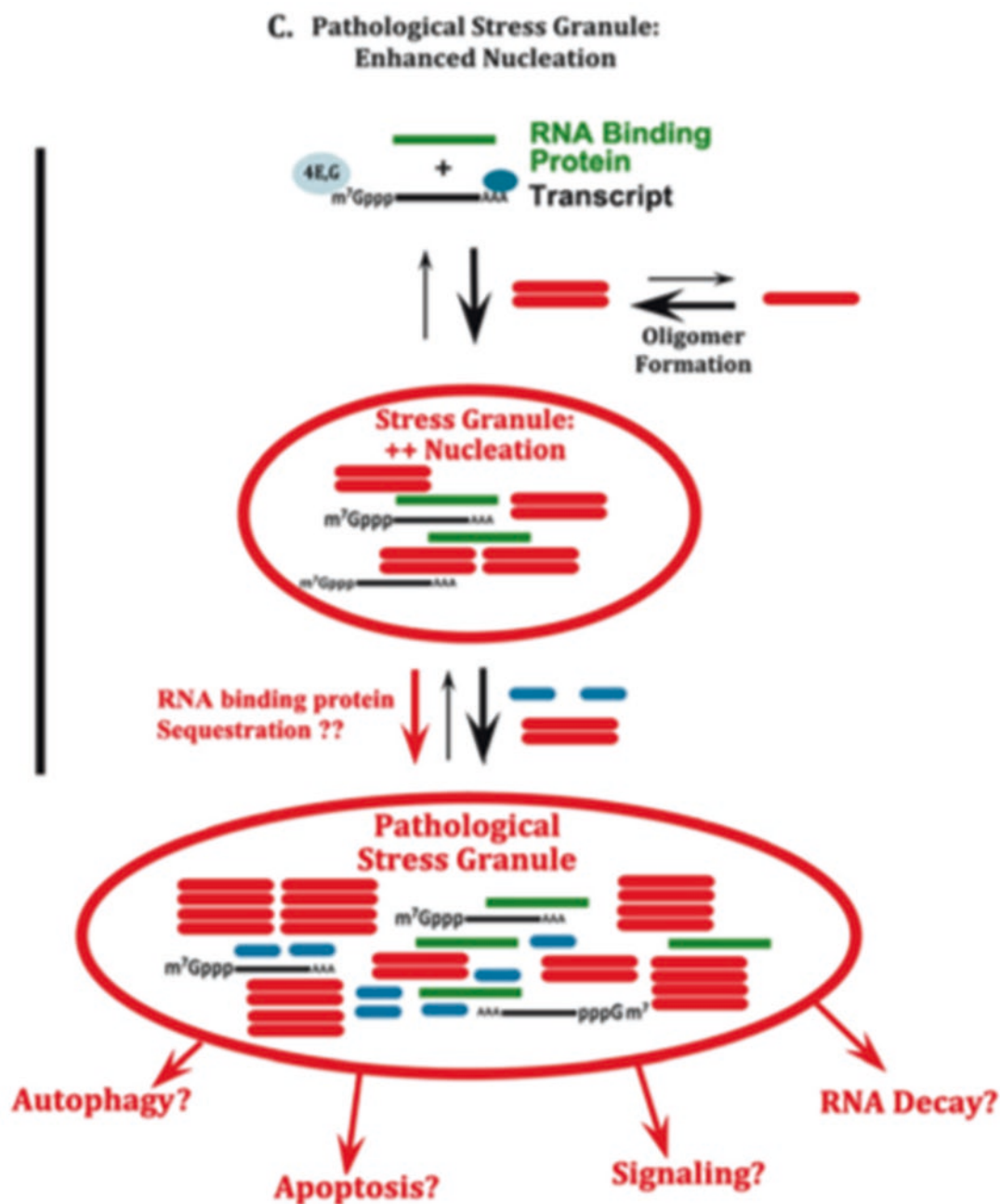


Fig. 19.4 (continued)

19.7 Major Points

The biology of RBPs provides three novel concepts for the field of neurodegeneration:

1. “Regulated Protein Aggregation”—RNA binding proteins use protein aggregation as part of a normal regulated, physiological mechanism controlling protein synthesis. Increasing evidence indicate that dysfunction

of the RBP regulated protein aggregation pathway strongly contributes to the pathophysiology of neurodegenerative diseases.

2. Recent studies indicate that exposure to extracellular pre-aggregated tau and pre-aggregated α -synuclein can induce further aggregation of tau in cell culture (Guo et al. 2013). However, the initial mechanism through tau might become aggregated remains a mystery. The process of regulated protein aggregation of RBPs provides such a mechanism.

3. The link between tau pathology and RBPs raises the possibility that the pathophysiology of tauopathies affects patterns of RNA splicing, degradation and translation. How ribostasis is altered via the pathophysiology of tau represents a novel line of investigation for the field, which will undoubtedly identify many novel changes occurring during the course of tauopathies

19.8 Conclusions

The high prevalence of prion-like domains in RBPs creates a large genetic target for pathogenic mutations that create hyper-aggregation variants with downstream toxic effects. The role of RBPs in degenerative pathologies may be quite common, as 10 of the top 20 human RBPs predicted to have prionogenic domains have been linked to some form of degenerative disease (Ramaswami et al. 2013). This prevalence suggests that hyperassembly or persistence of SG aggregates may be a causative event in disease progression. The self-perpetuation of such toxic assemblies might be pathological, at least in part, because they disrupt RNA homeostasis. First, pathogenic SG formation could reduce the functional pool of the RBPs. Second, by sequestering associated mRNAs, SG aggregates could alter the population of mRNAs available for translation. Third, pathologic SG aggregates could sequester other mRNA-binding regulatory factors, including other mRNA-binding proteins, miRNAs, and recruited signaling proteins, such that they are not available for their normal functions (Wolozin 2012; Ramaswami et al. 2013). Thus the elucidation of the mechanisms of toxicity in RBP-linked diseases may provide opportunities for pharmacological intervention that will be broadly applicable to many neurodegenerative conditions.

The biology of RBPs and RNA granules presents a new paradigm for understanding the genesis of many neurodegenerative diseases, including motor neuron diseases and tauopathies, which focuses on regulated protein aggregation, implicates tau in this process, and provides a biological context in which these misfolding processes occur. It is well known that aggregation-prone and prion-related domains from different proteins have a propensity to interact with one another (Wolozin 2012). That prion-like aggregation of RBPs appears to provide a seed for the misfolding and aggregation. In disease, aberrant post-translational modifications or other signaling pathways might cause RBPs to lose their normal functions, resulting in their aggregate forming persistent, pathological stress granule complexes. Thus, the discovery intersecting the fields of tauopathies and RNA granule biology shedding light on critical unanswered research questions that may be highly relevant to a further understanding the pathophysiology of neurodegeneration.

19.9 Review Questions

1. What is the role of RNA granules in the stress response?
2. What are the conserved domains in RNA binding proteins and what are their functions?
3. How are mutations in RNA binding proteins thought to contribute to neurodegenerative diseases?
4. What are anti-viral granules?
5. List three core nucleating RNA binding proteins, two RNA binding proteins linked to RNA transport and an RNA binding protein involved in activity dependent translation.

19.10 Answers

1. SGs serve as centers of mRNA triage by dynamically sorting sequestered mRNAs for re-initiation, storage, or degradation, and may be required to allow optimal translation of stress-responsive anti-apoptotic mRNAs and thus appear to be protective. SGs have been shown to associate with PBs, with TTP proposed to play an integral role in the shuttling of mRNAs destined for decay from SGs to PBs.
2. RRM: RNA recognition motif, The RRM domains confer high-affinity binding to specific RNA sequence motifs.
Prion like domain: glycine-rich, low complexity domain that allows RNA binding proteins to reversibly aggregate.
NLS: Nuclear localization signal
3. Mutations in genes coding for SG associated proteins appear to lead to enhanced SG formation. The formation of long-lived stable insoluble protein aggregates seen in disease may lead to accelerated, long-lived SG formation. These highly insoluble aggregates could also serve as a nidus for further aggregation of SGs, by binding with other RBPs and also binding RNA as part of the process of SG maturation. The persistent, pathological SGs appear to be detrimental partly by sequestering RNA binding proteins which prevents them from doing their normal physiological activity.
4. Anti-viral granules are types of RNA granules that form in response to viral infections. In some cases they represent the cellular defense against the virus, which acts by binding viral RNA binding proteins. In other cases they represent the viral machinery used to take over the cellular protein synthesis and anti-viral machinery.
5. Core nucleating RNA binding proteins: TIA1, TTP and G3BP.

RNA binding proteins linked to RNA transport: Staufen, fragile X mental retardation protein (FMRP) and Pumilio (PUM1)

RNA binding proteins involved in activity dependent translation: FMRP

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Abstract

Exosomes are extracellular vesicles and regulators of tissue homeostasis, immune effectors, disease biomarkers and drug delivery vehicles. Primarily they serve as vesicular carriers for intercellular communication by bringing nucleic acids, proteins, carbohydrates and drugs to injury action sites. As such, by acting as vesicles for tissue repair and delivery for disease combating therapies they can ameliorate tissue injuries and attenuate microbial infections. On balance, they can perpetuate disease by serving as carriers for infectious agents. In contrast, they can control inflammatory responses through the delivery of tumor necrosis factor alpha (TNF- α) and interleukin-6, ultimately affecting survival during a myriad of infectious, inflammatory and degenerative diseases. Exosomes may also speed engulfment of apoptotic cells and clearance of debris, and reduce systemic inflammatory responses. How exosomes are synthesized, secreted and regulate cellular and tissue function is a key part of understanding immunity and the nervous system. To such ends this review devotes itself to an understanding of the biology and function of exosomes in both health and disease with a special focus on their role as new therapeutic delivery vehicles.

Keywords

Apoptotic cells • Biomarkers • Exosomes • Human immunodeficiency virus • Oligodendrocytes

20.1 Introduction

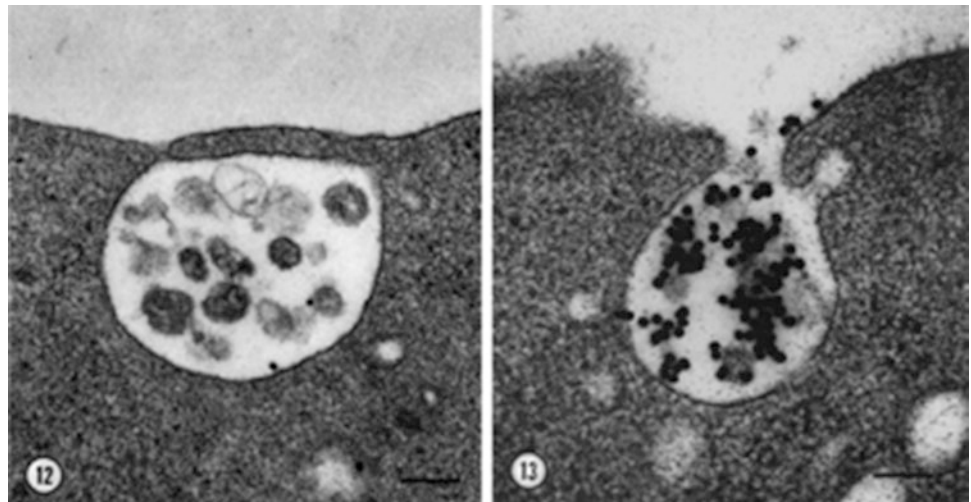
Exosomes are cell-secreted microvesicles that serve as drug, RNA, microbial and protein transporters. Transport is enabled through bodily fluids and actions facilitate intercellular communication and immune homeostasis responses. Both the cell source and the physiological state and can determine composition and cargo of exosomes, and thus how cells communicate with one another. Exosomes are released into the extracellular environment when multi-vesicular bodies (MVBs) fuse with

the plasma membrane. The first images of a multivesicular body (MVB) exocytotic event appeared in Harding et al. (1983): an example is shown in Fig. 20.1. Exosomes carry cellular components with specialized functions for intercellular signaling, cell homeostasis, growth, differentiation, antigen presentation, microbial infection and tumor proliferation. The first notion for the existence of exosomes was based on an observed vesicular release from maturing reticulocyte endosomes into the extracellular environment (Harding et al. 1983; Lachenal et al. 2011; Pan and Johnstone 1983). Previously thought to be purely waste management in function, exosomes have recently generated intense interest largely based on their role as signaling mediators.

Exosomes are small, 30–150 nm membrane-bound vesicles, that are constitutively secreted by most cell types in the body. These vesicles are found in most bodily fluids including blood, saliva, urine, colostrum, semen and

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Fig. 20.1 Exocytosis of MVEs releases exosomes containing transferrin receptor. (*Left*) View of an MVE from a fixed reticulocyte sparsely labeled with AuTf. The apparent fusion of the MVE and the plasma membrane may represent incipient MVE exocytosis. Bar, 100 nm. (*Right*) View of MVE exocytosis in a reticulocyte labeled with AuTf, quick frozen without prior fixation, and freeze substituted. Bar, 200 nm. ©1983 Harding et al. The Journal of Cell Biology. 97: 329–339. doi:10.1084/jem.138.3.607



cerebrospinal fluid (Johnstone et al. 1987; Street et al. 2012; Madison et al. 2014; Dear et al. 2013). Of the exosomes in circulation, an estimated two-thirds originate from platelets (Hunter et al. 2008). After they are released into extracellular spaces, exosomes can interact with adjacent cells, travel an intermediate distance, or enter the circulation. Exosomes then exert their effects by interacting with specific target cells. Their major role is cell-to-cell communication that occurs by binding then signaling through cell receptors and/or by transferring functional proteins, RNA, metabolites and lipids. Delivery of these bioactive molecules is essential for normal function of the target cells but may also contribute to the progression of disease. Because of these features, exosomes appear attractive as both a biomarker source and therapeutic delivery vehicle. Furthermore, exosomes exhibit immunomodulatory and regenerative properties that encourage their application for other therapeutic purposes.

20.1.1 Exosome Biogenesis

Exosomes are defined by their specific physical properties as part of greater populations of secreted vesicles. Notably, they differ from other shedding vesicles on their mode of biogenesis. Three main types of vesicles were described so far: microvesicles (100 nm–1 μ m) which directly bud from the plasma membrane, apoptotic blebs (50–500 nm), which are released by cells undergoing apoptosis, and exosomes (30–100 nm), released by exocytosis from MVBs of late endosomes (Urbanelli et al. 2013).

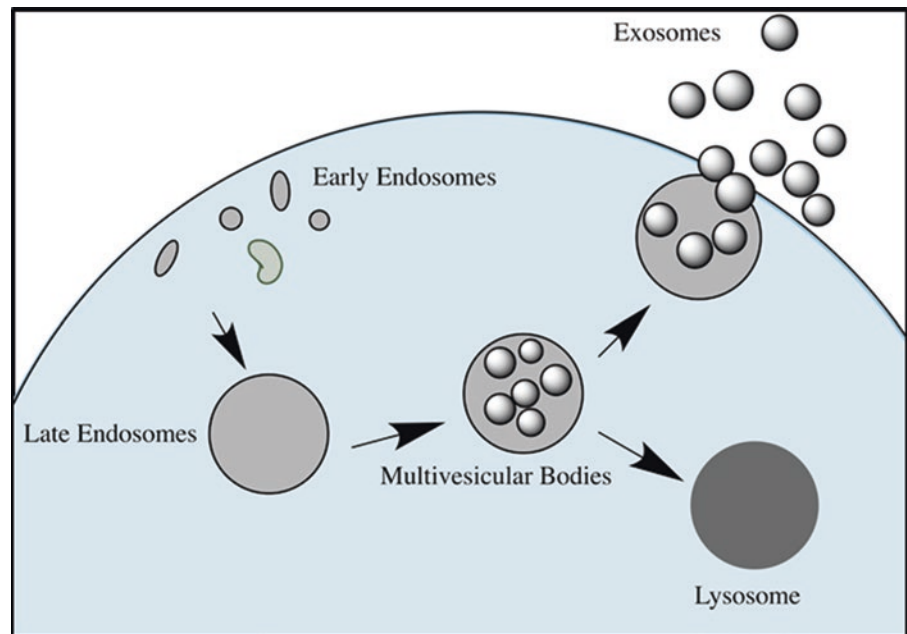
The current dogma of exosome biogenesis involves their generation through endocytic pathways. First, the plasma membrane invaginates to form an intracellular vesicle. The resulting vesicle fuses with endosomal compartments. When the endosomal membrane bubbles

inward, the MVB forms, an organelle characterized by multiple internal vesicles enclosed within a single outer membrane. The MVBs generated in the endocytic pathway move along microtubules and fuse with the plasma membrane. Upon fusion of the MVB with the plasma membrane the intraluminal vesicles (ILVs) are released as exosomes into the extracellular environment (Dragovic et al. 2011; Théry et al. 2002b).

The mechanisms underlying exosome biogenesis and trafficking are an area of active investigation. Currently, two models have been proposed. One mechanism of exosome biogenesis involves the endosomal-sorting complex required for transport (ESCRT) system. In this model, MVB formation occurs when membrane proteins are ubiquitinated and internalized into the early endosome. Here, ubiquitinated to be recognized by ESCRT-0, -I and -II and enter MVBs (MacDonald et al. 2012). Exosomes are also generated through an ESCRT-independent pathway. The concentration of various membrane lipids may allow for raft-based segregation of cargo within the endosomal membrane. Presence of a ceramide micro-domain has been shown to trigger budding of the endosomal membrane, and consequently the formation of the MVB (Trajkovic et al. 2008) (Fig. 20.2).

Physiological conditions such as increased intracellular calcium, UV irradiation, decreased membrane cholesterol, or free radical stress can increase exosome secretion. Conditions dictating exosome release are thought to be cell-type dependent. Upon release from cells, exosomes can follow one of these three pathways: (1) they can be captured by nearby cells, (2) they can be internalized by cells within an intermediate distance, or (3) they can enter circulation and travel to distant tissues (Pant et al. 2012). Exosomes' mode of action ultimately occurs by affecting intercellular communication. This occurs through transfer of bioactive molecules to their target cells.

Fig. 20.2 Schematic representation of exosome biogenesis. Exosome biogenesis occurs when plasma membrane proteins and lipid are endocytosed. Endocytotic vesicles are delivered to the early endosomes, which fuse with each other, forming late endosomes. When the late endosomal membrane blebs inward, multivesicular bodies (MVB) are formed. MVB may mature into lysosomes, where the degradation of their content occurs, or fuse with the plasma membrane and release internal vesicles (exosomes) to the extracellular space



20.1.2 Composition

20.1.2.1 Lipid Content

The exosomal membrane is enriched with lipid-rafts including cholesterol, sphingolipids, ceramide and glycerophospholipids containing long and saturated fatty-acyl chains (Théry et al. 2009). The concentration of various membrane lipids may allow for raft-based segregation of cargo within the endosomal membrane. Presence of a ceramide micro-domain has been shown to trigger budding of the endosomal membrane, and consequently the formation of the MVB (Trajkovic et al. 2008). Exosomes contain cell-type specific lipids that reflect host cell identity. Oligodendrocytes release exosomes that include the lipids galactocerebroside, sulfatide, and cholesterol which are also prominent in oligodendroglial lipid rafts and characteristic myelin lipids (Krämer-Albers et al. 2007). Similar to plasma membranes, exosomes are organized in a bilayer. But unlike plasma membranes, the exosomal membrane is relatively rigid at pH 7. This rigidity may protect exosomes from lipolytic or proteolytic degradation in circulation (Sampey et al. 2014).

20.1.2.2 Protein

Exosomes of different cellular origins sequester a common set of molecules involved biogenesis, fusion, transport, and vesicle structure. They also carry cell-type specific components, which may reflect the biological function of the parent cell. Proteins from incorporated from the plasma membrane retain the same topological orientation as at the cell surface (Simpson et al. 2008).

Because exosomes are generated in the endocytic pathway, neither nuclear, mitochondrial, nor endoplasmic proteins have been observed in exosomes (Keller et al. 2006). Also due to

their endosomal origin, exosomes contain proteins involved in membrane transport and fusion such as flotillin, Rab-GTPases, and annexin. The presence of such proteins may also be important for fusion of the exosomal membrane with the target cell (van Niel et al. 2006). Adhesion molecules such as tetraspanins and intercellular adhesion molecules (ICAMS) may also be functionally important for the fusion and trafficking of exosomes (Théry et al. 2002b).

Exosomes contain cell-type specific proteins from the originating cell. For instance, exosomes derived from antigen presenting cells also carry MHC I and II on the surface as well as co-stimulatory molecules. Additionally, neuronal exosomes carry the cell adhesion molecule L1, the GPI-anchored prion protein, as well as the GluR2/3 subunits of the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) receptor (Fauré et al. 2006; Lachenal et al. 2011) (Table 20.1).

Normal cellular components such as cytoskeletal proteins and metabolic enzymes are also common to the exosomal membrane. Proteome studies have revealed that exosomes contain a conserved set of proteins across species. For example, nearly all exosomes contain cytoplasmic proteins such as tubulin, actin, as well as the signal transduction proteins, kinases, heterotrimeric G-proteins. Proteins most commonly found associated with exosomes include heat shock proteins such as Hsp70 and Hsp 90, and certain members of the tetraspanin superfamily, especially CD9, CD63, CD81, and CD82 (Théry et al. 2002b). For this reason, the trinity of CD9, CD63, and CD81 is often used as an exosome marker.

Proteins may be trafficked to exosomes in a variety of mechanisms. As previously discussed, ubiquitinated proteins are internalized and transported to the endosome where they interact with members of the ERSCT complex involved in

Table 20.1 Common exosome surface markers

Heat shock proteins
Hsp 70, 90
Adhesion molecules
Integrins
Tetraspanins (CD9, CD63, CD81 and CD82)
Immunoglobulin family members
Membrane transport and fusion
Rab GTPases
Annexin
Flotillin
Endosomal sorting proteins
Alix
Tsg101
Lysosomal proteins
LAMP
Antigen presentation
MHC class I
Cytoskeletal components
Actin
Tubulin
Profilin
Cofilin
Metabolic enzymes
GAPDH
Pyruvate kinase
Membrane proteins from cell of origin

MVB formation. Due to the location of MVB biogenesis, components of the endosomal compartments are also likely to be incorporated into ILVs. Last, it has been shown that proteins that are capable of higher-order oligomerization and membrane binding target proteins to the exosomal membrane (Fang et al. 2007).

20.1.2.3 Exosome Cargo

Proteins, mRNA, miRNA and DNA have all been identified in exosomal cargoes (Valadi et al. 2007; Kadiu et al. 2012; Kahlert et al. 2014). Cargoes are both cell-type and cell-state specific. Studies have shown that physiological conditions and the cellular microenvironment heavily influence content of exosomes. For example, it was shown that mouse mast cells exposed to oxidative stress released exosomes that differed in their mRNA content compared to their normal counterparts (Eldh et al. 2010). In another study, when retinal pigment epithelial cell cultures were stressed, α B-crystalline, an antiapoptotic protein, was released via exosomes (Biasutto et al. 2013). Release of the molecule provided neuroprotection for the light-sensitive photoreceptors in the retina (Biasutto et al. 2013).

The mechanisms underlying the sorting of nucleic acids and proteins into exosomes are far from being unraveled. Recent studies have demonstrated that lipid rafts have the ability to sort proteins into exosomes (de Gassart et al. 2003).

Alternatively, the ESCRT complex may also be able to sort exosomal cargoes. Reports have demonstrated that the sorting of trans membrane proteins into the MVB pathway relies on the activity of various ESCRT components (Katzmann et al. 2001; Babst et al. 2002). Though, ESCRT machinery has not yet been studied specifically in relation to exosomal cargo packaging, this finding encourages further investigation.

A mechanism for exosomal microRNA sorting has also been suggested. Recent studies indicate that the shuttling of miRNAs into exosomes is a selective process where specific miRNA motifs are recognized by the heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNP A2/B1), an ubiquitously expressed RNA binding protein (Villarroya-Beltri et al. 2013). Other reports find that 3'-end uridylated miRNA isoforms are over-represented in exosomes and facilitate selective miRNA sorting into exosomes, while 3'-end adenylation increases the probability for retention within the cell (Koppers-Lalic et al. 2014). Consistent with these findings, exosomal miRNAs are composed of a distinct set of miRNAs, different from the miRNA signature of the parent cell (Squadrito et al. 2014). In all, the evidence may imply that certain miRNAs have evolved to be packaged into exosomes to carry out a specific biological function.

20.1.3 Functions

Exosomes have pleiotropic biological functions and are involved in a wide variety of physiological processes. Both cell autonomous and cell non-autonomous activities are mediated by exosomes. Cellular processes such as establishment of cell polarity and differentiation involve exosomes. In cancer, exosomes mediate Wnt-directed cell polarization (Luga et al. 2012). During reticulocyte maturation, exosomes are used in the shedding of the transferrin receptor (Johnstone et al. 1989). Cell non-autonomous functions include intercellular communication, a topic to be detailed in the length of the chapter. In brief, exosomes mediate intercellular communication through the shuttling of active biomolecules to recipient cells. Currently, interaction of exosomes with target cells under physiological conditions is not well understood. Three different models of exosome function have been proposed: (1) internalization by target cells, (2) binding to the cell surface and triggering second messenger pathways, and (3) releasing the components into the extracellular matrix (Frühbeis et al. 2012).

20.1.4 Exosomes and Immune-Modulation

Through a variety of mechanisms, exosomes regulate host immunity. As previously detailed, exosomes derived from antigen presenting cells (APCs) express MHC class I and II,

and co-stimulatory molecules. Because exosomes carry immunorelevant structures, they are suggested to participate in immune response (Admyre et al. 2007). Studies have demonstrated that exosomes are involved in both innate and adaptive immune responses.

Exosomes may directly or indirectly stimulate T-cell activation. Several studies have reported activation of CD4⁺ and CD8⁺ T cells by exosomes originating from APCs (Théry et al. 2002a; Andre et al. 2002; Morelli et al. 2004). Interestingly, exosomes may elicit an immune response using two methods, either through direct antigen presentation or by an indirect presentation through transfer of antigenic peptides to APCs. One study found that peptide-bearing exosomes could not induce antigen-dependent T-cell stimulation unless mature CD8⁺ DCs were also present in the cultures. This suggests that in addition to carrying antigen, exosomes promote the exchange of functional peptide-MHC complexes between DCs (Théry et al. 2002a). Several studies have also reported instances of direct antigen presentation without the need of antigen transfer to an APC. For example, exosomes derived from mature DCs were observed to induce an APC-independent antigen-specific T-cell response in vitro (Segura et al. 2005). Similarly, monocyte derived DC-exosomes can induce IFN- γ production in CD8⁺ T cells in the absence of DC (Admyre et al. 2006). B cell exosomes isolated from patients with birch pollen allergy have also demonstrated an ability to induce T-cell proliferation and TH2-like cytokine production independent of peptide transfer to APCs (Admyre et al. 2007).

Some exosomes have inherent immunological activity. For instance, natural killer (NK) cells release exosomes that carry granzyme and perforin, and exert a cytotoxic activity against target cells (Lugini et al. 2012). Mast-cell (MC)-derived exosomes also elicit immune response. In a study, MC-exosomes stimulated immature DCs to up-regulate antigen presentation molecules such as MHC class II, CD80, CD86, and CD40 (Skokos et al. 2003). This demonstrates that exosomes can modulate immune responses by increasing antigen presentation capacity.

During infection, exosomes may be able to recruit or activate host immune cells. Hollow fiber-based experiments indicate that macrophages treated with exosomes released from *Mycobacterium tuberculosis* (*Mtb*)-infected cells can promote recruitment of macrophage and CD11b⁺ cells into the lung in vivo (Singh et al. 2012). This suggests that exosomes may participate in recruiting and regulating host immune cells during infection.

Exosomes can induce immune activation. One study showed that Hsp70 present in renal tumor-derived exosomes could induce production of pro-inflammatory cytokines and tumor growth factors in myeloid-derived suppressor cells (MDSCs). Furthermore, the exosomal Hsp70 was able to suppress MDSC activity via phosphorylation of Stat3; the tran-

scription factor activated through a TLR2-MyD88 pathway (Diao et al. 2015). Lancaster and Febbraio also determined that exosomal Hsp70 could activate NK cells and macrophages (Lancaster and Febbraio 2005). In all, exosomes may be able to initiate host immune responses.

Exosomes may also play a role in immune tolerance. In a recent study, investigators found that exosomes derived from CD8⁺ suppressor T cells were able to act in an antigen-specific manner to suppress allergic cutaneous contact sensitivity in mice. The authors found that the surface of exosomes collected from tolerized mice was coated in immunoglobulin light chains, accounting for antigen specific targeting (Bryniarski et al. 2013).

Inflammatory responses may also be mediated by exosomes using a variety of mechanisms. For example, exosomes may accelerate engulfment and clearance of apoptotic cells, and in this manner serve to reduce systemic inflammatory responses. Additionally, in vitro studies suggest that exosome-delivered miR-155 can increase inflammatory response to endotoxin in primary bone marrow-derived DCs (Alexander et al. 2015). Exosomes may also modulate inflammatory responses through integrin trafficking, a process crucial for the regulation of leukocyte extravasation and migration, a mechanism essential for immune surveillance. One study demonstrated that exosomes released from human macrophages have the ability to negatively regulate endothelial cell migration through integrin trafficking in primary HUVECs. The macrophage-derived exosomes promoted internalization and lysosomal degradation of integrin β 1 (Lee et al. 2014). Because leukocyte recruitment is dependent on the association of epithelial cell and leukocyte integrins, modulation of integrin trafficking suggests that exosomes may mediate the balance of leukocyte recruitment and inflammation expansion.

20.1.5 Exosomes in CNS Communication

In the CNS, neurons, microglia, astrocytes and oligodendrocytes secrete exosomes into the extracellular environment (Glebov et al. 2015), raising the possibility that communication mediated via extracellular vesicles is a common mechanism in the CNS. It has been reported that exosomes are involved in the normal development and physiology of the nervous system (Hooper et al. 2012; Goetzl et al. 2016). One study demonstrated that mature hippocampal and cortical neurons released exosomes in response to calcium and glutamatergic synaptic activity, which suggests a role in normal physiology (Lachenal et al. 2011).

Additionally, oligodendrocytes release exosomes upon stimulation with the neurotransmitter glutamate. The exosomes are readily internalized by neurons, and enhance neuronal tolerance to oxidative stress and oxygen-glucose

deprivation through the transfer of superoxide dismutase and catalase (Fröhlich et al. 2014). Oligodendroglial exosomes also have an impact on neuronal physiology. Electrophysiology studies revealed an increased firing rate of neurons exposed to oligodendroglial exosomes (Fröhlich et al. 2014). Exosomes not only altered neurons physiologically, but also on a molecular level. Differential gene expression and altered signal transduction pathways were observed in neurons following exosome treatment (Fröhlich et al. 2014). Oligodendrocytes also secrete exosomes to deliver neuroprotective proteins, glycolytic enzymes, mRNA, and miRNA to axons in response to neuronal stress signals (Frühbeis et al. 2013).

Astrocytes also respond to neuronal stress signals by releasing exosomes containing neuroprotective Hsp70 and synapsin 1, facilitating the neuronal survival (Taylor et al. 2007). In primary culture, cortical astrocytes and microglial cells have been shown to release exosomes via ATP activation of P2X7 receptors and the downstream activity of acid sphingomyelinase (Bianco et al. 2009). Additionally, microglial microvesicles have been reported to interact with neurons and enhance spontaneous excitatory transmission through the stimulation of sphingolipid metabolism (Antonucci et al. 2012).

Proteomic and functional analyses of microglial exosomes indicate that they may have a role in neuronal metabolic support and neuropeptide catabolism. One study concluded that microglia-derived exosomes expressed metabolically active CD13, an aminopeptidase involved in cleaving leucine- and methionine-enkephalins peptides. The CD13-cleaved neuropeptides were unable to bind to the neuronal opioid receptor as assessed by cAMP response (Potolicchio et al. 2005). In all, exosomes may represent a previously unrecognized form of CNS communication, a significant finding in an organ restricted by cellular motility.

20.2 Exosomes in Disease Pathogenesis

Though exosomes mediate processes in the CNS required for normal function, they also contribute to the pathogenesis of several neuroinflammatory disorders, cancer, and viral infections. Several studies have demonstrated that exosomes play a role in prion and virus trafficking. Proposed by Gould, the “Trojan exosome” hypothesis describes that exosomes have the capacity to facilitate viral spread, a receptor-independent, and Env-independent mode of infection. Currently, this Trojan exosome model is also being evaluated in protein-misfolding neurodegenerative diseases as a possible mechanism for the spread of pathogenic proteins involved in neurodegeneration. Understanding the role of exosomes in the progression of human disease is important for developing novel therapeutic strategies. Potentially, exosome biogenesis, release, and fusion pathways could become novel pharmacological targets.

20.2.1 Exosomes and Infectious CNS Diseases

20.2.1.1 Transmissible Spongiform Encephalopathies (TSE)

Transmissible spongiform encephalopathies (TSE) or prions are transmissible, fatal neurodegenerative disorders that include Creutzfeldt-Jakob disease, fatal familial insomnia, Gertsmann-Straussler-Scheinker syndrome, kuru, and variably protease-sensitive prionopathy in humans, as well as bovine spongiform encephalopathy and scrapie in sheep (Monari et al. 1994; Collinge et al. 2006; Gambetti et al. 2008; Prusiner 1982; Wells et al. 1987). It has been a long-standing question as to how prions spread between cells and throughout the body. Currently, three mechanisms have been proposed, including (1) direct cell-cell contact, (2) tunneling nanotubes, and (3) extracellular vesicles such as exosomes (Kanu et al. 2002; Gousset et al. 2009; Fevrier et al. 2004). The ability of exosomes to travel over relatively long distances within the body makes them an ideal vehicle for infectious pathogens to exploit. Indeed, a number of studies have implicated exosomal vesicles with prion infectivity. Cellular prion protein (PrP^C) is a known constituent of the protein cargo in blood-circulating extracellular vesicles. Moreover, exosomes isolated from conditioned media of TSE-infected cells produced disease when injected into mice (Saá et al. 2014). Another study concluded that the dissemination of prions in N2a cell culture is mediated through the exosomal pathway (Alais et al. 2008).

Recent *in vitro* studies elucidate how PrP^C and PrP^{Sc} are packaged into exosomes (Fevrier et al. 2004). In cultured cell lines, PrP^C is tethered to the plasma membrane by a glycosylphosphatidylinositol anchor and predominantly associated with lipid rafts, especially microdomains of sphingolipids and cholesterol. The conversion of PrP^C to PrP^{Sc} has been suggested to occur in these lipid raft regions (Taylor and Hooper 2006). The presence of such lipid rafts in exosomes suggests a possible role in prion transmission (Baron et al. 2002). Studies have demonstrated that ESCRT machinery is essential for prion trafficking in exosomes. In Mov neuroglial cells, silencing of the HRS-ESCRT-0 or Tsg101-ESCRT-I subunits drastically impairs the formation of cellular infectious prions. Altered trafficking of cholesterol or impaired production of ceramide also significantly decreases infectious prion release (Vilette et al. 2015). Reports also indicate that inhibition of the neutral sphingomyelinase (nSMase), an enzyme associated with lipid-raft dependent endosomal sorting, impairs both exosome formation and prion packaging, suggesting that the nSMase pathway regulates both exosome formation and packaging of infectious prions (Guo et al. 2015).

20.2.2 Viral Infections

Retroviruses exploit the host cell's intercellular communication machinery to evade immune surveillance and facilitate viral spread. This can be accomplished through several mechanisms including tunneling nanotubes and extracellular vesicles (Kadiu and Gendelman 2011). HIV-1 virus has developed many exosome-mediated strategies to manipulate the host's cell machinery. Recently, exosomes have emerged as novel facilitators of HIV-1 infection (Kadiu et al. 2012; Nguyen et al. 2003). Exosomes may mediate infection in a variety of ways. One mechanism follows the "Trojan exosome" hypothesis, in which exosomes have the capacity to facilitate viral spread through a receptor-independent, and Env-independent mode of infection. This model provides retroviruses the ability to exploit intracellular pathways of vesicular biogenesis and transport, allowing for viral dissemination via exosomes (Nguyen et al. 2003). It is worthwhile to note that HIV and exosome bear a great number of similarities. For instance, HIV and exosome particles share similar lipid content, size, topology, carbohydrate profile, and host proteins. The physiochemical overlap between exosome and viral particles may in part explain how retroviruses are able to seize host machinery within the endosomal compartments to facilitate spread.

It has been shown that exosomal proteins can be sorted on the basis of both higher-order oligomerization (the oligomerization of oligomers) and plasma membrane association. Orthoretroviridae Gag proteins are known to bind the plasma membrane and assemble into higher-order oligomeric complexes (Coffin et al. 1997). This higher-order oligomerization is sufficient to target plasma membrane proteins to HIV virus-like particles, suggesting that Gag proteins possess inherent exosomal-sorting information. One study demonstrated this concept using the HIV Gag protein. Due its plasma membrane anchor and oligomerization domains, the HIV Gag protein, targeted to sites of exosome budding on the PM, and co-localized with exosomal markers CD81 and CD63 at PM cap domains (Fang et al. 2007). Similar patterns have also been observed for Gag proteins other retroviruses including equine infectious anemia virus (EIAV), murine leukemia virus (MLV), HTLV-1, Rous sarcoma virus (RSV), Mason-Pfizer monkey virus (MPMV), and HERV-K (Fang et al. 2007). Retroviruses may also hijack the exosomes biogenesis pathway using other means. The generation of ILVs/exosomes results from the binding of the hepatocyte growth factor tyrosine kinase substrate (Hrs) to ubiquitinated cargo, which recruits Tsg101. HIV-1 Gag is known to mimic Hrs, thereby taking over Tsg101 and other components of the MVB machinery to facilitate viral budding (Pornillos et al. 2003).

Though viral spread through exosomes is thought to be a low-efficiency process, it remains a mechanistically important and previously overlooked mode of infection. The

Trojan exosome model has important implications for the host immune response. This process would allow the virus to access innate and adaptive immune cells, and may help to explain resistance to neutralizing antibodies, a phenomenon observed in HIV infection. The failure of protective HIV vaccines may also be attributed to this model. When viral particles are camouflaged within exosomes, retroviruses are able to bypass the host adaptive immune response. This may help to explain why retroviral antigen vaccines are unlikely to provide prophylactic protection. Given their endosomal origin, exosome-bearing retroviral particles should be sensitive to inhibitors of exosome biogenesis and fusion, allowing for better response to inhibitors of the classic Env-dependent infection (Nguyen et al. 2003).

Other aspects of HIV pathology may be induced by exosomes. Specifically, CNS inflammation observed in HIV associated neurodegenerative disorder (HAND) may also be mediated by exosomes. Previously, investigators have identified that SIV and HIV infection upregulates certain microRNAs (miRNAs) in macaque and human brains (Chaudhuri et al. 2013; Noorbakhsh et al. 2010). A recent study demonstrated that exosomes isolated from rhesus macaque SIVE brains contained miR-21 that could activate TLR7, leading to neurotoxicity (Yelamanchili et al. 2015).

Exosomes play a role in other viral infections. During Human T-lymphotropic virus type 1 (HTLV-1) infection, exosomes that contain the protein Tax cause phenotypic changes in unaffected cells, potentially contributing to pathogenesis of adult T-cell leukemia (Jaworski et al. 2014). Likewise, a correlation between exosome release and viral spread has also been observed for Hepatitis B, C, and E viruses (Bukong et al. 2014), Human Herpes Virus and Herpes simplex (Temme et al. 2010), and Epstein-Barr virus (Pegtel et al. 2010).

20.2.3 Exosomes and Neurodegeneration

Exosomes have become an increasingly popular topic in the field of neurodegeneration. Several studies have demonstrated that exosomes' capacity to transport pathogenic proteins such as alpha-synuclein and amyloid precursor protein. Recently key proteins involved in neurodegenerative diseases such as Alzheimer's (AD) and Parkinson's (PD) disease, and Amyotrophic lateral sclerosis (ALS) have demonstrated the potential for induced spreading of misfolded forms of these proteins in a manner similar to that of prions (Brundin et al. 2010; Goedert 2015; Prusiner 2013).

In addition to transmission of infectious proteins, exosomes may also contribute to pathobiology of neurodegenerative diseases via immune modulation. As previously discussed, exosomes mediate various innate and adaptive immune responses. Among neurodegenerative disorders, one commonality is the involvement of innate and adaptive

immune responses in the CNS. The interplay between peripheral and resident CNS immunity can affect neuroinflammatory responses and exacerbate neurodegeneration (Anderson et al. 2014). Thus, it is possible that inappropriate immune activation induced by exosomes contributes to the pathogenesis of a broad range of neuroinflammatory and neurodegenerative disorders including ALS, AD and PD.

Likewise, exosome cargo RNA may also play a role in neuroinflammation and neurodegeneration. A relationship between exosomes, miRNAs and TLR activation may exist in the nervous system. The interplay of exosomes and TLRs possibly contributes to the pathophysiology of AD, PD, and other related disorders. Several studies have shown that exosomes carry miRNAs associated with pathological alterations during the course of many neurological diseases. miRNAs entering the cells via exosomes may regulate the activation of TLRs, leading to outcomes such as inflammation or apoptosis (Paschon et al. 2016).

20.2.3.1 Parkinson's Disease

Alpha-synuclein (α -syn) aggregation plays a central role in Parkinson's disease pathology, and the transmission of alpha-synuclein from diseased to healthy neurons may be important in the spread of PD through the nervous system. Currently, exosome-based models of α -syn transmission are of particular interest. It has been reported that exosomes released from α -syn over-expressing cells contain α -syn and are capable of transferring α -syn to unaffected cells. The study also demonstrated that lysosomal dysfunction also led to an increase in α -syn production in exosomes and transmission to recipient cells (Alvarez-Erviti et al. 2011a). α -syn can affect neurons via exosomes. Treatment of microglia cells with alpha-synuclein increased exosomal secretion. Exosomes derived from alpha-synuclein treated mouse microglia cell line BV-2 cells were found to express a high level of MHC class II molecules and membrane TNF- α , and were observed to increase apoptosis of rat cortical neurons in vitro (Chang et al. 2013). In all, microglial exosomes produced in response to α -syn may be an important mediator of neurodegeneration in PD. Following the discovery of exosomal α -syn, it was recognized that α -syn might be produced through different pathways, and the mode of secretion may affect α -syn transmission and toxicity. One study concluded that exosomal α -syn oligomers not only are taken up more readily by recipient cells, but can also induce greater toxicity compared to free oligomers (Danzer et al. 2012).

In addition to the transmission of protein aggregates, exosomes may also contribute to PD pathology by propagating an improper immune response. In the inflammatory model of neurodegeneration, chronic activation of microglia is thought to result in loss of dopaminergic neurons (Lull and Block 2010). As previously discussed, exosomes have the ability to affect many aspects of immunity, including antigen presentation and

T-cell activation. Given this, exosomes may mediate the cross talk between that innate and adaptive immune response in the CNS. To date, the role of exosomes in neuroinflammation and Parkinson's has yet to be elucidated.

20.2.3.2 Alzheimer's Disease

Exosomes may contribute to neurodegeneration seen in AD. It has been shown that primary astrocytes secrete exosomes associated with ceramide and PAR-4 in response to A β exposure in vitro. This process is dependent upon ceramide generation by neutral sphingomyelinase 2 (nSMase2). The activation of nSMase2 and expression of PAR-4 is critical for the secretion of apoptotic exosomes and glial apoptosis. Thus, these exosomes may contribute to glial apoptosis, and therefore, neurodegeneration in AD (Wang et al. 2012).

Currently, the role of exosomes in AD is unclear in regards to A β plaque clearance. While some evidence suggests that exosomes interfere with A β clearance, while other studies imply that exosomes may enhance uptake of A β fibrils. The processing of amyloid precursor protein (APP) occurs in early endosomes. Given that exosomes generation also occurs at the endosome, small fractions of A β peptides can be secreted from the cells via exosomes (Rajendran et al. 2006). Pathogenic proteins, such as A β and tau are secreted from the exosomes into the extracellular space where they can interact with TLR2, 4 and 9, receptors overexpressed in AD animal models. Interaction of pathogenic proteins with the TLRs may activate the microglial inflammatory response, leading to clearance of AB plaques (Richard et al. 2008). Likewise, another study demonstrated that exosome secretion increases A β fibrils associated with exosomes, enhancing A β uptake by microglia and eventually resulting in the reduction of extracellular A β (Yuyama et al. 2012). It is thought that the neuronal exosomes capture A β through their enriched glycosphingolipids (Yuyama et al. 2015). Conversely, there is evidence to show that exosomes contribute to the propagation of aggregated proteins. Exosomes have been shown to interfere with uptake of A β by primary cultured astrocytes and microglia in vitro (Dinkins et al. 2014). More recently, Asai and colleagues demonstrated that microglia spread tau via exosomes, and inhibition of exosome biogenesis through pharmacologic blockade of sphingomyelinase-2 reduced tau transmission in vitro and in vivo (Asai et al. 2015).

20.2.3.3 Amyotrophic Lateral Sclerosis

Both mutant and misfolded wild-type SOD1 can traverse cell-to-cell either as protein aggregates that are released from dying cells and taken up by neighboring cells via the surface of exosomes (Grad et al. 2014). Propagation and transmission of misfolded wild-type SOD1 via exosomes is therefore a potential mechanism in the systematic progression of ALS pathology.

20.2.3.4 Chronic Mental Diseases

The study of aggregated proteins can contribute to our understanding of the chronic and progressive mental illness diseases. Dysbindin, a protein encoded by the schizophrenia susceptibility gene DTNBP1 has previously been reported to aggregate with DISC1 (Ottis et al. 2011). Exosomes have been shown to propagate transmission of these neurotoxic dysbindin-1B aggregates in mice (Zhu et al. 2015).

20.2.4 Cancer: T and B Cell Malignancies, Glioblastoma and Neuroblastoma

While exosomes are involved in a myriad of different cancers including melanoma (Hood et al. 2011), breast (Luga et al. 2012), and prostate (Corcoran et al. 2012), their participation in cancers of the CNS and immune system are of particular interest within the field of neuroimmune pharmacology.

Glioblastoma exosomes contain angiogenic proteins and stimulate tubule formation by endothelial cells, and mRNAs with the capacity to remodel the tumor stroma and encourage tumor growth (Skog et al. 2008). Glioblastoma exosomes have a network of nanofilaments on their surface, a structural feature absent in normal human astrocyte exosomes. It is thought that these nanofilaments may increase the probability of exosome binding to target cells (Sharma et al. 2014). If exosomes contain tumor-promoting proteins, and RNAs, this feature may increase their malignant effect. Malignant properties of neuroblastoma may also be modulated by exosomes. Proteomic studies have revealed that neuroblastoma-derived exosomes contain proteins such as prominin-1 (CD133), B7-H3 (CD276), basigin (CD147 or EMMPRIN), and GD2 ganglioside, which may promote tumor-supportive microenvironment and promote tumor progression (Marimpietri et al. 2013).

Exosomes derived from malignant T and B cells have similar properties to those of glioblastoma and neuroblastoma. For instance, Hypoxic leukemia cells secrete exosomes containing miRNAs including miR-210, which modulates tumor microenvironment in a way that promotes endothelial cell tube formation (Tadokoro et al. 2013). Additionally, exosomes from a chronic myeloid leukemia cell line (K562), induced angiogenic activity (Mineo et al. 2012). Both oxidative stress and thermal stress were shown to increase the release of exosomes bearing Natural Killer Group 2 member D (NKG2D) ligands such as MICA/B, ULBP1 and ULBP2 from human T cell leukemia Jurkat- and B cell leukemia/lymphoma Raji cells (Hedlund et al. 2011). These NKG2D ligands prevent NK cell activation, and are upregulated under stressed conditions, allowing immune evasion through the suppression of the NKG2D-dependent NK cell cytotoxicity (Raulet 2003). In B-cell lymphoma, exosomes may contribute to the cellular composition of the tumor, specifically the populations of clonogenic side

population cells and non- side population cells. Exosome-, mediated Wnt signaling enables transitions between clonogenic states, and thus tumor progression (Koch et al. 2014).

20.3 Applications in Nanomedicine

Recently, the diagnostic and therapeutic potential of exosomes has generated immense interest. The ubiquitous nature of exosomes, and their differential miRNA expression in disease states encourages their use as non-invasive biomarkers. Furthermore, their inherent ability to carry metabolically active cargo and travel in the body makes exosomes attractive as therapeutic delivery vehicles.

20.3.1 Biomarkers

As exosomes can be isolated from circulating fluids such as serum, urine, and cerebrospinal fluid (CSF), they provide a potential source of biomarkers for neurological conditions (Street et al. 2012). Analysis of exosomal RNA or protein content may be used to both diagnose disease and predict patient response to therapy. Exosomes appear attractive as biomarkers due to their prevalence in bodily fluids, non-invasive isolation from patients, and differential enrichment of RNAs in pathological conditions. Exosomes isolated from patient bio-fluids may have the potential to diagnose a plethora of diseases or predict patient outcomes. Phosphorylated tau associated with exosomes is present in CSF samples from human AD patients and age-matched controls. Significant and selective enrichment of exosomal CSF phospho-tau was observed in mild (Braak stage 3) stages of AD (Saman et al. 2012). In another example, glypican-1 (GPC1) positive circulating exosomes isolated from the serum of patients and mice with pancreatic cancer were able to distinguish healthy subjects, those with benign pancreatic disease, and those with early and late stage pancreatic cancers. Cohort data found that the levels of GPC1⁺ circulating exosomes correlated with both tumor burden and the survival of patients pre-/post-surgery. Additionally, mutant Kras mRNA was detected in GPC1⁺ exosomes isolated from mice and humans with spontaneous pancreatic cancer (Melo et al. 2015). As the previous study suggests, exosomes may be able to predict patient response to therapy. Response to chemotherapy in patients diagnosed with acute myeloid leukemia (AML) may be predicted through analysis of exosome cargo. One study demonstrated that changes in exosomal TGF- β 1 content are correlated with stage of treatment in AML patients. The investigators found that low exosomal TGF- β 1 levels might be predictive of long-term survival, because exosomal protein levels of patients who achieved long-term remission were not significantly elevated (Hong et al. 2014).

20.3.2 Cell-Free Therapeutic

Exosomes have generated immense interest as a cell-free therapeutic. They offer many advantages over current nanoscale delivery vectors. One such advantage is that exosomes are able access immunologically restricted tissues. While some exosomes actively participate in immune function, other exosomes are able to avoid immune-surveillance, perhaps due to the nature of self-tolerance. Their ability to circumvent immunological barriers and traffic to privileged sites, such as the CNS, is especially relevant in the field of neuroimmune pharmacology. Several studies have demonstrated the capacity of exosomes to mediate delivery of therapeutic bioactives to immune privileged sites. In a report, exosomes were used to encapsulate both curcumin and an activator of Stat3 inhibitor, JSI124. Intranasal administration of these exosome formulations led to rapid delivery drug to the brain, where microglial cells selectively took them up. Treated mice showed protection from LPS-induced brain inflammation (Zhuang et al. 2011). In another study, when exosomes loaded with catalase were administered intranasally in PD mouse model, exosomes were readily taken up by neuronal cells, and significant neuroprotective effects in *in vitro* were observed (Haney et al. 2015). In all, these studies suggest exosome based therapeutics may be a novel therapeutic approach for treating brain inflammatory-related diseases.

Exosome-based delivery systems also allow for targeted delivery. Several studies have also described various methods that allow targeted delivery of exosomal cargo. For instance, exosomes isolated from tolerized CD8⁺ suppressor T cells target effector T cells in an antigen-specific manner via surface coating of antibody light chains. Upon this finding, investigators used this antigen-specific system to deliver inhibitory miR-150 to contact sensitivity effector T cells (Bryniarski et al. 2013). The synthesis of second-generation “biomimetic” exosomes may also represent an efficient approach to develop antigen carriers for specific targeting (Li et al. 2015). Targeting molecules may also be synthetically introduced to exosomes. To achieve delivery of short interfering siRNA to the mouse brain, self-derived dendritic cell exosomes were engineered to express Lamp2b fused to the neuron-specific RVG peptide3. Following intravenous injection, the targeted exosomes delivered GAPDH or BACE1 siRNA to neurons, microglia, and oligodendrocytes in the brain, and resulted in a specific gene knockdown (Alvarez-Erviti et al. 2011b). This method shows great potential in exosome-targeting strategies, however such peptides are subject to proteolysis in circulation. To overcome this, chemical modifications of the peptide may be required. One study demonstrated that glycosylation of peptides on the exosome surface allows for enhanced delivery. This study not only suggests that glycosylation stabilizes targeting peptides, but

also that such modifications do not impede peptide interactions with the target receptor (Hung and Leonard 2015).

Exosomes can either have a stimulatory or inhibitory effect on the immune system; because of this attribute they are attractive as immunotherapy agents. This natural capacity can be utilized to modulate immune response for therapeutic purposes. Exosomes have been investigated for immunotherapeutic treatment of cancer, autoimmune diseases, and as a novel vaccination strategy. In regards to cancer therapy, one approach involves direct interaction between the cancer cells and exosomes. This idea was demonstrated using THP-1 macrophages, which were transfected with chemically modified miR-143 *ex vivo*. Following intravenous injection into in xenografted nude mice, exosome-like vesicles containing the modified miR-143 were secreted, and miR-143 was detected in the in the serum, tumor, and kidney of the host animals. These results study suggests the possible therapeutic use of exosomes in an RNA drug-delivery system (Akao et al. 2011). Apart from directly targeting exosomes to tumor cells, immune cells could also be primed by exosomes containing genetic material, enabling the immune system to recognize and destroy cancer cells. Several studies have utilized DC- exosome bearing functional MHC class I/ peptide complexes to stimulating a specific CTL anti-tumor response (Zitvogel et al. 1998; André et al. 2004). Tumor-derived exosomes have also been shown to act in a similar manner, promoting CD8⁺ T-cell-dependent antitumor effects *in vitro* and *in vivo* (Wolfers et al. 2001).

Immune cell priming via exosomes could also be applied for the development of retroviral vaccines. As discussed previously, failure of protective prophylactic retroviral vaccines, like HIV, may be attributed to the fact that HIV is internalized into CD81⁺ DC intracellular compartments, converging with the exosome biogenesis pathway and allowing for effective viral transmission to CD4⁺ T-cells (Izquierdo-Useros et al. 2010). In all, the proposed exosomal origin of retrovirus predicts that HIV poses an unsolvable paradox for adaptive immune responses (Nguyen et al. 2003). Exosomes-based vaccines may help to overcome this problem. One study demonstrated that DC exosomes bearing HIV_{Gag} were capable of stimulating Gag-specific effector CD8⁺ CTL responses, and resulted in some degree of protective immunity (Wang et al. 2014). Conversely, some exosome have demonstrated the capacity to inhibit CTL responses, which may prove beneficial for autoimmune diseases. Exosomes derived from CD8⁺25⁺ T regulatory (Treg) cell-secreted are capable of inhibiting *in vivo* DC-induced CTL responses (Xie et al. 2013). Because CD4⁺25⁺ and CD8⁺25⁺ regulatory Treg have been shown to inhibit autoimmune diseases (Zwar et al. 2006), CD4⁺25⁺ and CD8⁺25⁺ Treg derived exosomes may become an alternative for immunotherapy of autoimmune diseases. Overall, exosomes may represent a new approach in the development of immunotherapies.

Exosomes have potential use in the field of regenerative medicine. Stem cell transplantation has been thoroughly studied as a means of regenerative therapy. However, exosomes offer several advantages over stem cell transplantation namely; exosomes lack the ability to self-replicate. Due to this fact, exosome therapy would not lead to teratoma formation, a risk associated with stem cell transplantation. Additionally, exosomes can be readily isolated from patients, minimizing immunogenic reactions such as graft versus host disease associated with hematopoietic stem cell transplantations (Storek et al. 1997). Several studies have demonstrated the exosomes' regenerative capacity in neurological and demyelination disorders. One study found that nasal administration of young serum-derived exosomes increase myelin in aging rats through the delivery of miRNA 219 (Pusic and Kraig 2014). Another demonstrated that INF- γ stimulated DC exosomes also increased brain myelin (Pusic et al. 2014). Following traumatic brain injury, exosomes isolated from multipotent mesenchymal stromal cells facilitated functional recovery in rats by promoting angiogenesis and neurogenesis while reducing inflammation (Zhang et al. 2015). In all, exosomes have potential application as cell-free therapeutics for neurodegenerative diseases.

Exosomes have a natural capacity to carry and transmit functional RNAs and DNAs (Valadi et al. 2007; Kahlert et al. 2014). Thus, they have become attractive as gene therapy vectors. In vitro, exosomes can transfer miRNAs from T cell to APCs during immune synapsis, modulating gene expression in recipient cells (Mittelbrunn et al. 2011). Several studies investigating the potential of exosomes as gene-therapy mediators have capitalized on this inherent ability to traffic RNA. Plasma exosomes have been used to transport exogenous siRNA to human monocytes and lymphocytes, effectively delivering siRNA into the blood cells and causing selective gene silencing of mitogen-activated protein kinase (Wahlgren et al. 2012). Exosomal delivered miRNA has also been used to target tumor cells (Akao et al. 2011). These studies suggest that exosomes may be efficient gene delivery vectors, providing cells with therapeutic RNAs.

In addition to the transmission of therapeutic RNAs, exosomes may also allow new opportunities for enzyme therapeutics. Certainly, one could deliver mRNA of interest to the target for later translation into a functional protein. Another approach would involve the loading of functional proteins into exosomes, since the vesicles provide proteolytic protection. Genome editing tools, specifically the CRISPR Cas9 system, have demonstrated the ability to eradicate latent HIV infection (Hu et al. 2014). However, the use of such technology has limited translational applications due to its rapid degradation in vivo. Thus, exosomes might allow technologies such as the CRISPR-Cas9 system to be used therapeutically in humans, allowing for simultaneous delivery of both the enzyme and the RNA components of CRISPR.

20.3.3 Exosome as a Theranostic Tool

Exosomes are unique in their capacity to function as both biomarkers and therapeutic vehicles. The concept of a dual therapeutic and diagnostic agent is termed theranostics in the field of nanomedicine. Chemical modifications to the exosome surface may allow exosomes to be used both diagnostically and therapeutically. One study utilized click chemistry to functionalize the surface of exosomes with azide-fluor 545. Surface modification did not appear to alter uptake into cells, suggesting that the bioconjugation of the fluorescent molecule was functionally inert (Smyth et al. 2014). Reactions like the one described here could theoretically be used to decorate exosomes with a variety of compounds such as radionuclides, fluorescent moieties, or contrast agents. This would also allow for precise in vivo tracking of administered exosomes, potentially useful implication for pharmacokinetic studies of exosome therapeutics.

20.4 Concluding Remarks

Previously thought to be purely waste management in function, exosomes have recently generated intense interest based on their role as signaling mediators. Through the delivery of bioactive cargo such as RNA and protein, exosomes are involved in a wide range of normal and pathogenic processes including CNS function, immunity, inflammation, and cancer. Understanding the role of exosomes in both normal physiology and human disease is important for developing novel therapeutic strategies. Potentially, exosome biogenesis, release, and fusion pathways could become novel pharmacological targets. The ubiquitous nature of exosomes, and differential miRNA expression in disease states encourages their use as non-invasive biomarkers. Furthermore, their inherent ability to carry metabolically active cargo and travel in the body makes exosomes attractive as therapeutic delivery vehicles. The field of exosome biology is rapidly expanding, with exciting discoveries being made in areas of basic research, biomarker discovery, drug delivery and therapeutics.

20.5 Review Questions

1. How was the idea of exosomes first hypothesized?
2. Describe the differences between microvesicles, apoptotic blebs, and exosomes.
3. Describe the contents of the exosomal membrane.
4. What are the three different proposed models of exosome function?
5. Explain Gould's "Trojan exosome" hypothesis
6. How do Glioblastoma exosomes potentially increase the effects of cancer cells?
7. How can an exosome-based drug delivery system be used successfully?

20.6 Answers

1. The first notion for the existence of exosomes was based on an observed vesicular release from maturing reticulocyte endosomes into the extracellular environment (Harding et al. 1983; Pan and Johnstone 1983).
2. Microvesicles (100 nm–1 µm) directly bud from the plasma membrane; apoptotic blebs (50–500 nm) are released by cells undergoing apoptosis; exosomes (30–100 nm), released by exocytosis from MVBs of late endosomes (Urbanelli et al. 2013).
3. The exosomal membrane is enriched with lipid-rafts including cholesterol, sphingolipids, ceramide and glycerophospholipids containing long and saturated fatty-acyl chains.
4. (1) internalization by target cells, (2) binding to the cell surface and triggering second messenger pathways, and (3) releasing the components into the extracellular matrix.
5. Exosomes have the capacity to facilitate viral spread, a receptor-independent, and Env-independent mode of infection. Currently, this Trojan exosome model is also being evaluated in protein-misfolding neurodegenerative diseases as a possible mechanism for the spread of pathogenic proteins involved in neurodegeneration.
6. Glioblastoma exosomes contain angiogenic proteins and stimulate tubule formation by endothelial cells, and mRNAs with the capacity to remodel the tumor stroma and encourage tumor growth (Skog et al. 2008). Glioblastoma exosomes have a network of nanofilaments on their surface, a structural feature absent in normal human astrocyte exosomes. It is thought that these nanofilaments may increase the probability of exosome binding to target cells (Sharma et al. 2014). If exosomes contain tumor-promoting proteins, and RNAs, this feature may increase their malignant effect.
7. Exosomes isolated from tolerized CD8⁺ suppressor T cells target effector T cells in an antigen-specific manner via surface coating of antibody light chains. Upon this finding, investigators used this antigen-specific system to deliver inhibitory miR-150 to CS effector T cells (Bryniarski et al. 2013).

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Abstract

The importance of non-coding RNAs that regulate cellular processes has become evident in the recent past. The most prominent among this class of RNAs are microRNAs (miRs) that are 20-22-nucleotide long single stranded RNAs. miRs are transcribed from the genome as longer transcripts called primary miRs that are processed sequentially by RNase III enzymes DROSHA and Dicer to generate the mature form. In the cytoplasm, miRs recruit a protein complex called the RNA-induced silencing complex (RISC) and mediates down-regulation of translation from target mRNAs. miRs are expressed in a tissue, cell-type and developmental stage specific manner. Aberrant expression of miRs therefore contributes to disease pathology as a consequence of dysregulation of target protein levels. In this chapter we review the discovery, biogenesis, mechanism of action of miRs and their role in neuronal function and dysfunction with emphasis on HIV-associated neurocognitive disorders.

Keywords

DICER • DROSHA • HIV • Inflammation • microRNA • Neurodegeneration • Non-coding RNAs • RISC

21.1 Background and Discovery

The central dogma of molecular biology assigned ribonucleic Acids (RNAs) with the sole function of deciphering the genetic code, stored in deoxyribonucleic acids (DNA). The three types of RNAs that had been identified at that point, namely, messenger RNAs (mRNAs), transfer RNAs and ribosomal RNAs, were all found to play a role in protein synthesis leading to the conclusion that the sole role of RNA was as the intermediate between transcription and translation. This set of assumptions left proteins responsible for all subsequent cellular functions.

However, shortly after the central dogma was proposed, a series of experiments by Howard Temin and David Baltimore led to the discovery that RNA can also serve as a template for DNA synthesis, and provided the first evidence for broader functional roles of RNAs (Temin and Mizutani 1970; Baltimore 1970). Subsequently, the characterization of ribonuclease (RNase) P as a ribozyme, that is, an RNA molecule with catalytic function (Stark et al. 1978), and the discovery of small nuclear RNAs (snRNAs) that function in mRNA splicing (Lerner et al. 1980; Rogers and Wall 1980) promoted RNA to share some of the role of proteins in carrying out cellular functions. The diversity and complexity of the RNA world became evident with the completion of the human genome project that showed that while only 1 % of the genome is translated into proteins, the vast majority of the genome is transcribed into RNA. Several new species of RNAs, for example, small nuclear RNAs, responsible for post-transcriptional modification of nucleotides in other RNA molecules, long non-coding RNAs that regulate chromatin structure and genomic imprinting and microRNAs (miRs) that regulate mRNA translation were soon discovered

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added to this growing list of ‘non-classical’ RNAs (Eddy 2001). The first miRs were co-discovered by the laboratories of Gary Ruvkun and Victor Ambros. During the investigation of *lin-4*, a gene responsible for temporal regulation embryonic development in *Caenorhabditis elegans* (*C. elegans*), it was found that *lin-4* lacks traditional start or stop codons. Introducing mutations into the putative open reading frame for *lin-4* also failed to recapitulate the phenotype induced by its loss-of-function mutations. This led to the conclusion that *lin-4* cannot encode a protein (Wightman et al. 1993; Lee et al. 1993). Rather, the *lin-4* RNA appeared to be its functional form, as it was found to oppose the function of *lin-14* protein in embryogenesis of *C. elegans* (Lee et al. 2004b). Wightman et al. demonstrated that *lin-4* RNA binds to complementary sequences on the 3′-untranslated region (UTR) of its target mRNA *lin-14* resulting in reduction of *lin-14* protein levels (Wightman et al. 1993). Lee et al. showed that *lin-4* primary transcript is processed to generate two smaller RNAs: a 61-nucleotide long *lin-4L* and a 22-nucleotide long *lin-4s*. This 22-nucleotide long *lin-4s* is now recognized as the founding member of a new class of small non-coding RNAs known as miRs (Lee et al. 1993). For 7 years *lin-4* remained an exception, the only RNA of its kind, capable of binding to and down-regulating its target mRNA, until in 2000, it was discovered that *let-7* operates by the same mechanism (Reinhart et al. 2000). Unlike *lin-4*, the *let-7* sequence was conserved evolutionarily (Pasquinelli et al. 2000). This provided the impetus for a systematic search for similar small non-coding RNAs in other organisms culminating in the discovery of several such RNAs in *C. elegans*, *Drosophila melanogaster* (*D. melanogaster*) and HeLa cells. This new class of small regulatory RNAs was named by common consent of the three research groups as microRNA (miRs) (Lagos-Quintana et al. 2001; Lee and Ambros 2001; Lau et al. 2001).

21.2 Biogenesis of miRs

Genes encoding miRs are usually found in clusters. Based on their location, miRs genes can be classified as intergenic or intronic. Intergenic miR genes are located in between traditional protein-coding genes and independent promoters regulate their transcription. On the other hand, intronic miR genes are located within introns of protein-coding genes and are transcribed along with the host gene by the same promoter. The majority of the miRs are transcribed by RNA polymerase II, however certain miRs with *Alu* (*Arthrobacter luteus*) repeat sequences are transcribed by RNA polymerase III (17). The long primary transcript initially generated is called primary-miR (pri-miR). Similar to mRNAs, pri-miRs have 7-methyl guanosine caps and poly-A tails. The secondary structure of pri-miRs represents a stem-loop along with single-stranded RNA (ssRNA) tails at the 5′ and 3′ ends (Lee et al. 2004a).

Pri-miRs generated from intergenic miR genes are processed co-transcriptionally in the nucleus by the microprocessor complex which consists of the class 2 RNase III enzyme Drosha and along with its associated protein: the DiGeorge syndrome critical region 8 protein (DGCR8) in humans or Pasha in *D. melanogaster* and *C. elegans* (18, 19, 22, 23). Drosha contains two RNase III domains and a double-stranded RNA (dsRNA) binding domain. However, the dsRNA-binding domain is not sufficient for its interaction with the pri-miR. Loading of Drosha to the pri-miR is achieved with the assistance of DGCR8/Pasha. These proteins recognize the dsRNA-ssRNA junction in the pri-miR and anchor the microprocessor complex by interacting with it both the ssRNA tails, as well as the dsRNA stem. Once loaded, Drosha cleaves the pri-miR stem 11 base pairs from the ssRNA-dsRNA junction to generate a smaller stem-loop precursor-miR (pre-miR) (Zeng and Cullen 2005; Han et al. 2006). Pri-miRs that are generated from intronic sequences do not require this Drosha processing step (Kim and Kim 2007). Instead, these pri-miRs are spliced out of the host pre-mRNA with the help of spliceosomal proteins.

The pre-miR is next transported out of the nucleus into the cytoplasm by the exportin 5-RanGTP complex that not only facilitates cytoplasmic transport but also prevents nuclear degradation of pre-miRNA (Lund et al. 2004). In the cytoplasm, the pre-miR is cleaved by Dicer (Hutvagner et al. 2001), a class 3 RNase III containing two RNase III domains, one dsRNA binding domain, one PAZ domain, and a helicase domain. Dicer recognizes the 3′ overhang in the pre-miR with the help of its PAZ domain, and the dsRNA binding domain enables Dicer to bind to the pre-miR without the help of any of its associated proteins. The Dicer-associated proteins TAR-RNA binding protein (TRBP or loquacious in *D. melanogaster*) and protein activator of the interferon induced protein kinase (PACT) appear to help load the mature miR into the RNA-induced silencing complex (RISC) (Chendrimada et al. 2005; Lee et al. 2006). The distance between the PAZ domain and RNase III domain of Dicer fixes the cleavage site at exactly 22 nucleotides away from the Drosha cleavage site thereby generating a 22-nucleotide long mature miR (Hutvagner et al. 2001). Cleavage of pre-miR by Dicer is ATP-dependent and relies on ATP to undergo the necessary protein conformational changes for enzyme turnover and product release.

After the synthesis, the mature microRNA duplex is loaded onto the RISC complex by Dicer, TRBP, and one of the Argonaute (Ago) proteins, that together form the RISC loading complex (RLC) (MacRae et al. 2008). The exact mechanism of RISC loading by RLC is unknown. However, in *Drosophila*, another associated protein called R2D2 acts as a thermodynamic sensor (Tomari et al. 2004). Relative thermodynamic stability governs the orientation of miR duplex loading onto the RISC. The end of the duplex that is

thermodynamically more stable is bound to Dicer and/or TRBP, while the other end is bound to Ago. Additionally, thermodynamic stability also appears to determine which of the two strands of the duplex will be loaded into the RISC. The strand with less stable base-pairing at the 5' end is incorporated into the RISC. This is the functional strand of the mature miR and is known as the guide strand. The remaining strand, known as the passenger or star (*) strand, is degraded (Khvorova et al. 2003; Schwarz et al. 2003). Therefore, mature miR duplexes that have similar thermodynamic stability at both ends can generate two functional miR strands because both have equal probability of being incorporated into the RISC. It has been demonstrated that Ago2 has endonuclease activity and is responsible for the degradation of the passenger strand (Matranga et al. 2005; Rand et al. 2005). However, the mechanism of degradation of the passenger miR strands associated with the other three isoforms of human Ago (Ago1, 3 and 4) is yet to be determined.

21.3 Regulation of MIR Biogenesis

RNA polymerase-associated transcription factors regulate transcription of miR genes (Rao et al. 2006; Conaco et al. 2006). Expression of certain miR genes, such as miR-203, is regulated by differential DNA methylation (Bueno et al. 2008). Mature miR levels can be altered by factors that regulate the expression and/or activity of the miR generating enzymes, Drosha and Dicer, or the associated proteins, DGCR8 and TRBP. Drosha and DGCR8 form an autoregulatory loop. Interaction with DGCR8 stabilizes Drosha, while Drosha cleaves DGCR8 mRNA and decreases DGCR8 protein level. This autoregulatory loop helps maintain the microprocessor activity homeostasis. Additionally, post-translational modifications can affect both level of expression and localization of miR generating proteins. Glycogen synthase kinase 3 beta-mediated phosphorylation is required for nuclear localization of Drosha. Acetylation prevents degradation and stabilizes Drosha. Similarly, extracellular signal-related kinase (ERK)-mediated phosphorylation stabilizes DGCR8, while deacetylation increases its affinity for binding pre-miRs. Additionally, phosphorylated methyl-CpG-binding protein 2 (MECP2) binds to and sequesters DGCR8, thereby preventing miR processing. In contrast, the bone morphogenetic protein/transforming growth factor beta signaling pathway increases Drosha processing activity and may influence miR expression levels (Davis et al. 2008a). Similarly, post-translational modifications can also regulate pre-miR processing in the cytosol. Phosphorylation of TRBP by ERK leads to preferential upregulation of growth-promoting miRs by an unknown mechanism. Several RNA binding proteins, for example, KSRP and lin-28, also modulate Dicer activity by binding to the pre-miR.

miR stability is sometimes determined by 5' and 3' end variations. Such variations may arise as a result of non-template directed addition of nucleotides (in case of 3' end variations) or alternative processing by Drosha (in case of 5' end variations). The non-template directed addition of 14 uridine nucleotides to the 3' end of pre-let-7 has been shown to interfere with processing of this pre-miR by Dicer (Heo et al. 2008). The 5' end variations can arise from alternative processing by Drosha as shown with miR-142 (Wu et al. 2009). In addition to affecting miR stability, such variations can also alter the miR target repertoire. Further, RNA-editing enzymes, such as adenosine deaminase acting on RNAs (ADARs), alter the pri-miR sequence by converting adenosine to inosine thus making them poor substrates for Drosha (Yang et al. 2006).

21.4 Mechanism of Action of miRs

miRs regulate the expression of about 60% of all protein-coding genes (Friedman et al. 2009). They do so by binding to the target mRNA and typically decreasing the level of protein product. First the guide strand of the mature miR binds to the target mRNA, most commonly in the 3'UTR, although some miRs have been reported to bind to the 5'UTR and coding sequence (Forman and Collier 2010; Reczko et al. 2012; Grey et al. 2010). The nucleotides in the position two to seven base pairs from the 5' end of the miR are known as the seed sequence. The seed sequence of a miR is perfectly complementary to the miR recognition element (MRE) in the target 3'UTR (Lewis et al. 2003). The miR seed sequence-MRE interaction is often stabilized by base-pairing at the 3' end of the miR (Grimson et al. 2007), presence of an adenine residue at position 1 or an adenine/uridine residue at position 9 from the 5' end of the miR (Lewis et al. 2005). Additionally, presence of multiple MREs on the target 3'UTR increases the probability and extent of inhibition of the target by the miR (Filipowicz et al. 2008). Besides the traditional seed sequence described above, atypical seed sequences have also been reported that exhibit extensive base pairing of the 3' end of the miR to the target 3'UTR (Lewis et al. 2005; Bartel 2009).

Binding of miR to its target mRNA is followed by either inhibition of translation or degradation of the target. RISC-mediated inhibition of translation can occur at the initiation step or at the elongation step. Ago proteins are structurally similar to the translation initiation factor eIF4E and compete for binding to the 7-methylguanosine cap of the target mRNA to prevent initiation of translation (Humphreys et al. 2005; Kiriakidou et al. 2007). Further, RISC interacts with eIF6, blocking the association of the 60s and 40s ribosomal subunits to prevent elongation (Chendrimada et al. 2007). Additionally, miRs can stimulate the premature release of the targets from the ribosome during elongation or decrease the speed of elongation (Petersen et al. 2006). The miR-RISC can

also cause accelerated degradation of its target by recruitment of the CCR4-NOT complex followed by deadenylation of the mRNA poly-A tail and decapping (Wu et al. 2006). The net result of all such processes is a decrease in the cellular level of the protein product of the target mRNA. Although most miRs-mRNA interaction leads to decrease in levels of the target mRNA or its protein product or both, some miRs have also been reported to activate translation of their targets (Vasudevan and Steitz 2007).

21.5 Degradation of miRs

While most of the research thus far has focused on how miRs are generated and what downstream cellular pathways they regulate, our knowledge about the processed leading to their degradation is limited. Only a few miR degrading enzymes have been identified. In *Arabidopsis*, miRs are degraded by small RNA degrading nucleases (SDN1-4) (Ruegger and Grosshans 2012). However, it is not known whether SDN homologs in other eukaryotes also function as miR degrading enzymes. In *C. elegans*, XRN1 and XRN2 help in miR turnover by virtue of their 5'–3' exoribonuclease activity. The miR degrading function of XRN1 appears to be conserved in human cell lines. Polynucleotide phosphorylase (PNPT1) has also been reported to degrade certain mature miRs in humans. Additionally, several pre-miR degrading enzymes have been identified in humans. These include the following: MCPIP that cleaves and inactivates immune system related pre-miRs, IRE1 α that degrades miRs that regulate apoptosis, Dis3l2 that degrades poly-uridylated let-7 and translin/trax complex that competes with Dicer/TRBP leading to degradation certain pre-miRs (Asada et al. 2014). It appears that the miR degrading enzymes identified thus far act only on specific sets of miRs/pre-miRs. In the case of translin/trax, this specificity is conferred by pre-miR secondary structure, trax being a single-strand-specific endonuclease is able to degrade only those pre-miRs that have unpaired bulges in their stem. Our knowledge about regulation of miR turnover is still nascent and requires further investigation.

21.6 Methods in miR Research

Differential expression of miRs can be identified by performing miR microarrays and can then be confirmed by performing qRT-PCR or Northern blot. Localization of miR can be determined by performing in situ hybridization. Because most miRs act by downregulating expression of specific targets, putative miR targets can be identified *in silico* from expression data using various algorithms. The most commonly used tools for such analysis are TargetScan (Friedman et al. 2009), microRNA.org (miRanda) (Betel et al. 2008),

PicTar (Krek et al. 2005), and DianaMicroT (Maragkakis et al. 2009a, b). Most of these algorithms use the miR seed sequence (nucleotide 2–7 from the 5' end) to search for potential matches on the 3'UTR of mRNAs. However, such *in silico* analysis yields hundreds of potential targets for a single miR. To narrow down on physiologically relevant targets, it is necessary to perform further bioinformatic analysis and/or literature review. For example, gene ontology (GO) analysis can be performed to group the predicted targets according to the biological processes (BP) in which they are involved, cellular components (CC) in which they are expressed, or their molecular functions (MF). If GO analysis identifies an overrepresentation of certain BP, CC or MF, it is reasonable to hypothesize that the miR of interest regulates that particular pathway. Direct action of the miR of interest on the 3'UTR of the predicted targets in that group can then be tested experimentally. 3'UTR-luciferase reporter assays are commonly used for this purpose (Aldred et al. 2011). In this experimental paradigm, the 3'UTR of the target of interest is cloned into a luciferase reporter vector and co-transfected into a cell line along with the miR of interest. If this miR indeed binds to the 3'UTR, this should result in a decrease in expression of the luciferase as compared to co-transfection with a scrambled miR sequence. The decrease in luciferase expression is detected as a reduction in light signal (luminescence) in the assay. Downregulation of the target by the miR can again be confirmed by performing qRT-PCR or Western blot. However, a negative result in qRT-PCR should be interpreted with caution, as some miRs prevent translation without affecting mRNA levels.

21.7 miRs and Neuronal Physiology

Given the widespread influence of miRs on cellular protein levels, it is not surprising that they participate in regulation of development of the central nervous system (CNS), neuronal differentiation and function. The first indication of the importance of miRs in brain development came from studies that disrupted expression of proteins involved in miR biogenesis (Dicer, DGCR8) or function (Ago). Deletion of Dicer in zebrafish resulted in impaired miR biogenesis and led to abnormal neural tube development, neural positioning and touch-induced behavioral response (Giraldez et al. 2005). Surprisingly, overexpression of a single miR, miR-430, was sufficient to rescue most of the neural deficits of dicer deficiency. miR-430 belongs to a subset of miRs that are specifically enriched in the brain (Sempere et al. 2004). Expression levels of these brain-enriched miRs are regulated in a developmental stage and cell-type specific manner (Miska et al. 2004). Similar to the findings in zebrafish, deletion of Dicer in *D. melanogaster* and HeLa cells led to accumulation of abnormal protein aggregates characteristic of neurodegenera-

tive disorders (Bilen et al. 2006). Complete deletion of Dicer is embryonically lethal in mice (Murchison et al. 2005). To overcome this limitation, researchers have focused on conditional deletion of dicer in specific brain regions. Dicer deletion in the forebrain led to motor impairments and early post-natal death accompanied by multiple defects in brain development including microcephaly, enlarged lateral ventricles, increased neuronal apoptosis, altered dendritic spine length, abnormal dendritic morphology, and axonal pathfinding defects (Davis et al. 2008b). Similarly in the midbrain and hindbrain, dicer deletion resulted in reduction in cerebellum size, impaired development of dopaminergic neurons and increased apoptosis (Huang et al. 2010). Both of these studies demonstrated that dicer deletion was associated with reduced expression of brain-specific miRs.

Similar to the Dicer-knockout, it was found that complete deletion of DGCR8 also resulted in embryonic lethality, and caused DGR8-knockout mice to die around embryonic day 6.5. The DGCR8 heterozygous mice exhibited reduced dendritic spine width, reduced complexity of dendritic spines, and impaired working memory (Stark et al. 2008).

Knocking down or mutating genes that encode argonaute proteins offers a second mechanism of disrupting the cellular miR machinery. Loss-of-function mutations of Ago1 in *D. melanogaster* have been found to cause malformation of the nervous system with reduction in number of neurons and disruption of axons (Kataoka et al. 2001). Just as in the Dicer and DGCR8 knockouts, homozygous knockout of Ago2 in mice were found to cause embryonic lethality and heterozygous deletion of Ago2 were found to cause severe developmental defects, including failure of neural tube closure (Liu et al. 2004). These studies emphasized the importance of maintaining a normal global miR expression profile during CNS development and function.

Specific miRs that regulate neuronal development have also been identified. miR-124 appears to be the most abundantly expressed miR in adult brain (Lagos-Quintana et al. 2002). Transfection of miR-124 into HeLa cells alters the cellular gene expression such that it begins to resemble a neuronal profile (Lim et al. 2005). miR-124 functions in an inhibitory feedback loop with the repressor element 1-silencing factor (REST) to promote differentiation of neurons from neural progenitor cells (Conaco et al. 2006; Visvanathan et al. 2007). Another brain-enriched miR, miR-137 increases neuronal differentiation and regulates adult neurogenesis (Silber et al. 2008; Szulwach et al. 2010). miR-9, a brain-specific miR expressed in the neurogenic niche in both embryonic and adult brain, regulates neural stem cell proliferation and neural differentiation by controlling the expression level of several transcription factors (Zhao et al. 2009; Shi et al. 2010). The let-7 family of miRs, although not brain-specific, is highly expressed in the brain and also induces neuronal differentiation (Sempere et al. 2004). miR-184 on the other hand, promotes neural stem

cell proliferation and inhibits neuronal differentiation by downregulating its target mRNA, NUMBL (Liu et al. 2010). Other miRs that regulate neurogenesis include miR-125b and miR-128 (Lang and Shi 2012).

Differential sub-cellular localization of miRs in mature neurons provides an efficient method of regulating local mRNA translation. Besides the cell body, in mature neurons, miRs and RISC are found at dendritic spines and axon terminals as well (Hengst et al. 2006; Cougot et al. 2008). At the synapse miRs can regulate expression levels of key proteins in response to neuronal activity or neurotrophic and growth factors in a rapid and bidirectional manner without affecting the overall expression of these proteins. miR-134 was one of the first miRs found to be localized within dendrites where its expression increased with synaptic activity. miR-134 was found to regulate dendritic spine morphology (Schratt et al. 2006). In addition to its role in neuronal differentiation, miR-124 has also been detected in the presynaptic compartment (Rajasethupathy et al. 2009) and it regulates neurite outgrowth and serotonin induced long-term plasticity (Yu et al. 2008; Rajasethupathy et al. 2009). In *Drosophila*, neuronal activity relieves miR and RISC mediated inhibition of translation of Ca²⁺/Calmodulin dependent protein kinase II (CaMKII) mRNA thereby enhancing neuronal plasticity (Ashraf et al. 2006). A similar mechanism of activity-dependent relief of miR-138-mediated translational repression of CaMKII, LIM domain Kinase I, and lysophospholipase 1 mRNAs occurs in rats (Banerjee et al. 2009). Expression levels of certain miRs, e.g., miR-132, are increased in response in neuronal activity. Upregulation of miR-132 in turn increases dendritic spine formation and synaptic excitability (Magill et al. 2010). Other miRs that have been reported to regulate dendritic growth and synaptic function including miRs-34a, -375, -125b, and -188 (McNeill and Van Vactor 2012).

Finally, although most of the studies so far have concentrated on the expression and function of miRs in neurons in the CNS, miRs also play an important role in glial cell genesis and function. In this context, miRs-219 and -338 downregulate transcription factors Sox6 and Hes5 to promote differentiation of oligodendrocytes (Zhao et al. 2010). The miR-17-92 cluster promotes proliferation of oligodendrocyte precursors (Budde et al. 2010). Postnatal deletion of Dicer in astrocytes led to ataxia, cerebellar degeneration, seizures, and premature death. These Dicer-deficient astrocytes displayed a gene expression profile characteristic of immature and reactive astrocytes, and contributed to excitotoxic neuronal damage (Tao et al. 2011).

21.8 miRs in CNS Pathology

Differential miR expression has been reported in several neurodegenerative, neurodevelopmental, and neuropsychiatric disorders. This differential expression could either be a cause or

consequence of the observed pathological changes. miRs may target proteins that have been implicated in these disorders and contribute directly to disease pathogenesis. Alternatively, changes in miR expression downstream of changes in the disease-causing pathways may add on to the pathological manifestations by modulating neuronal survival or death pathways.

Accumulation of β -amyloid ($A\beta$) plaques is a hallmark of Alzheimer's disease (AD). This could be due to an increase in expression of the amyloid precursor protein (APP), increased processing of APP to $A\beta$ or decrease in $A\beta$ degradation. The miR-29 family can regulate expression of β -site APP cleaving enzyme 1 (BACE1), an enzyme required for $A\beta$ production. Decrease in expression of the miR-29 family may therefore lead to increased expression of BACE1 resulting in more $A\beta$ production and accumulation, as seen in AD (Hebert et al. 2008). Other studies have reported decrease in miR-107 expression in brain of AD patients in the early stages of the disease accompanied by increase in BACE1 protein levels (Nelson and Wang 2010; Wang et al. 2011). miR-107 can also bind to the 3'UTR of BACE1 and reduce its expression.

A point mutation in the 3'UTR of fibroblast growth factor 20 (FGF20) has been identified as a risk factor for Parkinson's disease (PD). FGF20 regulates expression of α -synuclein, a protein that accumulates in PD. The point mutation on the FGF20 3'UTR disrupts a miR-433 binding site (Wang et al. 2008). The loss of downregulation of FGF20 by miR-433 may therefore contribute to increase in α -synuclein levels. miR-133 is another miR that may be associated with PD. Expression of miR-133 is lower in post-mortem brain tissue of PD patients (Kim et al. 2007). miR-133b is highly enriched in midbrain dopaminergic neurons, and the degeneration of these neurons in PD may be the reason of the observed decrease in miR-133b levels. Decreased expression of miRs-34b and -34c has also been observed in PD in the frontal cortex, amygdala, substantia nigra, and cerebellum. In vitro, downregulation of miRs-34b and -34c in cell lines led to cell death, oxidative stress, and mitochondrial dysfunction—all characteristics of the neuronal dysfunction found in PD (Minones-Moyano et al. 2011). A number of miRs, including miR-7 and -153, downregulate expression of α -synuclein in vitro and in animal models of PD (Junn et al. 2009; Doxakis 2010). Reduced expression of these miRs may lead to increased synthesis and accumulation of α -synuclein characteristic of PD.

Amyotrophic lateral sclerosis (ALS) is associated with mutations in several RNA-binding proteins including Transactivating response element (TAR) DNA-binding protein 43 (TDP-43) and fused in sarcoma (FUS). Both TDP-43 and FUS regulate biogenesis of miRs. TDP-43 enhances miR biogenesis at the Drosha and Dicer processing steps (Kawahara and Mieda-Sato 2012). FUS interacts with Drosha to promote miR biogenesis (Morlando et al. 2012). Aberrant biogenesis of miRs may therefore contribute to pathogenesis of

ALS. Expression of miR-155 was found to be increased in animal models of ALS and inhibition of this miR increased survival in this model (Koval et al. 2013). miR-155 is an inflammatory miR, that increases secretion of pro-inflammatory cytokines by targeting suppressor of cytokine signaling 1 (SOCS1) mRNA (Wang et al. 2010) and prevents the immunosuppressive action of transforming growth factor β (TGF β) by targeting its downstream effectors Sma- and Mad-related protein (SMAD) 2 (Louafi et al. 2010) and SMAD5 (Rai et al. 2010). miR-206, a skeletal muscle-specific miR, inhibits translation of histone deacetylase 4 (HDAC4) thereby inducing secretion of fibroblast growth factor binding protein 1 (FGFBP1) from muscles (Williams et al. 2009). FGFBP1 promotes presynaptic differentiation at the neuromuscular junction. miR-206 may therefore be beneficial in ALS and knockdown of miR-206 has been shown to be detrimental in animal models of ALS (Williams et al. 2009).

In Huntington's disease (HD), decreasing levels of expression and miR-9 and miR-9* have been observed with disease progression (Packer et al. 2008). These miRs regulate the expression of transcription factors REST and its co-repressor. Upregulation of the REST repressor complex due to reduced levels of miR-9 and miR-9*, leads to a decrease in expression of pro-survival genes including brain derived neurotrophic factor (BDNF).

In fronto-temporal lobar dementia (FTLD), a single nucleotide polymorphism (SNP) in the 3'UTR of the granulin (GRN) gene was found to be associated with a familial form of the disease. This SNP introduces a miR-659 recognition element in the GRN 3'UTR. miR-659 that is normally expressed in the brain can bind to this MRE and reduce expression of the pro-survival and anti-apoptotic protein GRN (Rademakers et al. 2008).

Fragile X-syndrome (FXS) is an inherited neurodevelopmental disorder caused by triplet repeat expansion in the 5'UTR of the Fragile X mental retardation protein (FMRP) leading to hypermethylation and reduced expression of this gene. FMRP is a RNA-binding protein that has been found to associate with Dicer and Ago. In *Drosophila*, loss of function of FMRP led to defects in miR processing as evidenced by a decrease in mature miR-124a and accumulation of pre-miR-124a (Xu et al. 2008b). Expression of miR-124a appeared to be important for dendrite branching. Several miRs have now been shown to be associated with FMRP. Examples include miR-9, 124, -125a, -125b, -128, -132 and -219, which are localized in dendrites and control synaptic plasticity (Edbauer et al. 2010; Xu et al. 2008b). Interestingly, it was shown that FMRP acts a bidirectional switch controlling local RISC activity at the dendritic spine. Phosphorylated FMRP associates with miR-125a-RISC and enhances binding to and inhibition of translation of the target post-synaptic density 95 (PSD95) mRNA. In response to glutamate receptor signaling, FMRP was dephosphorylated and could no longer bind to miR-125a-RISC, which caused the

release the PSD95 mRNA from the inhibitory complex (Muddashetty et al. 2011). Loss of FMRP function could therefore impair synaptic activity dependent protein synthesis that is required for the formation of long-term memory.

Rhett syndrome is an X-linked neurodevelopmental disorder that has been associated with the mutation of the gene encoding methyl-CpG-binding protein 2 (MeCP2). In primary cortical neurons, expression of this protein is regulated by miR-132, a brain-enriched miR whose expression is induced by BDNF (Klein et al. 2007).

Genome-wide association studies have also indicated involvement of miRs in autism spectrum disorders (ASD). Two of the most significant copy number variations detected include microduplication of the 22q11.2 locus that includes the DGCR8 gene and imbalance at 15q13.2-15q13.3 that contains the miR-211 gene (Glessner et al. 2009; Marshall et al. 2008; Miller et al. 2009). The latter was associated with intellectual disability, epilepsy and electroencephalographic abnormalities of ASD.

In Down's syndrome (trisomy 21), five miRs, whose genes are located on chromosome 21, were found to be upregulated in the fetal brain. Of these, miRs-155 and -802 downregulate expression of MeCP2 and may partially contribute to the neurological manifestations of the disease (Kuhn et al. 2008).

The deletion of a part of human chromosome 22 (22q11.2 microdeletion) is one of the most important risk factors for schizophrenia. The deleted region includes the gene encoding the Drosha-associated protein DGCR8, indicating that miR biogenesis may be dysregulated in the disease. In a mouse model where the syntenic region harbored on mouse chromosome 16 was deleted, downregulation of a specific subset of miRs including miR-134 was observed and could be attributed to heterozygous loss of DGCR8 (Stark et al. 2008). Genome-wide association studies for SNPs in schizophrenia patients have identified duplication of a region encompassing the Dicer1 gene and association of the miR loci for miR-137, miR-206 and miR-198 (Schizophrenia Psychiatric Genome-Wide Association Study (GWAS) Consortium 2011; Hansen et al. 2007; Xu et al. 2008a). Additional evidence of altered miR expression in schizophrenia comes from miR expression analyses of post-mortem brain tissue from schizophrenia patients. These show evidence of dysregulation of miR-7, -24, -26b, -29b, -30a-5p, -30b, -30e, -92, -181b and -195 (Mellios et al. 2009; Beveridge et al. 2008; Perkins et al. 2007).

Major depression is associated with deficits in serotonergic neurotransmission and miRs that regulate synthesis, reuptake, or degradation of this neurotransmitter may be involved in pathogenesis of this disorder. miR-16, for example, targets the serotonin transporter, and overexpression of this miR can lead to antidepressant like effects (Baudry et al. 2010). Polymorphisms in genes encoding miRs-30e and -183 have also been associated with increased susceptibility to depression, although the downstream pathways regulated by these miRs are yet to be identified (Xu et al. 2010; Saus et al. 2010).

21.9 miRs and HIV-Associate Neurocognitive Disorders (HAND)

Expression profiling has provided some insight into the role of miRs in the pathogenesis of HIV-associated neurocognitive disorder (HAND). Noorbakhsh et al. compared the miR expression profile in the frontal cortex from autopsied brain of four individuals with HIV-encephalitis (HIVE) to four uninfected controls (Noorbakhsh et al. 2010). Using miR microarray and quantitative real-time PCR (qRT-PCR) they found expression levels of fourteen miRs were significantly altered greater than two-fold between HIVE and control sample. Of these, six were upregulated and eight were downregulated in HIVE samples. The authors then performed an *in silico* search for miR targets. Functional clustering of the predicted targets led to identification of caspase pathway as a common target for many of these miRs. Since most of the miRs targeting the caspase pathway were downregulated in HIVE samples, they concluded that derepression of the caspases may contribute to neuronal apoptosis.

In a second study, Yelamanchili et al. investigated the miR expression profile in the caudate nucleus of HIVE samples, as well as in the caudate nucleus and hippocampus of Simian immunodeficiency virus encephalitis (SIVE) samples using miR microarray (Yelamanchili et al. 2010). Six miRs were found to be upregulated in the caudate nucleus of SIVE samples compared to uninfected controls. Four of these miRs were also found to be upregulated in the caudate nucleus in HIVE and in the hippocampus of SIVE samples. Post-validation using qRT-PCR confirmed significant upregulation of three of these miRs (miRs-21, 142-3p and -5p). Interestingly, upregulation of miR-142-3p and -5p was confirmed by both groups of researchers. Of these, miR-21 was found to regulate the transcription factor Mef2c and alter potassium current in human neurons grown in vitro. miR-142, on the other hand, was found to target and downregulate SIRT1, which eventually led to decrease in the level of dopamine metabolizing enzyme monoamine oxidase A.

A third study by Tatro et al. utilized a PCR-based miR microarray platform to compare miR expression in the frontal cortex of uninfected control individuals, HIV infected individuals and those with HIV infection and major depression (Tatro et al. 2010). However, it was not indicated whether any neurocognitive impairment was present independent of depression, and as such no conclusions could be drawn about miR expression changes in HAND from this study.

How does HIV alter neuronal and glial miR levels? Studies in cell culture models have indicated that HIV proteins Tat and Vpr regulate miR expression in cultured human neurons and neuronal cell lines (Mukerjee et al. 2011; Chang et al. 2011). However, whether these viral proteins can regulate neuronal miR expression in vivo

remains to be determined. In microglial cells, HIV can subvert cellular defense mechanisms by increasing the expression of miR-146a, which in turn downregulates CCL8 (Rom et al. 2010), a chemokine that normally inhibits viral entry by binding to the receptor CCR5.

In addition to intracellular miRs, a large body of research has focused on miRs secreted in body fluids because these miRs can serve as potential biomarkers for diseases and drug treatments. One study compared the miR levels in the cerebrospinal fluid (CSF) of HIV infected patients with and without encephalitis to those of uninfected controls (Pacifi et al. 2013). Eleven miRs were found to be significantly differentially expressed across the three groups, out of which, six were also differentially expressed in the frontal cortex. The same study found a general trend of downregulation of miR expression in HIV infected group without encephalitis compared to uninfected controls. However this trend was reversed in the HIV infected group with encephalitis, in whom most of the miRs were upregulated compared to those without encephalitis. Comparison of the HIV encephalitis group to uninfected controls showed that most of the miRs were relatively unchanged in the CSF.

21.10 Future Directions

Since their discovery a just over a decade ago, numerous studies have established the importance of miR expression and function, both in normal physiological state, as well as in pathological conditions. However, several aspects of miR biology need further investigation. Each miR has several hundreds of potential target mRNAs. The principles that govern preferential target selection are largely unknown. One important criteria would be co-expression of the miR and its target mRNA in the same cell at the time frame being assessed. But, controlling for co-expression still leaves us with a large target pool for one miR. Other factors that could help determine target selection and efficiency of target downregulation need to be determined. Investigators are just beginning to study the stability and mechanisms of degradation of miRs. From initial reports it appears that miR degrading enzymes are specific for certain groups of miRs, the specificity being determined, at least in one case, by miR secondary structure (Asada et al. 2014). While most miRs bind to the 3'UTR of their target mRNA by complementary base pairing between the traditional miR seed sequence and MRE, several instances of miR binding to the coding sequence and 5'UTR have now been documented. We need to determine whether such binding is an exception to the general rule, or a common occurrence that has been overlooked.

21.11 Review Questions

1. Briefly describe the mechanism of biogenesis of miRs. What factor determines the strand selection for mature miRs while incorporation into the RISC?
2. How do miRs regulate level of the protein product of their target mRNAs?
3. List 5 miRs that are upregulated in the brain in HIV. Explain the probable mechanism through which any one of these miRs contributes to pathogenesis of the disease.
4. You suspect that 'disease X', a neurodegenerative disease, is associated with defects in miR biology. What method will you use to identify and thereafter validate miRs with altered expression in the disease?
5. You identify that miR-9999 is downregulated in 'disease X'. While performing qRT-PCR for validating the level of expression of miR-9999, by mistake, you add the primer for pre-miR-9999 instead of the mature form. However, your results show that there is no change in pre-miR-9999 expression. How can you explain your result?

21.12 Answers

1. Section 21.2 and Fig. 21.1 describes in detail the mechanism of miR biogenesis. Relative thermodynamic stability of the two ends of the miR duplex determines which of the two strands will be loaded into the RISC. The strand with less stable base-pairing at the 5' end is incorporated into the RISC. This is the functional strand of the mature miR and is known as the guide strand. The other strand, known as the * strand is degraded.
2. miRs decrease the level of protein product of the mRNAs that they target. They may do so by two mechanisms: inhibition of translation or degradation of the target mRNA. Section 21.4 describes the mechanism of action of miRs.
3. Table 21.1 lists the miRs that are upregulated in HIV. The probable mechanism of action of these miRs is described in Sect. 21.9.
4. Section 21.6 describes the methods used in miR research. Generally, miR microarrays can be used to identify global changes in miR expression. qRT-PCR is used to validate the level of expression of candidate miRs.
5. Expression of the mature form of miR-9999 is decreased while pre-miR-9999 expression level did not change in 'disease X'. This implies that there may be a defect in the miR biogenesis machinery. Particularly Dicer/TRBP processing step or at the export of pre-miR by exportin 5 may be defective.

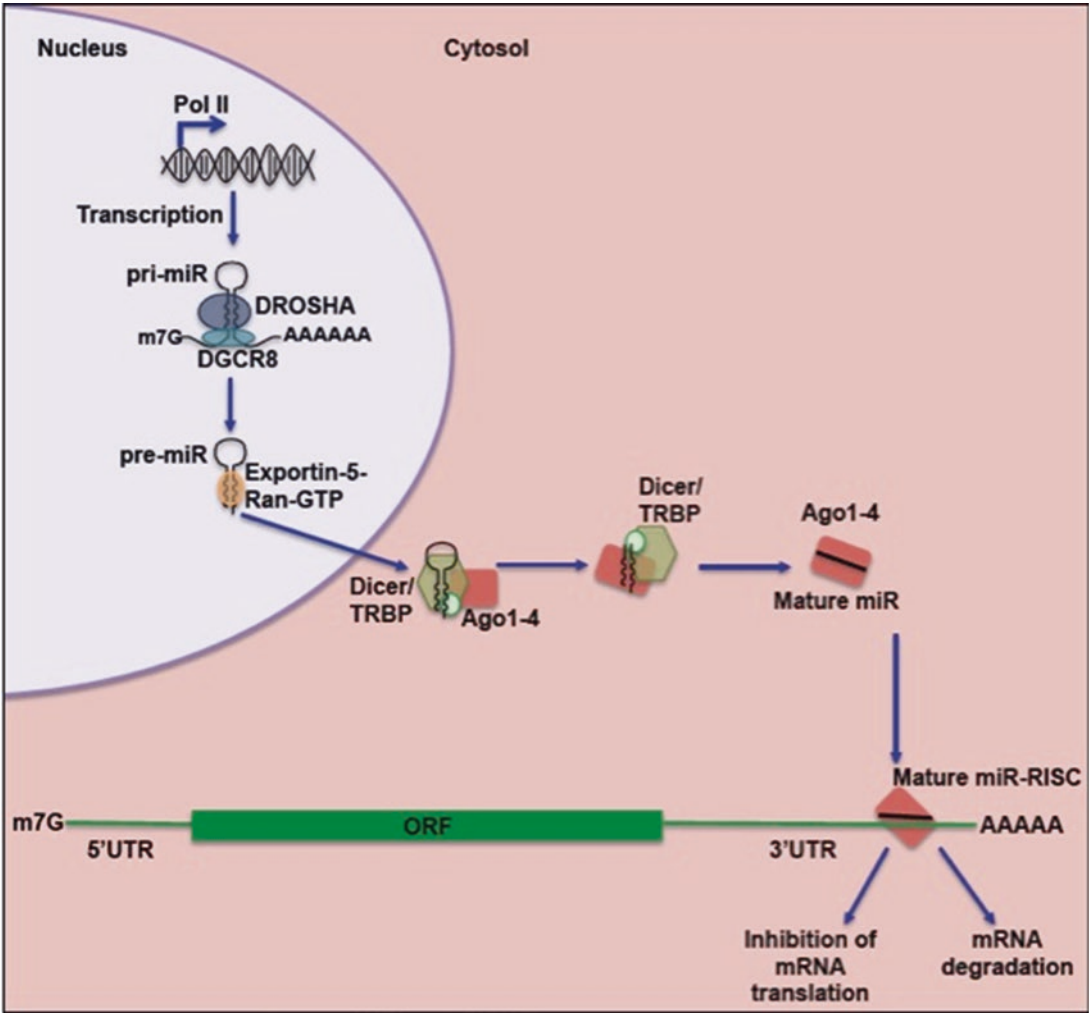


Fig. 21.1 Biogenesis and mechanism of action of miRs. Pri-miRs are transcribed by RNA polymerase II and processed in the nucleus by DROSHA/DGCR8 complex to pre-miRs. Pre-miRs are exported to the cytoplasm by exportin-5-Ran-GTP. In the cytosol pre-miRs are cleaved

by Dicer/TRBP complex to generate mature miRs that are incorporated into RISC complex consisting of Argonaute proteins (Ago1-4). Mature miR-RISC binds to 3'UTR of target mRNA leading to inhibition of translation or degradation of target

Table 21.1 MicroRNAs in HIVE

miRs-135a, -128, 129-3p, -129*, -130a, -7, -218, -329	Downregulated in frontal cortex in HIVE compared in uninfected control
miRs-338-5p, -181a, -142-5p, -142-3p, -584, -219-3p	Upregulated in frontal cortex in HIVE compared to uninfected control
miRs-21, -142-3p, -142-5p	Upregulated in caudate nucleus in HIVE, caudate nucleus and hippocampus in SIVE compared to uninfected control
miRs-1203, -1224-3p, -182*, -19b-2*, -204, -362-5p, -720, -744, -934, -937	Upregulated in CSF in HIVE compare to HIV infection without encephalitis
miRs-484	Downregulated in CSF in HIVE compare to HIV infection without encephalitis
miRs-1224-3p, -204, -720, -934	Upregulated in frontal cortex in HIVE compare to HIV infection without encephalitis

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Part II

Immunology of Neurodegenerative, Neuroinflammatory, Neuroinfectious and Neuropsychiatric Disorders

Serge Przedborski

Abstract

Neurodegeneration is any pathological condition in which the nervous system or nerve cell (i.e. neuron) loses its function, structure, or both. On a medical point of view however, the term neurodegeneration is used in a more restricted sense. Typically, it represents a large group of heterogeneous disorders in which affected neurons belong to specific subtypes, within specific anatomofunctional territories of the nervous system. Often, but not always neurodegenerative diseases arise for unknown reasons and progress in a relentless manner. Within the context of this definition diseases of the nervous system can be catalogued into three board categories: (i) pathologies which are restricted to the nervous system and which are *primary neuronal* diseases (i.e. neurodegenerative diseases *per se*); (ii) pathologies which are restricted to the nervous system but are not *primary neuronal* diseases, such as brain neoplasm or cerebral edema and hemorrhage; and, (iii) pathologies provoked by systemic causes which damage the nervous system, such cardiovascular arrest, carbon monoxide poison, or infections due to herpes simplex. Based on this simple categorization, hundreds of disorders of the nervous system including Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis clearly fulfill the criteria of neurodegenerative disorder and are unanimously regarded as such.

Keywords

Alzheimer's disease • Amyotrophic lateral sclerosis • Asynchronous death • Basal ganglia • Cell autonomy • Cerebral cortex • Creutzfeldt-Jakob disease • Comorbidity • Dystonia • Etiology • Friedreich ataxia • Huntington's disease • Leucine-rich repeat kinase-2 (LRRK2) • Multiple sclerosis • Multisystem atrophy • Neurodegeneration • Neuropathology • Non-cell autonomy • Olivopontocerebellar atrophy • Pathogenesis • Parkinson's disease • PD-ALS-dementia complex of Guam • Prion diseases • Progressive supranuclear palsy • Schizophrenia • Shy-Drager syndrome • Superoxide dismutase-1 • Striatonigral degeneration • Synucleinopathies • Tauopathies • Tourette's syndrome

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22.1 Introduction

An examination of the literature indicates that since 1982 the term *neurodegeneration* appears in the title of over 4100 indexed publications. Neurodegeneration is a mentioned topic of virtually all neurological science textbooks. Thus, it is safe to assume that the meaning of the word *neurodegeneration* is universally understood. Most relevant textbooks however will not actually define neurodegeneration but will discuss the

issue in bits and pieces as part of the discussion of various diseases of the nervous system rather than as a single chapter. As discussed in Przedborski et al. (2003) and in the previous iteration of this chapter, neurodegeneration is composed of the prefix “neuro-,” which denotes relationship to a nerve or the nervous system (<http://tropmed.org/dictionary/coverpage9.htm>) and of “-degeneration,” which here is synonymous of devolution, meaning a process of declining from a higher to a lower level of effective power, vitality or essential quality (<http://www.wordreference.com>). Thus, neurodegeneration is any pathological condition in which the nervous system or nerve cell (i.e. neuron) loses its function, structure, or both. On a medical point of view however, the term neurodegeneration is used in a more restricted sense. Typically, it represents a large group of heterogeneous disorders in which affected neurons belong to specific subtypes, within specific anatomofunctional territories of the nervous system. Often, but not always neurodegenerative diseases arise for unknown reasons and progress in a relentless manner. Within the context of this definition diseases of the nervous system can be catalogued into three broad categories: (i) pathologies which are restricted to the nervous system and which are *primary neuronal* diseases (i.e. neurodegenerative diseases *per se*); (ii) pathologies which are restricted to the nervous system but are not *primary neuronal* diseases, such as brain neoplasm or cerebral edema and hemorrhage; and, (iii) pathologies provoked by systemic causes which damage the nervous system, such cardiovascular arrest, carbon monoxide poison, or infections due to herpes simplex. Based on this simple categorization, hundreds of disorders of the nervous system including Alzheimer’s disease (AD), Parkinson’s disease (PD), Huntington’s disease (HD), and amyotrophic lateral sclerosis (ALS) clearly fulfill the criteria of neurodegenerative disorder and are unanimously regarded as such. Aside from these unambiguous neurodegenerative diseases, others such as essential tremor, torsion dystonia, Tourette’s syndrome, or schizophrenia represent an interesting nosological challenge, as they do not show any distinct neuronal loss. Perhaps they have been traditionally included in this category because they are chronic diseases of the nervous system with an unknown cause. Finally, diseases such as multiple sclerosis (MS) also represent a perplexing situation. Conventionally, MS has been linked to pathology of the myelin that ensheath neuronal axons and not of the neuron *per se*. Several experts however have argued that MS is not only a demyelinating disease but also a disease where the neurons die due to a destruction of their axons. Worth noting, long-term disability in MS has a higher correlation with axonal damage than with the degree of demyelination (Bjartmar et al. 2003). For these reasons, several scientific authorities, as stressed in Trapp and Nave (2008), are now wondering whether inflammatory demyelination in MS might not be secondary in the disease process and that MS would be, in fact, a true neurodegenerative disease as defined above.

22.2 Frequency, Lifespan, and Co-morbidity

The two most prevalent neurodegenerative diseases are AD and PD, and according to a report from the World Health Organization published about a decade ago, AD (together with other forms of dementias) and PD made up more than 14 % of the global burden of neurological illnesses (2006). Scientists estimate that up to 4.5 million Americans suffer from AD and 1.2 million from PD. This represents roughly 2.5 % of the entire population in the United States. This estimate is likely valid for most, if not all countries of the world as epidemiological studies have found roughly comparable incidence and prevalence rates of AD and PD around the globe. Nevertheless, some geographic and temporal clusters of neurodegenerative conditions have been described. Two such examples include the marked increase in cases of parkinsonism in connection to the influenza pandemic of 1918 (Casals et al. 1998) and the high incidence of PD-ALS-dementia complex confined to the Chamorro Indians who live on the Western Pacific Island of Guam (Chen and Chase 1986). These clusters while quite enlightening must be regarded as the exceptions rather than the rules. The most consistent risk factor for developing a neurodegenerative disorder, especially in regards to AD and PD, is increasing age (Tanner 1992) and this fact may have far-reaching implications for the generations to come. As previously noted (Przedborski et al. 2003), over the past century, the growth rate of the population ages 65 and above in industrialized countries has far exceeded that of the population as a whole. Therefore, it can be anticipated that, over the next generations, the proportion of elderly citizens will double. Consequently, unless effective preventive strategies are soon found, the number of persons suffering from a neurodegenerative disorder will rise dramatically and the epidemiological numbers provided above may have to be amended to higher percentages.

It is also important to emphasize the fact that nearly all neurodegenerative disorders shorten the life expectancy of affected patients even if medications which alleviate the symptoms are available. This is well illustrated in the case of PD in which symptoms worsen over time. As discussed elsewhere (Dauer and Przedborski 2003), before the introduction of the potent symptomatic treatment levodopa, the mortality rate among PD patients was three times that of the normal age-matched subjects. Unexpectedly, population-based surveys suggest that PD patients continue to display a decreased longevity compared to the general population despite the fact that motor problems can be controlled by levodopa (Levy et al. 2002; Morgante et al. 2000; Hely et al. 1989). Yet, only a few neurodegenerative disorders are fatal *per se*. Indeed, only those diseases in which the affected neurological structures are implicated in controlling or driving vital physiological functions such as respiration or circulation are truly lethal.

Amongst these ALS is found, where the losses of lower motor neurons innervating the intercostal and diaphragm muscles lead to fatal respiratory paralysis. In Friedreich ataxia, the association of neurodegeneration with heart disease (Harding 1981) can also be a cause of death, although, in this case, death is not due to any neurodegenerative event but instead to cardiodegenerative problems such as congestive heart failure. In most other neurodegenerative disorders, death is attributed neither to the damage of the nervous system nor to any associated extra-nervous system degeneration but rather to medical problems including fatal falling, aspiration pneumonia, pressure skin ulcers, malnutrition, and dehydration, whose occurrence is favored by immobility, impaired balance, and cognitive decline. The leading causes of death in our industrial societies include cancers and cardiovascular problems. As a result it is worth mentioning that medical co-morbidity such as atrial fibrillation and cancer doubles the chances of death in nursing homes of demented patients (Van Dijk et al. 1996). Cardiovascular problems such as high blood pressure have also been suggested to stimulate the dementing process (Doraiswamy et al. 2002). Thus, medical co-morbidities appear to aggravate the long-term prognosis of patients inflicted with a neurodegenerative disease. However, whether or not patients suffering from a neurodegenerative disease are at greater (or lower) risk for cancer, stroke, or heart attack, remains to be demonstrated.

22.3 Classification

The estimated number of different neurodegenerative diseases is a few hundred and, among these, many appear to overlap with one another clinically and pathologically. Classification of neurodegenerative diseases, while useful, is quite a complicated task. In neurodegenerative diseases, it is typical that several areas of the brain are affected. Yet, the degrees in which these different brain areas are damaged often vary from one case to another, thus giving rise to different phenotypes. For example, in a disease such as multisystem atrophy where typically parkinsonism is a prominent feature, it may be accompanied with either severe ataxia, autonomic failure, or both depending on whether, in addition to the basal ganglia, the cerebellum and the intermediolateral column of the thoracic spinal cord also degenerate (Wenning et al. 2004). Even in the case of a defined genetic defect, a similar mutation may also produce varied phenotypes. For instance, in familial parkinsonism linked to mutations in the leucine-rich repeat kinase-2 (LRRK2) gene a striking pleomorphic pathology has been reported with some members of the affected family, developing a motor neuron disease superimposed to dementia and/or parkinsonism (Zimprich et al. 2004). Despite these difficulties, it remains that the most popular categorization of neurodegenerative disorders

is based on either the main clinical feature or the location of the predominant lesion, or often on a combination of the two. This proposed classification has been discussed previously (Przedborski et al. 2003) and will be repeated here for the sake of completeness of this chapter. We have proposed that neurodegenerative disorders may be grouped into diseases of the main anatomical division of the central nervous system: cerebral cortex, basal ganglia, brainstem/cerebellum, and spinal cord. Within each anatomical group, diseases may be sub-grouped based on their main clinical features.

Based on this clinico-anatomical classification, diseases of the cerebral cortex may be divided into cortical diseases associated with dementia (e.g., AD) and without dementia. In theory, circumscribed degeneration of the cerebral cortex can occur in absence of dementia as reported in some cases of progressive primary aphasia (Kirshner et al. 1987). However, because the cerebral cortex is so heavily involved in cognition, neurodegeneration, over time, often spreads beyond the initial locus of pathology and the majority of these patients end-up with dementia (Le Rhun et al. 2005). It is thus not surprising to find that neurodegenerative diseases of the cerebral cortex are usually equated to dementia. While AD in this group of diseases is by far the most frequent (Sulkava et al. 1983), about 50 other and less prominent dementing cortical diseases can be found (Tomlinson 1977).

Diseases of the basal ganglia are essentially characterized by abnormal motor activity. Based on the type of the motor problem, diseases of the basal ganglia can be classified into hypokinetic or hyperkinetic groups. Hypokinetic basal ganglia disorders include PD, in which the amplitude and velocity of voluntary movements are diminished or, in extreme cases, non-existent. Aside from PD, parkinsonism—which refers to an association of at least two of the following clinical signs: resting tremor, slowness of movements, stiffness, and postural instability—is also found in a variety of other diseases of the basal ganglia (Dauer and Przedborski 2003). In some, there is only parkinsonism (e.g., striatonigral degeneration) but in others, often called Parkinson-plus syndromes, there is parkinsonism plus signs of cerebellar ataxia (e.g., olivopontocerebellar atrophy), orthostatic hypotension (e.g., Shy-Drager syndrome) or paralysis of vertical eye movements (e.g., progressive supranuclear palsy). Because early on, Parkinsonism may be the only clinical expression of Parkinson-plus syndromes, it is difficult to formulate a definitive and accurate diagnosis before the patient reaches a more advanced stage of the disease. This problem is particularly well depicted by the fact that more than 77% of patients with parkinsonism are diagnosed in life as having PD (Stacy and Jankovic 1992), but as much as a quarter of the diagnosed patients are found at autopsy to have lesions incompatible with PD (Hughes et al. 1992). Of note, it is customary to classify the stiff-person syndrome among the hypokinetic diseases. However, available evidence would argue that the

stiff-person syndrome while being a hypokinetic condition is an autoimmune disease involving the nervous system and not a neurodegenerative disease.

Found at the other end of the spectrum are the hyperkinetic basal ganglia disorders, which are represented by HD and essential tremor. In these two conditions, excessive abnormal movements such as chorea or tremor are superimposed onto and interfere with normal voluntary movements. Although hyperkinetic basal ganglia disorders are probably as diverse as hypokinetic basal ganglia disorders, their specific disease markers such as gene mutations, which exist for several of the hyperkinetic syndromes, create accurate classifications, which classify this group as less problematic.

All neurodegenerative diseases of the cerebellum and its connections are clinically associated with ataxia, meaning that the main criteria of classification will rely not on the clinical presentation but on the loci of pathology. In order to utilize a meaningful and functional classification it is particularly a challenging task. Some diseases of the cerebellum and connections can easily be grouped into three main neuropathological types: cerebellar cortical atrophy (lesion confined to the Purkinje cells and the inferior olives); pontocerebellar atrophy (lesion affecting several cerebellar and brainstem structures); and Friedreich ataxia (lesion affecting the cerebellum and to a much greater extent the posterior column of the spinal cord, peripheral nerves, and the heart). However, several other diseases of the cerebellum and its connections fall somewhere in between these three well delineated categories such as, dentatorubral degeneration, in which the most conspicuous lesions are in the dentate and red nuclei, and Machado-Joseph disease, in which degeneration involves the lower and upper motor neurons, substantia nigra, and the dentate system.

Among the neurodegenerative disorders predominantly affecting the spinal cord are, ALS and spinal muscular atrophy, in which the most severe lesions are found in the anterior part of the spinal cord, and the already cited Friedreich ataxia, in which the most severe lesions are found in the posterior part of the spinal cord. Finally, there is one group of neurological diseases which are often, but not always, considered neurodegenerative because of their chronic course and unknown etiopathogenesis but, unlike those described above, these show no apparent structural abnormalities. These include torsion dystonia, Tourette syndrome, essential tremor, and schizophrenia. However, structural abnormalities are referred to here as a loss of neuronal cell bodies, but it is unknown whether or not structural damage restricted to synaptic connections occurs in torsion dystonia or schizophrenia, which could account for the disease phenotype. Moreover, in all of these singular neurodegenerative disorders, various brain imaging studies and electrophysiological investigations have revealed significant functional abnormalities. Thus, could it be that some neurological diseases

labeled as neurodegenerative disorders such as torsion dystonia may be caused by a loss of function of specific neural networks with minimal or no loss of neuronal viability?

Collectively, the above discussion was aimed at stressing some of the obtrusive shortcomings of the current classification of neurodegenerative diseases. To circumvent these problems researchers have tried over the recent years to reconstitute and complete the clinical/pathological-based classifications by using molecular information obtained from various neurodegenerative diseases. Based on this novel approach, some neurodegenerative diseases, which used to belong to very distinct categories, are now brought together because of a common molecular alteration. For example, HD, spinal-cerebellar atrophy, and myotonic dystrophy now fall under the category of the trinucleotide repeat disorders (Cummings and Zoghbi 2000); Creutzfeldt-Jakob disease, Gerstmann-Straussler-Scheinker syndrome, and fetal familial insomnia fall under the category of the prion diseases (Prusiner 1998); PD, progressive supranuclear palsy, and diffuse Lewy body dementia fall under the category of the synucleinopathies (Galvin et al. 2001); and AD, corticobasal degeneration, fronto-temporal dementia with parkinsonism linked to chromosome-17, and Pick's disease fall under the category of tauopathies (Goedert and Spillantini 2001). Although the jury is still out on whether or not this new type of classification will alleviate the problems previously encountered, we strongly believe that a recasting of the conventional neuropathological-based classification to a molecular-based classification holds the promise of a more practical and less ambiguous standing for neurodegeneration from a clinical and therapeutic point-of-view.

22.4 Etiology of Neurodegenerative Diseases

The word *etiology* refers to the initiating factor of a disease. In some cases a given clinical entity is linked to a single etiological factor such as in HD. Interestingly, however, several others can be caused by various and quite distinct factors. PD, for example, can be caused by overexpression of the small synaptic protein alpha-synuclein (Singleton et al. 2003), loss of parkin E3-ubiquitin ligase activity (Shimura et al. 2000), or by an increase in kinase activity of LRRK2 (West et al. 2005), to cite a few. These observations raise an important issue that plagues the field of neurodegeneration which is whether prominent disorders such as PD or AD are indeed diseases or rather syndromes, i.e. very different diseases lumped together under a same name because they merely share the same clinical expression.

Regardless of this problem, it should be known that the causes of neurodegenerative diseases or syndromes are in most instances unknown. Even in some rare cases when the

etiology has been identified, the mechanism by which the etiological factor leads to neuronal death remains, at best, speculative. For example, while the etiology of HD was identified more than 20 years ago (Group THsDCR 1993), we still do not know with certainty how mutant huntingtin kills striatal neurons. The same circumstances are also true for superoxide dismutase-1 (SOD1) whose mutations are linked to a familial form of ALS (Rosen et al. 1993).

Among the neurodegenerative disorders, only a few arise as a familial condition, supporting a genetic basis. Within the affected members of a family, these genetic diseases can run as an autosomal dominant condition, which is the case for HD and dentatorubral-pallidoluysian atrophy. Less frequently, the disease can run as an autosomal recessive (e.g., familial spastic paraparesis), an X-linked (e.g., spinal and bulbar muscular atrophy), or even a maternally inherited trait (e.g., mitochondrial Leber optic neuropathy). In addition to these genetic-neurodegenerative diseases, others, while primarily sporadic, can also show a small contingent of patients in whom the illness is inherited. This is the case for PD, AD and ALS where less than 10 % of all cases are generally familial.

For those in whom the disease is truly sporadic, which is the vast majority of patients; it appears that any genetic contribution to the neurodegenerative process would be minimal (Tanner et al. 1999). In these cases, environmental factors may be the prime suspects in initiating neurodegenerative processes. Relevant to this idea is the observation that some neurodegenerative conditions as mentioned above might arise in geographic or temporal clusters. For instance, in the case of the PD-ALS-dementia complex of Guam which was introduced previously, it is believed that the pathology is caused by a toxic compound contained in the *Cycas circinalis*, an indigenous plant commonly ingested as food or medicine by the Chamorros Indians (Kurtland 1988). Intoxication with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, a by-product of the synthesis of a meperidine compound, is also known to produce a severe and irreversible parkinsonian syndrome, which is almost identical to PD (Przedborski and Vila 2001). Several large-scale epidemiological studies have failed however, to link in a definitive manner the environmental factors to diseases such as PD (Tanner 1989). Furthermore, most of the toxic exposures known to cause neurological problems occur within a specific geographic, social, or professional context and in absence of any significant association with an increased incidence of PD, AD or other prominent neurodegenerative diseases. Collectively, the aforementioned findings argue that sporadic cases are neither clearly genetic nor clearly environmental. Alternatively, this does not exclude the possibility that neurodegenerative disorders can result from a combination of both. Along this line, the demonstration of a non-syndromic familial deafness linked to a mitochondrial point mutation (Prezant et al. 1993) provides compelling support for this

view. In this study, family members who harbor the mitochondrial mutation develop a hearing impairment only upon exposure to the antibiotic aminoglycoside, thus illustrating the significant pathogenic interactions between genetics and environment. Also striking is the demonstration that the gene encoding for Ataxin-2, which is a known cause of spinocerebellar ataxia when its polyQ expansions is >34 glutamines, can also increase the risk of developing ALS when its polyQ expansions between 27 and 33 (Elden et al. 2010). The latter work illustrates quite-well the notion that genetic variants could predispose to environment factors.

22.5 Pathogenesis of Neurodegenerative Diseases

Compared to *etiology*, *pathogenesis* does not refer to the initiation of the disease process but rather to the cascade of cellular and molecular mechanisms set into motion by the etiological factor(s) and which ultimately leads to the demise of the affected neurons. As mentioned above, only in a very small group of so-called neurodegenerative conditions comprise of diseases with no apparent neuropathological changes. Along this line, it is worth remembering that the loss of neuronal cell bodies is often the cardinal feature used to define the neuropathology of a given neurodegenerative disorders. Yet, it is increasingly recognized that in neurodegenerative disorders such as AD, PD and ALS show, especially early on, damages predominate, not at the level of the cell body, but at the level of the synapse (Overk and Masliah 2014). While synaptic lesions may be challenging to detect, it may be critical to develop specific methods to survey the state of the synaptic fields as this may help to not only refine the classification of neurodegenerative disorders, including those which are associated with no apparent neuropathological changes, but also to shed light into pathogenic mechanisms of neurodegeneration. In all others, overt neuropathology, mainly in the form of a focal loss of neurons with gliosis, is discernible. Residual neurons may exhibit varying morphologies ranging from an almost normal appearance to a severe distortion with a combination of abnormal features such as process attrition, shape and size alterations of the cell body and nucleus, organelle fragmentation, dispersion of Nissl bodies, cytoplasmic vacuolization, and chromatin condensation. In several neurodegenerative disorders, spared neurons can also present with various types of intracellular proteinaceous inclusions, which, in the absence of any definite known pathogenic role, are quite useful in differentiating neurodegenerative disorders. As for the glial response it is comprised of innate resident immune cells including astrocytes and microglia. To a lesser extent, adaptive immune cells such as T-lymphocytes can also be found in the diseased areas of the brain.

All neurodegenerative disorders associated to cell death have in common the fact that specific subpopulations of neurons at the level of specific structures of the nervous system degenerate. This means that autopsy areas of the brain that are damaged can be surrounded by healthy tissues and that within the affected regions, degenerating and healthy neurons are intermingled. In some neurodegenerative diseases, such as olivopontocerebellar atrophy, multiple brain structures within the nervous system are affected. In these so called *system neurodegenerative diseases*, the spatial pattern of the lesions often becomes better defined as the disease progresses (Przedborski et al. 2003). In regards to the spatial pattern of the lesions it is essentially unrelated to the distribution of blood vessels. Conversely, as emphasized by Oppenheimer and Esiri (1997) the different lesions appear, at least in some cases, functionally and anatomically interconnected. Such a linked degeneration is observed in ALS, where both the corticospinal track and spinal cord lower motor neurons are affected, or in progressive supranuclear palsy, where both the globus pallidus and the subthalamic nucleus are lesioned. Although such trans-neuronal degeneration is a well-recognized phenomenon (Saper et al. 1987), still very little is known about its molecular basis except for the fact that this trans-synaptic demise occurs by programmed cell death (DeGiorgio et al. 1998; Ginsberg and Martin 2002). Relevant to this enigma is the increased attention paid to the possible self-propagating and spread, in a prion-like manner, of several neurodegenerative disorder-related misfolded proteins such as synuclein or Tau (Goedert et al. 2010). According to this interesting speculation, synuclein, for example, is prompt to adopt deleterious conformations; hence, once entering neighboring cells, misconformed synuclein promotes, perhaps by an alteration of free energy, will trigger a harmful protein stress. Trans-neuronal degeneration is, however, unlikely to account for all of the combinations of lesions that are found in system neurodegenerative diseases. In Friedreich ataxia there is degeneration of the spinocerebellar tracts and the dentate nuclei, but not of the Purkinje cells, which would have been the link between these two lesions. It is thus more probable to consider that etiological factors are ubiquitous, but that the pathological threshold is attained by specific structures of the nervous system at different times. Perhaps, in some neurodegenerative diseases, some areas of the nervous system may never reach this threshold and will thus remain unaffected throughout the span of the disease. Also, not in all neurodegenerative disorders are large numbers of areas of the nervous system at risk. In some specific neurodegenerative diseases, the lesions appear restricted to one or only a few brain regions. This is particularly well illustrated in spinal muscular atrophy, in which the degenerative process is limited to a loss of lower motor neurons, or in ALS, which is characterized by damage to the upper and lower motor neurons which may represent

the sole neuropathological change. Nevertheless, almost all neurodegenerative disorders, which initially appeared *monosystemic*, will eventually become *multisystemic* over the progression of the degenerative process. In ALS for example it is typical to observe overt neuropathological changes mainly at the level of the spinal cord and motor cortex in patients who died after the short course of the disease, for unrelated causes (i.e. car accident or heart attack). However, in ALS patients with a more protracted course of degenerative changes can now also be detected in the substantia nigra (Sasaki et al. 1992) and the oculomotor and trochlear nuclei (Hayashi et al. 1991). Recognition of this temporal spreading of the neurodegenerative process has led some neuropathologists (Braak et al. 2003) to propose that the neurodegenerative process starts on a specific circumscribed area of the nervous system where it begins a domino-effect: the initial affected area is responsible for the disease occurring in the next region, whereby propagating the degenerative process. Although quite appealing, this model is speculative and would only be true if the different regions affected in the neurodegenerative conditions were anatomic neighbors which, as mentioned above, is often not the case.

In most if not all neurodegenerative disorders the locations of the principal lesions have been well established. However, it often remains difficult to determine the extent of degeneration affecting more than one group of neurons and, consequently, to define the exact neuropathological topography of certain diseases. This problem stems from at least three issues. First, lesions are often missed through incomplete examination of the brain and spinal cord. Second, quantitative morphology in post-mortem samples seldom use the rigorous counting methods necessary to generate reliable neuron numbers (Saper 1996). The third issue pertains to sick neurons, which will not necessarily die. These compromised neurons often lose their phenotypic markers which are used for identification purposes in order to count them (Clarke and Oppenheim 1995). Accordingly, the use of phenotypic markers in pathological conditions may lead to erroneous conclusions about the status of a given group of neurons. For these reasons, reported estimations regarding the distribution and magnitude of neuronal loss in neurodegenerative disorders may, perhaps with a few exceptions, have to be taken with caution.

22.6 Onset and Progression of the Disease

As indicated above, prominent neurodegenerative disorders are sporadic and with the absence of pre-symptomatic markers for virtually all of them, patients are seeking medical attention only when the first symptoms of the diseases emerge. Because there is significant cellular redundancy in neuronal pathways, the onset of symptoms does not equate

with the onset of the disease. Instead, the beginning of symptoms merely corresponds to a neurodegenerative stage at which the number of residual neurons in a given pathway drops below the number required to maintain its endowed function. It is thus clear that the onset of the disease occurs at some preceding time, which, depending on how fast the neurodegenerative process evolves, it can range from a few months to several years. Our ability to determine the actual onset of the disease is at this point unfortunately undermined by the lack of pre-symptomatic markers and the little knowledge about the true kinetics of cell demise.

Occasionally there is a sudden worsening of a patient's condition. Although a sudden acceleration of the neurodegenerative process cannot be excluded, especially under the effect of intercurrent deleterious factors such as infection, it is more probable that the rate of neuronal death remains about the same throughout the natural course of the disease. However, the relationship between the clinical expression of a disease and the number of residual neurons does not have to be either linear or even constant. For instance, a patient may clinically remain unchanged during a prolonged period of time despite a loss of many cells and then abruptly their condition may deteriorate as the number of neurons drop below a functional threshold. Another important aspect is that virtually all neurodegenerative disorders progress slowly over time, often taking several years to reach end-stage. It must be remembered that neuronal degeneration corresponds to an asynchronous death (Pittman et al. 1999) in that cells within a population die at very different times. The correlate of this fact is that at any given time, only a small number of cells are actually dying and among these, they are at various stages along the cell death pathway. However, standard clinical, radiological, and biochemical measurements, which are critical to assessing the disease, generate information about the entire population of cells. Therefore, the rate of change in any of the usual clinical parameters reflects the decay of the entire population of affected cells and provides very little, if any insight into the pace at which the death of an individual cell occurs. Still, if one looks at the large body of *in vitro* data, it appears that, once a cell gets sick, the entire process of death proceeds rapidly. Given these facts, it may be possible that the protracted clinical progression is the reflection of only a small number of neurons dying at any given time from a rapid demise.

22.7 Non Cell-Autonomous Nature of the Degenerative Process

As indicated above, neurodegeneration refers to pathology of neurons. It is thus not surprising to find from an examination of the literature that all theories about neurodegeneration pathogenesis revolve around neurons. This rather *neuronocentrist* view is increasingly at odds however with

the current concept of neurodegeneration pathogenesis. As stressed above, in most prominent familial neurodegenerative diseases, the mutant proteins are ubiquitously expressed. This is the case for example with mutant alpha-synuclein in familial PD and of mutant SOD1 in ALS. While the underlying gene mutations are unquestionably pathogenic, ALS-linked SOD1 mutations expressed selectively in astrocytes (Gong et al. 2000) or in neurons (Pramatarova et al. 2001; Lino et al. 2002) fail to provoke the death of motor neurons in transgenic mice. Conversely, the expression of ALS-linked SOD1 mutations in all cells does cause an adult-onset paralytic phenotype in both transgenic mice and rats (Gurney et al. 1994; Nagai et al. 2001). Of further support to the idea that many different cell types collaborate to achieve the demise of neurons in neurodegenerative diseases comes from the work of Clement and collaborators (2003). In the study, the authors produced chimeric animals made of mixtures of normal and SOD1 mutant-expressing cells. They found that motor neurons that chronically expressed mutant SOD1 did not degenerate if they were surrounded by a sufficient number of normal non-neuronal cells. In addition, normal motor neurons surrounded by mutant-expressing non-neuronal cells showed to acquire intraneuronal ubiquitinated deposits consistent with the concept that mutant-expressing cells could transfer the diseases phenotype to normal neighboring cells. These data provide a compelling argument in that death of neurons in neurodegenerative diseases such as ALS may not be as cell-autonomous as previously thought. Accordingly, the cellular environment surrounding neurons may play a critical role in determining the demise of specific neurons within these types of diseases of the nervous system. Among the prime candidates that mediate this non-cell autonomous mechanism of neurodegeneration are glial cells. Along this line, neuroinflammation has indeed emerged over the past decade as a key contributor to neurodegeneration in diseases such as AD, PD and ALS (Wyss-Coray and Mucke 2002; Przedborski and Goldman 2004; McGeer and McGeer 2002). Based on both epidemiological and pre-clinical studies in animal models of human diseases (Chen et al. 2003; Wyss-Coray and Mucke 2002; Przedborski and Goldman 2004; McGeer and McGeer 2002), it is now proposed that neuroinflammation could stimulate neurodegeneration. Furthermore, the contribution of non-cell autonomous mechanisms mediated by non-neuronal cells, such as astrocyte or oligodendrocytes, to the demise of neighboring neuronal cells are increasingly recognized (Garden and La Spada 2012). Likewise, alterations in the neurovascular unit—which is comprised of neurons and glial cells as well as brain endothelial, pericytes and vascular smooth muscle cells—has received growing attention from the perspective of neurobiology of disease. For instance, Zlokovic (2010) is reminding us that the state of the blood-brain barrier, and more specifically, its non-neuronal cellular

constituents, by regulating the exchange between the circulating blood and the brain extracellular fluid may be instrumental in driving the neurodegenerative process.

22.8 Review Questions

1. Define neurodegeneration.
2. What are the three broad categories of diseases of the nervous system?
3. Provide examples of diseases from the three categories.
4. What is expected to occur with the prevalence of neurodegenerative diseases in the forthcoming generations and why?
5. What is the effect of neurodegeneration on the life expectancy and for what reason(s) typically patients with neurodegenerative disease die?
6. What is the common method used in classifying neurodegenerative diseases and what are the difficulties inherent with this type of classification?
7. Describe the different forms of genetic contributions among the neurodegenerative diseases.
8. What does multisystemic neurodegeneration refer to?
9. Why are neurodegenerative diseases progressive and what does it mean at the level of the whole population of affected cells and a single affected cell.
10. Define the notion of non-cell autonomous neurodegeneration and provide an example.

22.9 Answers

1. Neurodegeneration is any pathological condition in which the nervous system or nerve cell (i.e. neuron) loses its function, structure, or both.
2. Pathologies which are restricted to the nervous system and which are *primary neuronal* diseases (i.e. neurodegenerative diseases *per se*);
 - (a) Pathologies which are restricted to the nervous system but are not *primary neuronal* diseases
 - (b) Pathologies provoked by systemic causes which damage the nervous system
3. Alzheimer's disease, Parkinson's disease, Huntington's disease
 - (a) Brain neoplasm or cerebral edema and hemorrhage
 - (b) Cardiovascular arrest, carbon monoxide poison, or infections due to herpes simplex
4. It can be anticipated that, over the next generations, the proportion of elderly citizens will double. Consequently, unless effective preventive strategies are soon found, the number of persons suffering from a neurodegenerative disorder will rise dramatically and the epidemiological numbers provided above may have to be amended to higher percentages.
5. Nearly all neurodegenerative disorders shorten the life expectancy of affected patients. Deaths are often attributed to medical problems including fatal falling, aspiration pneumonia, pressure skin ulcers, malnutrition, and dehydration, whose occurrence is favored by immobility, impaired balance, and cognitive decline.
6. The most popular categorization of neurodegenerative disorders is based on either the main clinical feature or the location of the predominant lesion, or often on a combination of the two. Classification of neurodegenerative diseases, while useful, is quite a complicated task. In neurodegenerative diseases, it is typical that several areas of the brain are affected. Yet, the degrees in which these different brain areas are damaged often vary from one case to another, thus giving rise to different phenotypes.
7. Among the neurodegenerative disorders, only a few arise as a familial condition, supporting a genetic basis. Within the affected members of a family, these genetic diseases can run as an autosomal dominant condition, which is the case for Huntington's disease and dentatorubral-pallidoluysian atrophy. Less frequently, the disease can run as an autosomal recessive (e.g., familial spastic paraparesis), an X-linked (e.g., spinal and bulbar muscular atrophy), or even a maternally inherited trait (e.g., mitochondrial Leber optic neuropathy). In addition to these genetic-neurodegenerative diseases, others, while primarily sporadic, can also show a small contingent of patients in whom the illness is inherited. This is the case for Parkinson's disease, Alzheimer's disease and Amyotrophic Lateral Sclerosis where less than 10% of all cases are generally familial.
8. The neurodegenerative disease may initially begin in one location, but will eventually cause degeneration to other parts of the system.
9. The neurodegenerative disease will progressively cause more damage to the patient as its influence spreads throughout the body. The disease actively corrupts a single cell at a time.
10. Accordingly, the cellular environment surrounding neurons may play a critical role in determining the demise of specific neurons within these types of diseases of the nervous system. Among the prime candidates that mediate this non-cell autonomous mechanism of neurodegeneration are glial cells.

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Abstract

Demyelinating diseases encompass a range of acute and chronic diseases affecting both the central and peripheral nervous systems. Many of these diseases share elements of autoimmune etiology, such as the presence of autoreactive T cells and B cells producing autoantibodies, followed by cell- and complement-mediated tissue damage, often following viral infection. The most common chronic demyelinating disease is multiple sclerosis (MS), a neuroinflammatory disease affecting the central nervous system (CNS). Histologically, the disease is characterized by lymphocytic infiltration of the CNS with oligodendrocyte loss, but its etiology is complex and not fully understood. Epidemiological studies implicate both environmental and genetic factors in its pathogenesis. Approved pharmaceutical treatments are predominantly immunosuppressive agents targeting the inflammatory components of disease. This review will highlight the mechanisms by which these treatments are understood to interact with the complex cast of inflammatory mediators present in MS pathogenesis.

Keywords

Acute disseminated encephalomyelitis • Central nervous system • Cerebrospinal fluid • Multiple sclerosis • Experimental allergic encephalomyelitis • Immune system • T-cell receptor • Lymphocyte • Virus

23.1 Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS), in which episodes of oligodendrocyte loss result in focal regions of axonal demyelination disseminated in time and space ('t Hart and Massacesi 2009). The formation of these sclerotic demyelinated plaques in the cerebral white matter is associated with focal neurological deficits and progressive disability. MS is the primary cause of non-traumatic disability in young adults (Noseworthy et al. 2000; Ramagopalan and Sadovnick 2011), affecting

approximately 250,000–350,000 people in the U.S. (Jacobson et al. 1997; Simpson et al. 2011). MS most commonly presents as a relapsing-remitting disease (RR-MS), but approximately 50–60 % of patients with RR-MS eventually enter a secondary progressive phase (SP-MS), in which a relapse occurs and symptoms progressively worsen without subsequent remissions (Antel et al. 2012). In 10 % of patients, the disease may present with a primary progressive course (PP-MS), in which disease onset is followed by immediate progression without remission (Compston and Coles 2008). While the etiology of MS is not fully understood, both genetic elements and environmental exposures, such as antecedent viral exposure, have been identified as risk factors for MS, and autoimmune processes are recognized to contribute to its pathogenesis. Like many diseases with autoimmune etiology, MS predominantly affects women, and approximately 70 % of MS patients are female (Alonso and Hernan 2008).

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Primary progressive disease, however, lacks this female predominance and carries a worse prognosis in men (Antel et al. 2012). Geographically, MS prevalence increases with latitude and distance from the equator, with a frequency of 60 per 100,000 or more in the northern United States, northern Europe, New Zealand, and southeast Australia, as compared to 5–6 per 100,000 at tropical latitudes. An intermediate frequency of 30 per 100,000 is seen at intermediate latitudes in southern Europe and central Asia and Australia (Simpson et al. 2011).

23.2 Histopathology and Pathophysiology of MS

Histologically, MS is characterized by focal regions of demyelination known as plaques. These lesions show perivenular infiltrates of mononuclear cells, in particular, CD8+ T lymphocytes, macrophages and microglia, as well as B lymphocytes and plasma cells. These infiltrates are especially prominent in early lesions. In later lesions, inflammatory infiltrates are diminished and reactive gliosis becomes the more prominent histological feature (Popescu et al. 2013).

The demyelination seen in these lesions results from the separation of the outer lamellae of the oligodendrocyte processes surrounding the axon, which disrupts the integrity of the myelin sheath but does not necessarily indicate degeneration of the oligodendrocyte itself. Nonetheless, oligodendrocytes may still show variable levels of degeneration. Axons, however, are relatively spared (Popescu et al. 2013). Axonal loss may become more significant in irreversible phases of disease (Jadidi-Niaragh and Mirshafiey 2011).

This demyelination leads to loss of saltatory conduction, which results in slower conduction and longer conduction times. Sodium channels are upregulated and redistributed along the length of the demyelinated axonal membrane to compensate for the loss of saltatory conduction and maintain electrical transmission, but this also results in an increase in energy demand, which may cause the patient to experience fatigue, even when electrical transmission is fully compensated. As the disease progresses, increasing conduction capacity is no longer sufficient to compensate for lost saltatory conduction, and electrical transmission is compromised (Compston and Coles 2008). Conduction may be furthered compromised by heat, leading to conduction block at increased temperatures, a symptom known as Uhthoff's phenomenon (Humm et al. 2004).

23.3 Clinical Manifestations and Diagnostic Criteria

The optic nerve is the only cranial nerve to share an embryological origin with the CNS and, thus, is the only cranial nerve myelinated by oligodendrocytes rather than Schwann

cells (Wilson-Pauwels et al. 2010). Consequently, the optic nerve is preferentially affected in MS, and optic neuritis, or inflammation of the optic nerve, is both the most common lesion of MS patients, affecting approximately 50 % of patients over the course of a lifetime, and one of the most common presenting complaints, affecting 15–20 % of patients at presentation (Balcer 2006). The condition is typically unilateral and presents as a complete or partial loss of vision, sometime with pain on movement of the affected eye (Foroozan et al. 2002). Other clinical manifestations include focal sensory losses, such as numbness and paraesthesias, and motor deficits, such as weakness of the extremities (Compston and Coles 2008).

The loss of saltatory conduction may result in spontaneous electrical discharges producing the perception of flashing lights (Compston and Coles 2008). Lhermitte's sign is the sensation of an electrical shock descending through the spinal cord, typically upon flexion of the neck, and is described in a variety of pathologies of the cervical spinal cord but is most closely associated with MS (Kanchandani and Howe 1982). The previously described conduction block that occurs with increases in temperature following compromised saltatory conduction—Uhthoff's phenomenon—may result in a worsening of symptoms with exercise or after taking a bath (Humm et al. 2004).

Spinal cord involvement may result in sexual dysfunction and urinary symptoms, such as retention and frequency. In later stages of the disease, motor deficits progress from focal losses to full paresis and disability. Regardless of clinical course, the most common symptom of MS is fatigue, and many patients also experience depression and cognitive impairments (Compston and Coles 2008). Other diagnostic criteria include the increased synthesis of IgG in the intrathecal compartment and the detection of oligoclonal bands of IgG that are present in the CSF but absent from the sera ('t Hart et al. 2009).

23.4 Variants

Balo's concentric sclerosis is a demyelinating condition that presents radiologically with Balo lesions—large multilayered lesions consisting of multiple concentric rings of demyelination and remyelination. It is commonly thought to be a rare variant of MS but is sometimes observed in association with other neurological diseases, throwing its association with MS into question (Merkler et al. 2006).

Neuromyelitis Optica is a demyelinating disease that shares many features of MS but is distinguished serologically by its specific association with the presence of IgG autoantibodies against extracellular components of a water channel protein, aquaporin-4 (AQP4), found on the surfaces of astrocytes. These anti-AQP4 antibodies, also known as NMO-IgG, are thought to mediate the pathogenesis of NMO

by fixing complement. NMO-patient sera has been shown to effect the lysis of AQP4+ astrocytes in vitro in the presence of complement, sparing AQP4- cells (Saadoun et al. 2011). The success of plasma exchange therapy in treatment of NMO supports the centrality of NMO antibodies and associated complement activation in NMO pathogenesis (Compston and Coles 2008).

Clinically and radiologically, NMO shows preferential involvement of the optic nerves and spinal cord, with relative sparing of the brain. Histologically, lesions exhibit macrophage infiltration and perivascular granulocytes but little T cell involvement, in contrast to multiple sclerosis, in which lesions show significant CD8+ T cell infiltration (Saadoun et al. 2011).

NMO shows the highest prevalence in East Asian, African, and Aboriginal populations but more recently has been recognized in Northern European populations with the advent of serological testing for anti-AQP4 antibodies (Compston and Coles 2008).

Acute demyelinating encephalomyelitis (ADEM) is an acute inflammatory disease mediated by autoimmune attack of the central nervous system, most commonly in response to viral infection but rarely following vaccinations and bacterial infections. The condition manifests with disseminated lesions of the brain and spinal cord and associated focal neurological deficits. Symptoms arise typically 1–3 weeks following viral infection. Typically, patients are treated with high dose corticosteroids, such as methylprednisolone, to rapidly reduce inflammation. While the prognosis is typically good, with 57–81 % of patients making a full recovery, mortality may be as high as 10 %. Approximately 25 % of patients experiencing ADEM eventually develop MS (Dale and Branson 2005; Menge et al. 2007).

23.5 Radiological Manifestations

The gold standard imaging technique for the monitoring of neuroinflammatory disease is magnetic resonance imaging (MRI) (Politi et al. 2007). MS patients most typically exhibit significant involvement of the white matter of the CNS, particularly in the optic nerves, spinal cord, brain stem, and cerebellum (Popescu and Lucchinetti 2012). Gadolinium- and T2-hyperintense enhancing lesions of the cerebral hemispheres frequently exhibit a periventricular distribution clustering near the lateral ventricles and corpus callosum (Compston and Coles 2008). In patients with advanced disease, T1-weighted MRI may show “black holes,” formerly enhancing lesions that have become irreversibly demyelinated with subsequent axonal damage (Kamphorst and Ravid 1998; Laule et al. 2007; Paolillo et al. 2000; Zivadinov 2007).

Gray matter involvement may also be seen in the form of cortical demyelination, and is thought to be responsible for symptoms of fatigue and cognitive impairment (Compston and Coles 2008). Lesions predominantly affect the cortex

and cerebral nuclei (Brownell and Hughes 1962; Cifelli et al. 2002) and are characterized by apoptotic neurons (Peterson et al. 2001) and infiltration by ramified microglia (Peterson JW). Gray matter pathology is thought to relate to white matter pathology and the majority of gray matter lesions are classified as Type I lesions, which are contiguous with plaques of subcortical demyelination in the white matter (Pirko et al. 2007).

23.6 Pathogenesis

While the etiology of MS remains poorly understood, genetic, geographic, and demographic data suggest the synergistic interplay of environmental exposures and genetic susceptibility factors (’t Hart et al. 2004; Kawakami et al. 2012). Key features of pathogenesis include (1) the activation of self-reactive CD4+ Th1 and Th17 cells in the peripheral blood (Domingues et al. 2010), (2) the presentation of myelin antigen by dendritic cells in lymphoid tissues (Isaksson et al. 2009; ’t Hart and Massacesi 2009; Zozulya et al. 2009), and (3) the increased permeability of the blood brain barrier to infiltrating immune cells (’t Hart and Massacesi 2009) as contributing factors. However, the precise causal relationship between these events has yet to be fully understood.

T cell Immunity: Early theories have suggested that Th1 and Th17 cells initially sensitized to pathogenic antigens in the peripheral blood and lymphoid tissues subsequently cross-react with myelin antigens and cross the blood-brain barrier (BBB), initiating the cascade of inflammatory damage that leads to CNS damage (’t Hart et al. 2009; Domingues et al. 2010). While this response was initially believed to be purely Th1-driven, understanding of the disease has broadened to recognize the significant role that Th17 cells play in the permeabilization of the BBB. This disruption is mediated by the secretion of IL-17, a pro-inflammatory cytokine, which promotes the activation of matrix metalloproteinase-3 (MMP-3) as well as the recruitment of neutrophils. IL-17 levels are highly associated with active disease in MS, and IL-17 levels were found to be higher in the peripheral white cells of MS patients with active disease than healthy controls and patients with inactive disease (Jadidi-Niaragh and Mirshafiey 2011).

Other immunologic changes associated with MS include the functional impairment of T regulatory cells (T_{reg}) (Haas et al. 2005; Zozulya and Wiendl 2008). T_{regs} are CD4+, CD25+, and transcription factor forkhead box protein P3 (FoxP3+) cells, which play a role in the downregulation of autoreactive Th1 and Th17 cells (Zozulya and Wiendl 2008). Their dysregulation is implicated in a wide array of autoimmune diseases. The deletion of T_{regs} in mice has been found to produce spontaneous autoimmune disease, whereas the passive transfer of Treg cells has been shown to alleviate experimental models of autoimmunity, including animal

models of multiple sclerosis (Zozulya and Wiendl 2008). CD39 is another marker co-expressed by FOXP3+ Treg cells that is thought to play a role in tolerance induction, as CD39–Treg cells exhibit impaired ability to block allograft rejection (Fletcher et al. 2009). While MS patients have been found to have the same levels of CD4+, CD25+, and FoxP3+ Treg cells as healthy controls (Feger et al. 2007; Putheti et al. 2004), further studies have demonstrated reduced numbers of CD39+ Treg cells in MS patient sera (Borsellino et al. 2007) as well as functional impairment *in vitro* with diminished capacity to suppress T cell proliferation upon stimulation with anti-CD3 and anti-CD28 antibodies (Viglietta et al. 2004).

Environmental factors: Other theories of pathogenesis propose that the activation of Th1 and Th17 cells is not an initiating event but is instead an immune response to an intrinsic neurodegenerative process of unknown etiology ('t Hart et al. 2009). While no distinct triggering method has yet been identified, patient sera studies have shown the clonal expansion of T cells in reaction to common viruses such as cytomegalovirus (CMV) or Epstein-Barr virus (EBV), thus suggesting antecedent viral infections as an environmental factor ('t Hart et al. 2009). Other studies have identified vitamin D deficiency as a potential contributor (Holick 2004), which is supported by the increased prevalence of MS at latitudes where sun exposure is less direct and vitamin D deficiency more common. The strongest genetic association in MS is with the major histocompatibility complex type II (MHC-II) haplotypes HLA-DR15 and DQ6 ('t Hart et al. 2009). Closely associated genotypes include DRB1*1501, DRB5*0101, DQA1*0102, and DQB2*0602 (Compston and Coles 2008), with HLA-DRB1*1501 being the single most closely linked genotype ('t Hart et al. 2009). Because vitamin D is required for many immune cell functions and MHC-II is required for the presentation of endocytosed extracellular antigens by antigen presenting cells, both associations support the role of immune dysregulation in the pathogenesis of MS. However, risk has been observed to vary with migration, depending on the age of the subject. Subjects moving to high-risk areas prior to adolescence experience the increased risk associated with their destination. For subjects moving after the age of 15, risk remains unchanged (Compston and Coles 2008). This suggests MS susceptibility to depend on both environmental exposure and age at time of exposure.

B Cell Immunity: The inclusion of elevated IgG antibody levels and CSF-specific oligoclonal IgG bands as diagnostic criteria indicates B cell and plasma cell involvement as another crucial component of MS pathogenesis. Patient studies have revealed the expansion of clonally related B cells in the CSF (Harp et al. 2007; Qin et al. 1998; 't Hart and Massacesi 2009) but this change is not specifically associated with MS. Nonetheless, histological analysis has identified antibody-mediated complement deposition in the CNS

as a key pathological feature of disease progression ('t Hart et al. 2009). The binding of autoreactive antibodies to CNS tissues also can promote the recruitment of macrophages expressing the Fc receptor, triggering macrophage-mediated myelin breakdown and phagocytosis (Prineas and Graham 1981). Ultimately, the presence of B cell involvement further affirms suspicions of an infectious trigger in MS pathogenesis. Of particular interest are EBV-infected B-cells. Epstein Barr virus is a gamma-herpesvirus that demonstrates a tropism for B cells and subsequently establishes a latent infection in the memory B cells of infected individuals. These EBV-infected B cells have been implicated not only in lymphoproliferative disorders but also in the pathogenesis of MS. While EBV infection in early life is typically asymptomatic, approximately 40 % of EBV infections in adolescence and adulthood progress to infectious mononucleosis, an acute lymphoproliferative condition of several weeks duration that is associated with a several-fold increase in MS risk (Ascherio and Munger 2010; Levin et al. 2010). MS patients are almost universally seropositive for EBV, with >99 % of patients exhibiting seroconversion, as compared to only 90 % of healthy individuals of all ages and only 70 % of young, healthy individuals (Santón et al. 2011). While alternative theories suggest that the correlation of IM and MS may be explained by hygienic behaviors in early life delaying exposure to both EBV and other pathogenic triggers until adolescence, this theory is diminished by the low risk of MS in EBV-negative individuals. These individuals theoretically share the high hygiene practices that would delay their exposure to other potential infections leading to MS. Thus, they would be equally likely to develop MS if hygiene practices were truly a confounding factor (Ascherio and Munger 2010). Additionally, a 2003 study of military personnel performed showed that increased titres of antibodies against EBV nuclear antigen-1 (EBVNA-1) were associated with a 34-fold increase in risk of MS (Ascherio et al. 2005; Levin et al. 2003). While antibody titres showed no difference between cases and controls prior to age 20, significant differences were found between the ages of 25 and 29, where control titres remained unchanged but case titres increased, ultimately reaching a maximum (Ascherio et al. 2005). These associations have spurred interest in an EBV vaccine as a possible preventive strategy in the treatment of MS.

Regeneration: Additional theories of pathogenesis suggest the persistence of lesions in MS requires not only an initiating demyelinating event but also a deficit in the process of remyelination. In the healthy adult CNS, demyelination is normally followed by remyelination, but this capacity for remyelination normally declines with age and may be further compromised in patients with MS (Crawford et al. 2013). Studies of oligodendrocyte lineage attribute capacity for remyelination to a specific subpopulation of cells expressing nerve and glial antigen-2 (NG2) and platelet derived growth

factor- α (PDGFR- α) (Crawford et al. 2014; Franklin and Gallo 2014). These NG2-positive cells, termed oligodendrocyte precursor cells (OPC) or NG2-glia, are found to migrate in response to inflammatory injury and proliferate in sites of demyelination, where they subsequently differentiate. Studies in mouse models of focal demyelinating injury have identified distinct populations of dorsal and ventral OPC that migrate and proliferate in response to damage (Tripathi et al. 2014; Zhu et al. 2011). Their differentiation in particular is thought to play a crucial role in the repair process, as blocking differentiation with inhibitors of OPC differentiation such as HDAC inhibitors and semaphorins significantly impairs remyelination in vivo (Franklin and Gallo 2014).

The process of remyelination is also strongly linked to the surrounding milieu of immune cells. Inflammatory autoimmune diseases are associated with increases in the ratio of macrophages and microglia demonstrating an immunogenic M1 phenotype to those demonstrating a tolerogenic M2 phenotype. Similarly, in animal models of multiple sclerosis, peak clinical scores are associated with high M1:M2 ratios (Miron and Franklin 2014). A 2013 study by Miron et al. showed that M2 macrophage-conditioned media enhanced oligodendrocyte differentiation in vitro and that M2 cell density is increased in remyelinating MS lesions, suggesting that remyelination is favored by the conversion of macrophages and microglia from an M1 phenotype to an M2 phenotype (Miron and Franklin 2014; Miron et al. 2013).

23.7 Treatment

MS therapies generally adopt the strategy of (1) blocking the action of cellular and molecular mediators of inflammation to combat the progression of inflammatory damage or (2) promoting the repair of existing damage. Examples of the former include numerous immunosuppressive therapies, such as corticosteroid treatments and antibody-mediated therapies for the depletion of T cells and B cells (Constantinescu et al. 2011). Other pharmacologic strategies include immunomodulation via the induction of T regulatory cells that tolerize immune cells to myelin-derived self-antigens with glatiramer acetate (Zozulya and Wiendl 2008). Recognition of the potential role of EBV and B cells in MS pathogenesis, meanwhile, has spurred interest in EBV vaccination as a strategy of prevention (Ascherio and Munger 2010). Still other branches of research seek to promote oligodendrogenesis and myelin repair (Deshmukh et al. 2013; Kokaia et al. 2012). At the intersection of these preventive and reparative strategies stand cell-based treatments such as stem cell transplantation, which is hypothesized to play both a regenerative role in cell replacement and an immune regulatory role in the creation of a tolerogenic environment favoring repair.

23.7.1 Immunosuppressive Strategies

In this section, we will give a broad overview of the evolution of MS treatment, covering a range of therapeutic strategies, from early treatments and established mainstays to newer treatments, noting both successes and challenges.

23.7.2 Glucocorticoids

Glucocorticoids suppress inflammation both by inhibiting the synthesis of molecular mediators of inflammation and inhibiting lymphocyte proliferation. Glucocorticoid treatments such as intravenous prednisone and methylprednisolone are favored in the treatment of acute exacerbations, for which they have been shown to reduce recovery time. However, they have not been shown to improve the ultimate degree of recovery (Goodin 2014). These medications are also associated with significant side effects, including derangements in lipid and sugar metabolism, which may produce hyperglycemia, resulting in a condition known as “steroid diabetes.” The excess corticosteroid may produce Cushing’s syndrome, which is characterized by weight gain, fat redistribution, and skin changes. Other manifestations include osteoporosis, gastrointestinal disturbances, and psychosis. Patients are also at risk of adrenal insufficiency should they fail to recover adrenal function during steroid withdrawal (Shaikh et al. 2012).

23.7.3 Alemtuzumab (Campath)

Alemtuzumab is a human anti-CD52 monoclonal antibody used for the depletion of T-cells and monocytes. It is established clinically for the treatment of chronic lymphocytic leukemia (CLL) and, in 1994, was established for the prevention of relapse in RR-MS. More recently, alemtuzumab has been explored in the treatment of early multiple sclerosis and the prevention of secondary progression. A study by Coles et al. reported reduced accumulation of disability and reduced lesion burden on T2-weighted MRI in patients taking 24-mg doses of the drug. However, adverse effects associated with its use in this trial included the development of autoimmunity, with 23 % of patients developing autoimmune thyroid disease and 3 % developing immune thrombocytopenic purpura (Coles et al. 2008).

23.7.4 Rituximab

Rituximab is a chimeric anti-CD20 monoclonal antibody engineered for the depletion of CD20+ B cells. It has predominantly been used in the treatment of B-cell lymphoma but has

more recently been studied in the treatment of multiple sclerosis (Carson et al. 2014). A 2008 study demonstrate a significant decrease in disease relapse and reduced counts of gadolinium-enhancing lesions on T1-weighted MRI in patients treated with the antibody (Bourdette and Yadav 2008).

23.7.5 Natalizumab

The $\alpha 4, \beta 1$ -integrin, or very late antigen (VLA)-4 integrin, is a glycoprotein expressed by activated lymphocytes and monocytes that mediates their adhesion to the vascular endothelium of the blood-brain barrier and facilitates their transmigration into the CNS. This transmigration is mediated by the binding of VLA-4 to vascular cell adhesion molecule 1 (VCAM-1). Natalizumab is a human monoclonal antibody that binds the VLA-4 integrin and prevents its interaction with VCAM-1, thus blocking lymphocytic infiltration of the CNS. A placebo-controlled trial showed a reduction in both lesion load and frequency of clinical relapse over a 6-month period. Suppression of lymphocyte migration, however, may result in the re-activation of latent infections. The JC virus is a polyomavirus that typically persists latently in the bone marrow, kidneys, and spleens of immunocompetent individuals but may become active in the immunosuppressed (Constantinescu et al. 2011; Kleinschmidt-DeMasters and Tyler 2005; Langer-Gould et al. 2005). Historically, its reactivation in HIV and AIDS patients and, more recently, its reactivation in patients treated with natalizumab has been associated with the development of progressive multifocal leukoencephalopathy (PML), a diffuse demyelinating condition of the CNS resulting from spread of the virus to the CNS and infection of oligodendrocytes. The condition is named for its progressive course and the radiological observation of multifocal white matter lesions on MRI. Clinical manifestations include progressive weakness and sensory loss, loss of vision and speech, and eventual progression to disability and death (Kleinschmidt-DeMasters and Tyler 2005).

23.8 Immunomodulatory Strategies

Fingolimod (FTY720) is an oral medication derived from myriocin, a metabolite produced by *Isaria sinclairii*, a fungus (Schmitz et al. 2015). Once phosphorylated, fingolimod binds the sphingosine-1-receptor of lymphocytes and prevents lymphocyte migration from the lymph nodes to other compartments of the body, effectively blocking their infiltration of the CNS (Kappos and Radue 2010; Kappos et al. 2006). Fingolimod is thought to exert additional immunomodulatory effects through its action on the sphingosine-1-receptor and may also play a neuroprotective role by

promoting remyelination (Kappos et al. 2006; Schmitz et al. 2015). A 2010 placebo-controlled study on fingolimod for the treatment of RR-MS showed significant decreases in relapse, progression to disability and gadolinium-enhancing lesions on MRI for patients receiving the drug (Kappos and Radue 2010).

Dimethyl fumarate is a natural compound previously approved for the treatment of psoriasis that has shown moderate efficacy in the reduction of clinical scores in animal models of multiple sclerosis (Schmitz et al. 2015). In clinical trials, it has been associated with a significant reduction in active MRI lesions and relapse rate (Hutchinson et al. 2014).

Glatiramer acetate (GA) is an acetate salt of a polymer consisting of L-alanine, L-lysine, L-glutamic acid and L-tyrosine, an amino acid sequence found in myelin basic protein (MBP) (Aharoni et al. 2013). This synthetic polypeptide, formerly known as copolymer-1, has been found to induce T regulatory (Treg) cells promoting tolerance of MBP. Mechanistically, GA competes with MBP and is incorporated in its place at the MBP-binding site of the MHC-II molecule. The MHC-bound GA (MHC-GA) competes with MHC molecules presenting MBP (MHC-MBP) for binding to T-cell receptors (TCRs). Binding of MHC-GA to the TCR during antigen presentation induces a tolerogenic, regulatory Th2-like phenotype. In animal models of MS, GA has been shown to promote oligodendrogenesis and is suggested to play a role in neuroprotection and repair. However, while GA carries relatively little risk of significant side effects, its clinical efficacy has yet to be clearly determined. A recent multicenter, placebo-controlled trial of GA failed to show efficacy in slowing disease progression, but the lack of treatment effect in this study may be attributable to discontinuation of medication as well as low rates of disease progression in all study participants (Wolinsky et al. 2007).

Interferon- β -1a is a cytokine that is typically administered to patients intramuscularly or subcutaneously and decreases T cell infiltration of the CNS. It also increases nerve growth and repair via increases in nerve growth factor production. While a systematic review of clinical studies found a slight decrease in frequency of exacerbations in patients receiving interferon therapy (Filippini et al. 2003), a 2012 study reported no reduction in progression to disability with interferon administration (Shirani et al. 2014). Moreover, interferon therapy is associated with a number of adverse effects, which include flu-like symptoms and hyperthermia, likely due to cytokine release (Compston and Coles 2008).

23.9 Cell-Based Therapies

The transplantation of immune cells such as autologous tolerogenic dendritic cells (Zozulya et al. 2009) and attenuated anti-myelin T cells (Compston and Coles 2008) have been examined

as a means of targeting autoimmune processes in MS. Further attention has been paid to stem cell transplantation as a means of generating oligodendrocytes for remyelination and neurons for the replacement of cortical and axonal damage. Neural stem cell transplant therapies are currently of interest both as a means of direct cell replacement and a mediator of tolerogenicity, promoting an environment that downregulates the inflammatory response and favors remyelination and repair. Human neural progenitor cells (hNPCs) have been demonstrated to suppress the proliferation of MOG-specific T cells upon re-stimulation with MOG antigen as well as the proliferation of T cells and PBMC stimulated with anti-CD3 and anti-CD28 antibodies. Additionally, hNPCs were shown to impair the development of mature CD1a⁺ dendritic cells from monocytes in a dose-dependent manner under maturation conditions. These monocytes also exhibited dose-dependent decreases in the expression of MHC-II and the co-stimulatory molecules CD80 and CD86 with increasing concentrations of hNPCs (Pluchino et al. 2009). Adult NSCs (aNSCs), similarly, have been found to down regulate macrophage effector functions in vivo (Martino and Pluchino 2006). Mesenchymal stem cells have been shown to induce increased secretion of IL-10 and decreased secretion of TNF- α and IL-6 in co-cultured macrophages (Kim and Hematti 2009). Previously, Pluchino et al. have previously explored the transplantation of endogenous neural precursor cells (NPC) in the treatment of experimental autoimmune encephalomyelitis (EAE), the primary model of multiple sclerosis. Intravenously administered hNPC were found to significantly decrease mortality and EAE clinical scores (Pluchino et al. 2009).

The transplantation of endogenous neural precursor cells is limited however by its requirement for allogeneic fetal tissue sources, which provoke both ethical questions and practical concerns of tissue rejection (Takahashi and Yamanaka 2006). With the advent of iPS technologies, however, there has been particular interest in the generation of autologous patient specific stem cells for the regeneration of the CNS (Qiu and Farnsworth 2013). Induced pluripotent stem cells (iPSC) derived from somatic cells may be subsequently re-differentiated into neural precursor cells (Caiazzo et al. 2011; Pang et al. 2011). Alternatively, somatic cells may be directly transdifferentiated into cells of neural lineage (Caiazzo et al. 2011; Pang et al. 2011; Pfisterer et al. 2011; Thier et al. 2012; Vierbuchen et al. 2010). These lineage-restricted induced neural stem cells carry the advantage of reduced tumorigenicity relative to iPSC, which have also been found to form teratomas in vivo (Leten et al. 2014).

23.10 Concluding Remarks

MS is a complex inflammatory disease mediated by recognized autoimmune processes but potentially also by intrinsic neurodegenerative processes and endogenous defects in

immunoregulation and remyelination. Currently available treatments are predominantly immunosuppressive pharmaceuticals that target the autoimmune processes implicated in its pathogenesis through the depletion and downregulation of immune cells. These drugs are largely used for the treatment of RR-MS, in which the prevention of new inflammatory episodes is sufficient to maintain the disease in remission and damage is not yet extensive enough to produce lasting deficits in the absence of active inflammation. Many of these pharmaceuticals are also hypothesized to favor repair by promoting endogenous processes of remyelination while maintaining a tolerogenic environment that inhibits further inflammatory damage. However, there are currently no accepted drugs to show efficacy in the treatment of secondary progression, in which demyelination is extensive and irreversible and may be associated with significant neurodegeneration. Cell-based treatments such as the transplantation of autologous DC tolerized to myelin antigen or attenuated T cells are of interest in the quest for antigen-specific tolerance. Stem cell transplantation has attracted both scientific and public interest for its potential role as an immunoregulatory treatment and a source of cell replacement for CNS repair.

23.11 Review Questions

- Which of the following statements is true about gender differences in MS?
 - Men are more likely than women to develop MS.
 - Women are likely to have a later age of disease onset.
 - Women have a more rapid disease progression
 - Men with MS have a worse prognosis.*
- The geographic distribution of MS:
 - Follows an east-west gradient
 - Increases at higher altitudes
 - Increases with distance from the equator*
 - Is skewed toward northern latitudes in both hemispheres
- An environmental component in the etiology of MS is supported by:
 - The geographic distribution of MS
 - Migration studies
 - Relatively low concordance (25–30%) in identical twins
 - Reports of MS epidemics
 - All of the above*
- Oligoclonal bands represent:
 - Immunoglobulins directed against recently identified myelin epitopes in MS
 - Immunoglobulins directed against unknown CNS epitopes in MS
 - Immunoglobulins that have been shown to deposit around demyelinated plaques in MS*

- (d) Immunoglobulins often detected in the serum of individuals with MS
- (e) b & e
- 5. Optic neuritis is the presenting symptom in what percentage of MS patients?
 - (a) 10 %
 - (b) 20 %
 - (c) 30 %
 - (d) 40 %
 - (e) 50 %
- 6. The diagnostic criteria for MS:
 - (a) Depend upon the demonstration of white matter abnormalities in the brain or spinal cord by MRI.
 - (b) Require evidence for the presence of neurological dysfunction for the diagnosis in all cases.
 - (c) Require that there is no better explanation (other than MS) for the clinical presentation.
 - (d) None of the above
 - (e) a, b & c
 - (f) a & c
 - (g) b & c
- 7. MS plaques have been histologically demonstrated to include:
 - (a) Infiltration of CD8+ T-cells
 - (b) Infiltration of CD4+ T-cells
 - (c) Infiltration of B-cells
 - (d) IgG deposition
 - (e) Complement deposition
 - (f) *All of the above*
 - (g) a & b
 - (h) a, b & c
 - (i) a, b, c & d

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Guillain-Barré Syndrome, Chronic Inflammatory Demyelinating Polyradiculoneuropathy, and Axonal Degeneration and Regeneration

24

Ralf Gold and Klaus V. Toyka

Abstract

Current knowledge of the pathogenic mechanisms involved in Guillain-Barré syndrome (GBS) and chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) supports an autoimmune etiology. Some of the cellular and humoral immune responses that contribute to the development of GS and CIDP resemble those implicated in multiple sclerosis (MS). Also, the role of persistent axonal damage in these diseases has recently been revisited, similar to the situation in MS. Currently axonal damage is best understood in GBS associated with preceding infections. In all other subtypes, histopathological studies speak to the heterogeneity of disease mechanisms in GBS and CIDP. Several immunotherapies are available to treat GBS or CIDP. Intravenous immunoglobulin and plasma exchange are of comparable therapeutic efficacy for the acute phase of both diseases, whereas prednisone (or analogues) and immunosuppressive drugs are effective only to treat CIDP, which usually requires long-term therapy. Although some approaches have been made to support axonal regeneration in the model of experimental autoimmune neuritis, currently no promising specific therapeutic concept is available which can be transferred to treat the human disease.

Keywords

Autoantibodies • Axonal damage • Chronic inflammatory neuropathy • Gangliosides • Guillain Barré syndrome • Immune neuropathy • Myelin • Nerve conduction • Neuritis • Neurotrophin

24.1 Introduction

Guillain-Barré syndrome (GBS) and chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) are acquired demyelinating diseases of the peripheral nervous system (PNS), characterized by progressive or relapsing proximal and distal muscle weakness with possible sensory loss

(Saperstein et al. 2001; Hughes and Cornblath 2005). Historically GBS was described in 1916 in two French soldiers as an acute, postinfectious paralysis with elevated cerebrospinal fluid (CSF) protein but without cells. At that time also epidemics of poliomyelitis have occurred producing “Landry paralysis”, as a direct consequence of enterovirus infection, and in contrast to GBS is usually asymmetrical and associated with CSF pleocytosis. In the following decades, with the availability of electrodiagnosis the clinical hallmarks of GBS have been described more precisely. Estimated annual disease incidence in industrialized countries is up to 2/100,000. In contrast to CIDP, about 50 % of GBS cases are linked with preceding infections, typically diarrhea caused by *C. jejuni* (Enders et al. 1993) or infections of the respiratory tract (Hadden et al. 2001). In GBS acute respiratory failure or cardiac arrhythmias due to

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dysfunction of the autonomic nervous system cause at least 3 % mortality even in specialized centers, especially in elderly patients with rapid disease progression and those suffering from multimorbidity (Hughes and Cornblath 2005). In addition, both in GBS and CIDP, residual symptoms and chronic fatigue may lead to long-lasting disability in about half (Merkies et al. 1999).

The last years have seen remarkable progress in understanding cellular and molecular pathways that cause autoimmune damage in these disorders of the PNS (see reviews in (Gold et al. 2003; Kieseier et al. 2004; Gold et al. 2005; Mathey et al. 2015)). Based on the observations that a significant proportion of patients with CIDP are responsive to immunotherapy and that an inflammatory response is observed at sites of active disease (Schmidt et al. 1996; Sommer et al. 2005), it is generally accepted that GBS and CIDP are autoimmune disorder with myelin as the likely target for the immune response. There is increasing evidence that GBS and CIDP have heterogeneous pathomechanisms. The dominant subtype of GBS is the acute inflammatory demyelinating form (AIDP), a classical demyelinating disease, but an increasing number of axonal variants has been described after the first series linked to preceding infections in China with acute motor axonal neuropathy (AMAN) (McKhann et al. 1991). Whilst first data seemed to associate axonal damage to preceding *C. jejuni* infection, especially with Penner 19 strain (Ho et al. 1995), these regional specificities could not be confirmed in the largest multicenter GBS-study performed to date (Hadden et al. 1998, 2001). Acute motor-sensory axonal neuropathy (AMSAN) is clinically and electrophysiologically similar to AMAN, but has a detectable sensory involvement. Thus, there is probably no single or unique mechanism that leads to axonal damage, but several immunological components will be discussed which may contribute to neuronal dysfunction and degeneration. This heterogeneity of the disease is further underscored by the variant of GBS, the Miller Fisher syndrome (MFS), where cranial nerves and caudal spinal roots are involved, and the disorder is strongly linked with anti-ganglioside GQ1b immunoreactivity.

Chronic inflammatory demyelinating polyneuropathy (chronic polyneuritis, CIDP) characteristically presents as symmetrical weakness, with some impairment of distal sensation. Yet to our experience only about 50 % of CIDP patients display the classical phenotype. Variants include the sensory predominant CIDP, the progressive sensory ataxic CIDP, and motor dominant CIDP with often acute onset (Mathey et al. 2015). By electrophysiology both motor and sensory nerves are affected with slowing of nerve conduction velocity, especially in proximal nerve segments as reflected by abnormal F-wave examination. Cranial nerves may be involved, but less frequently than in GBS. The disease course may either be relapsing-remitting or stepwise progressive. If

the initial attack of CIDP is subacute, disease progression up to reaching a plateau level is longer than 8 weeks. The diagnosis is supported by an elevated CSF protein, and inflammatory demyelination in sural nerve biopsy.

24.2 Experimental Models

For many aspects of both diseases, the various models of experimental of autoimmune neuropathies (EAN) have helped us to better understand the immunological mechanisms (see review in (Gold et al. 2005)). The monophasic EAN of the Lewis rat has its greatest advantage for defining pathogenetic hallmarks and establishing innovative therapeutic principles, similar to the chronic EAN in dark agouti rats. Upon immunization with increasing doses of the neurotogen or when antigen-specific T cell lines are used for adoptive transfer (AT-EAN), many clinical and electrophysiological signs of the human diseases could be reproduced (Heininger et al. 1986; Hartung et al. 1988). In particular, a profound and rapidly evolving nerve dysfunction occurred dose-dependently which correlated with increased axonal damage due to Wallerian degeneration. In the high-dose adoptive transfer model, most likely endoneurial ischemia resulting from severe inflammation and edema was an admixed and critical pathogenic mechanism. In all these experimental autoimmune encephalomyelitis (EAE) models, the pathology was mediated by CD4+ T cells specific for myelin antigens. In contrast to these models induced by immunisation, a spontaneous EAN model in the non-obese diabetic (NOD) mouse developed when the costimulatory molecule B7-2 was knocked out (Salomon et al. 2001). Further analysis of pathogenic T cell populations showed that CD4+ T cells recognizing myelin protein P0 were crucial in mediating disease (Louvet et al. 2009).

Progress in molecular biology has allowed generation of an increasing number of transgenic mice, which are typically on the genetic background of C57BL/6. For these mouse strains defined peptides of the myelin protein P0 have been described as neuritogenic (Miletic et al. 2005; Visan et al. 2004). Besides the acute and chronic autoimmune models induced by immunization or adoptive transfer of T cells, the immune system has been shown to contribute to disease progression and expression in inherited myelinopathies, which was elucidated by Martini and colleagues (see review in (Martini and Toyka 2004)). When the immune system was paralyzed by backcrossing the P0 deficient mice on C57BL/6 background with recombination-activating gene (RAG) deficient knockout mice, a remarkable delay of myelin pathology was observed within 6 months. This was not limited to demyelination, but also axonal damage was reduced which may reflect trophic aspects of the axon-Schwann cell unit and possibly also immune responses to Schwann cell nodal

antigens. Recent *in vitro* studies have confirmed that indeed dorsal root ganglia cultures from a rat model for Charcot Marie Tooth disease undergo axonal atrophy over a period of time (Nobbio et al. 2006). This model may be utilized to study the molecular changes underlying demyelination and secondary axonal impairment, in particular in the chronic disorders such as CIDP. As axonal damage may occur after just 3 months and tissue cultures represent a strictly controlled environment, this model may also be ideal for testing neuroprotective therapies.

24.3 Pathogenesis of Axonal Damage in GBS and CIDP

The presence of prominent demyelination, with “onion bulb” formations in chronic disease, and of perivascular inflammatory infiltrates are both hallmarks of CIDP pathology (Bosboom et al. 2001b; Sommer et al. 2005), although they may not always be found on small nerve samples from human biopsies. The “onion bulb” formation is a manifestation of excessive Schwann cell processes and is often produced by segmental repetitive demyelination and remyelination (Krendel et al. 1989; Bosboom et al. 2001a). As the disease progresses, secondary axonal degeneration becomes more established, but it also may occur early in the disease course (Dalakas 1999). This is very similar to the situation in multiple sclerosis (MS), where the work of Trapp’s group (Trapp et al. 1998) quantified progressive axonal damage in late disease stages, complemented by the findings of axonal damage in early biopsied MS lesions (Kuhlmann et al. 2002). The precise mechanisms that lead to demyelination and (early) axonal damage in the CNS and PNS are not known, but are thought to be mediated by both cellular and humoral immune factors. In MS, axonal damage has been associated with cytotoxic CD8 T cells (Neumann et al. 2002), nitric oxide (NO) (Smith et al. 2001) and free oxygen radicals. Similar observations relate to the detrimental role of focal nitric oxide in the PNS (Kapoor et al. 2003) although in the whole animal, the opposite effect has also been described (Kahl et al. 2003).

Importantly, nodal and paranodal antigens in the non-compacted myelin have received increased importance as mediators of humoral immune destruction. In CIDP, these regions are often disrupted (Devaux et al. 2012) have described that up to 30% of patients’ sera bind to cellular adhesion molecules such as neurofascin (NF), gliomedin, and contactin. In another study 4% of serum samples from GBS and CIDP showed antibodies to human recombinant NF 155 and NF 186. The majority of identified autoantibodies were reactive against the glial neurofascin isoform NF155. These were IgG3 or IgG4 subtypes, and the patients with this high-titre immunopattern responded well to plasma exchange therapy (Ng et al. 2012).

A novel mechanism has been described for antiganglioside antibodies in the axonal subtype of the GBS namely AMAN: uptake of these antibodies in nerve terminals and its endocytic processing may have an impact on axonal pathogenicity (Fewou et al. 2012; Buchwald et al. 1998). Similar mechanisms may also apply to the more chronic inflammatory neuropathies.

24.3.1 Neurotrophic Factors and Survival in the Inflamed Nervous System

Experimental models have revealed that the immune system is not the only denominator for the extent of damage in the inflamed nervous system. In EAE the neurotrophic cytokine CNTF (ciliary neurotrophic factor) has clear neuroprotective role on survival of oligodendrocyte progenitor cells and mature oligodendrocytes (Linker et al. 2002). This in turn leads to enhanced demyelination in mice where CNTF was lacking, and ultimately was associated with axonal damage. In contrast to other neurotrophins CNTF does not have an effect on immune cells, so that these findings could be attributed only to neuroprotection. A retrospective study in MS patients gave additional evidence that CNTF is also relevant in the human disease MS: those MS patients which have a genetic deficiency of CNTF appeared to exhibit earlier motor symptoms and a more severe disease course (Giess et al. 2002).

Since CNTF has also been described as lesion factor in the PNS (Sendtner et al. 1992), it seemed attractive to substitute neurotrophic factors by exogenous administration. This is a major problem with CNTF, which is mostly absorbed in the liver upon *s.c.* injection (Dittrich et al. 1994), and did not show protective effects on regeneration of the inflamed nerve in the Lewis rat EAN model (Gold et al. 1996). In contrast LIF (leucemia inhibitory factor), another family member of this IL6-cytokine group turned out to augment survival of oligodendrocytes during EAE (Butzkueven et al. 2002) as confirmed by signalling studies and molecular histology. The administration of LIF had positive effects on the disease course both in a preventive and in a therapeutic setting. Yet LIF also interferes with the immune system and affects T cell priming thus contributing to a different quality of cellular inflammation with fewer macrophages in the inflamed neural structure. In addition, the chemokine pattern was changed with an increase of CXCL1 early and a decrease of CCL2, CCL3, and CXCL10 later in the disease (Linker et al. 2008).

Studies with *s.c.* administration of the neurotrophin BDNF have been performed in Lewis rat EAN (Felts et al. 2002). Treatment of Lewis rats with BDNF (10 mg/kg day) did not significantly affect the neurological deficit, nor significantly improve survival, motor function or motor innervation. The weight of the urinary bladder was significantly increased in control animals with EAN, but remained

similar to normal in animals treated with BDNF. These results speak for a very limited effect of exogenous administration of BDNF. For thorough interpretation it would be required that pharmacological tracking studies are performed to verify the effect of BDNF on the target organ. With the use of conditional knockout mice for BDNF and inducing EAE, its role has become much clearer in central demyelination. C57BL/6 mice lacking BDNF in CD4⁺ T lymphocytes and monocytes developed more severe chronic EAE, coinciding with more pronounced demyelination and axonal damage. A partial rescue could be performed with BDNF-transfected T lymphocytes carrying a transgenic T cell receptor (Linker et al. 2010). These findings underline the idea that immune cell-associated BDNF may imply a putative neurotrophic survival effect.

It is currently not clear how local factors in the target tissue modulate recovery from an immune attack in the PNS. As yet unknown genetic susceptibility factors may modulate the inflammatory process itself and the response of axons and of myelinating Schwann cells to the inflammatory assault. As an example, axonal degeneration occurring in autoimmune neuropathies clearly affects prognosis (Dalakas 1999). Indeed, parallel expression of neurotrophic factors and their receptors in CIDP may reflect such survival mechanisms in the PNS (Yamamoto et al. 2002).

Besides neurotrophic factors also small molecules mediating survival in the inflamed PNS have been described recently. Pitarokoili used laquinimod, a derivative of linoimide, to treat EAN in the Lewis rat and observed maintained integrity of axonal function (Pitarokoili et al. 2014). Furthermore, when she treated EAN with dimethyl fumarate she detected a reduction of early signs of axonal degeneration through a reduction of amyloid precursor protein expressed in axons of the peripheral nerves (Pitarokoili et al. 2015). This correlated with an increase of nuclear factor (erythroid derived 2)-related factor 2 positive axons, supporting a direct neuroprotective potential of dimethyl fumarate. Furthermore, nuclear factor (erythroid derived 2)-related factor 2 expression in Schwann cells was only rarely detected and there was no increase of Schwann cells death during EAN. This supported the notion that this did not result from increased trophic support from Schwann cell contact.

24.3.2 Cellular Immune Factors

Inflammatory infiltrates as seen in nerve biopsies, consisting primarily of macrophages (Sommer et al. 2005) and T cells (Schmidt et al. 1996), suggesting that a T-cell-mediated delayed hypersensitivity reaction directed toward myelin antigens is a probable cause of inflammatory tissue damage in GBS and CIDP. Inflammatory immune reactions are coordinated by a number of soluble chemical mediators and selective adhesion molecules, including the following: direct

differentiation and migration of T cells; translocation of T cells across the vascular endothelium; stimulation of protease release; and recruitment of macrophages and additional T cells to sites of inflammation (reviewed in (Gold et al. 2003)). In both CIDP and its CNS counterpart multiple sclerosis, dysregulation of these chemical mediators (i.e., cytokines, chemokines, and adhesion molecules) is postulated to play a role in the breakdown of the blood-nerve and blood-brain barriers, respectively. Furthermore, dysregulation of chemical mediators may be responsible for aberrant trafficking of T cells into the PNS and perpetuation of inflammatory responses that lead to demyelination. In both the PNS and CNS, inflammatory T cells are eliminated by apoptosis, occurring either during the natural disease course or after treatment with glucocorticosteroids (Zettl et al. 1995).

24.3.3 Humoral Immune Factors

In the last decades, postinfectious molecular mimicry has been postulated as the foremost putative mechanism underlying GBS. This implies that antigenic determinants are shared between an infectious agent and the peripheral nervous system. Thus the initial antigenic stimulation by the infectious agent results in a secondary immune response directed against the nervous system. Several microbial organisms share antigens with the nervous system: *C. jejuni* serotypes O19 or Lior 11 have lipopolysaccharides (LPS) or lipo-oligosaccharides with ganglioside-like structures (causing anti-GM1 or anti-GQ1b immunoreactivity). In addition, *Haemophilus influenzae* has homologies with GM1 and GT1a, *Mycoplasma pneumoniae* with galactocerebroside, and cytomegalovirus has crossreactivity with GM2 gangliosides.

Antibody binding to major glycolipid or myelin protein antigens has been shown in both GBS-CIDP and MS. Antibodies may bind to macrophages via their Fc portion, activating phagocytosis and release of inflammatory mediators toward the myelin sheath. An alternative mechanism is through neuromuscular blocking antibodies. An early study of the functional activity of serum IgG antibodies demonstrated a slowing of nerve conduction in marmoset monkeys upon passive transfer of purified IgG from CIDP patients (Heininger et al. 1984). Recently, IgG antibodies that are capable of blocking neuromuscular transmission were identified in CIDP patient serum (Buchwald, Ahangari and Toyka, unpublished observations). This neuromuscular blockade by IgG antibodies has first been observed in GBS and its variant Miller Fisher syndrome (Buchwald et al. 1998, 2002). In the latter study a new mode of action has been defined for polyvalent immunoglobulin G from healthy donors namely neutralizing the pathogenic effects of autoantibodies. These findings were detailed using monoclonal antibodies to various gangliosides (Buchwald et al. 2007).

As described above, amongst these immune targets the antigens of the nodal and paranodal region in non-compacted myelin may also be of critical importance.

Human peripheral nerve myelin contains acidic glycosphingolipids such as sulfated glucuronyl paragloboside (SGPG) and sulfated glucuronyl lactosaminyl paragloboside (SGLPG) (Quarles 1997; Willison and Yuki 2002). One study found elevated IgM anti-SGPG antibody titers in six of nine patients (67 %) with CIDP (Yuki et al. 1996). In earlier studies, antibodies to a variety of glycolipid antigens were described, including LM1, GM1, GD1a (reviewed in (Willison and Yuki 2002)). Another candidate antigen is the HNK-1 carbohydrate epitope, which is common to some glycolipids and other cell adhesion molecules. More evidence for the role of GM1 as target has been given when rabbits were immunized with a ganglioside mixture (Yuki et al. 2001) : all of them developed high anti-GM1 IgG antibody titers, flaccid limb weakness of acute onset, and a monophasic illness course. Pathological findings for the peripheral nerves showed predominant Wallerian-like degeneration, with neither lymphocytic infiltration nor demyelination. IgG was deposited on the axons of the anterior roots, and GM1 was proved to be present on the axons of peripheral nerves. Sensitization with purified GM1 also induced axonal neuropathy, indicating that GM1 was the immunogen in the mixture and explaining the association of AMAN with anti-GM1 reactivity.

Over the last few years antibodies to complexes of two gangliosides were described forming new target epitopes in the cell membrane with higher avidity than single gangliosides. These autoantibodies may have profound pathogenic effects in GBS and MFS and may better correlate to clinical subtypes than those directed at single gangliosides (Kaida et al. 2004; Ogawa et al. 2013; Willison and Goodyear 2013).

EAN can be induced by immunization of an animal with myelin proteins such as P2 basic protein, P0 glycoprotein, and peripheral myelin protein 22 (PMP22) [see review in (Gold et al. 2005)]. Gabriel et al. (2000) investigated whether PMP22 may be important in inducing human inflammatory neuropathy. The sera of patients with GBS, CIDP, other neuropathies (ONP), and normal controls were evaluated for IgM and IgG antibodies against PMP22. Antibodies were detected in 52 % of patients with GBS, 35 % with CIDP, and 3 % with ONP; no antibodies were detected in normal controls (Gabriel et al. 2000). Furthermore, Ritz et al. (2000) reported PMP22 antibodies in three of six (50 %) CIDP patients. In contrast, Kwa et al. (2001) reported the absence of these antibodies in sera from 24 patients with CIDP. The discrepancy among the results of these studies may be due to differences in the PMP22 antigen used to test the sera. When linear peptide epitopes of PMP22 and purified PMP22 protein from overexpression in *E. coli* were used, a higher percentage of patient sera showed reactivity. However, when PMP22 protein was expressed in mammalian cells under native conditions, the sera failed to show any reactivity.

In any chronic autoimmune inflammatory condition, several antigens may be involved via epitope spreading (Lehmann et al. 1992), making it difficult to identify the culprit antigen in an individual patient. Moreover, antibodies display a variety of affinities and avidities and some may activate the complement cascade while others may not (Janeway et al. 2001). Therefore, it is not easy to define the pathogenic role of individual antigen binding specificities. Given the heterogeneity of GBS and CIDP, it is likely that different antibodies are sequentially or even collectively involved in the pathogenesis of this disease.

Recent evidence demonstrates that antibodies against myelin protein zero (P0), a major structural protein of myelin, may play a role in CIDP (Yan et al. 2001). There is indirect evidence that P0 may have immunologic relevance. In an experimental study of heterozygous P₀ knockout mice, an animal model of Charcot-Marie-Tooth disease, Schmid et al. (2000) showed that (1) myelin degeneration and impairment in nerve conduction were attenuated when the immune system was impaired, and (2) T cells isolated from these mutant mice demonstrated enhanced reactivity to myelin proteins such as P0 and P2. P0, an adhesive cell-surface molecule of the Ig superfamily and the most abundant protein of myelin on the peripheral nerves, has multiple functions in the development and maintenance of myelin (Martini et al. 1995). An increase in macrophages with a subsequent increase in T cells was observed within the nerves of heterozygous P0 knockout mice. It was hypothesized that a reduction in P₀ could result in an instability of myelin, which then could lead to an attraction of macrophages and a macrophage-mediated attraction of T lymphocytes because these were not tolerized due to lack of myelin protein P0 in thymus (Visan et al. 2004). Activation of autoimmune T cells by antigen-presenting macrophages could lead to cellular and humoral immune reactions, which ultimately could result in demyelination (Schmid et al. 2000).

Yan et al. (2001) studied the sera of 21 CIDP patients for antimyelin activity using immunofluorescence and for binding to myelin proteins using Western blot analysis. Results showed that the sera of six patients (29 %) contained anti-P0 IgG antibodies, and four of these caused conduction block and demyelination when injected intraneurally into experimental animals. These results suggest that P0 is an autoantigen in some patients with CIDP. More work is needed to ultimately define the precise nature of circulating antibodies and their pathogenic role in CIDP.

24.4 Summary

There are similarities in the importance of axonal damage in immune neuropathies and MS. Probably in both cases destructive immune responses are involved, presumably by a direct inflammatory assault. Recently new antigenic targets including nodal antigens and ganglioside complexes have been identified

that may exert a pathogenic role in GBS, MFS, and CIDP patients. The ultimate nerve damage, i.e. demyelination, axonal degeneration or both can experimentally be counteracted by endogenous survival factors. As yet only some of them have been identified, mostly belonging to the group of neurotrophins or neurotrophic factors. Their therapeutic application is limited because of their absorption in peripheral tissues such as liver, ultimately preventing them from access to the target organ. With the further progress in molecular and cellular gene therapy we may be able to overcome these obstacles. Currently available immune therapies such as plasmapheresis and intravenous immunoglobulins probably limit the damage by reducing the primary inflammatory assault, or by reducing titers of or neutralizing pathogenic autoantibodies.

24.5 Review Questions

- Describe clinical differences between GBS and CIDP
- Briefly summarize the different experimental neuritis models in relation to the human diseases
- Describe the key immunological factors contributing to axonal damage in GBS and CIDP
- Which factors contribute to chronification of the autoimmune inflammation in the animal model?
- Which potentially pathogenic humoral elements in CIDP patients have been identified?
- Which of the following myelin proteins is not involved in experimental neuritis?
 - MAG
 - P2 Protein
 - P0 Protein
 - MOG
 - PMP22
- Which neurotrophic cytokine has been identified as lesion factor in the PNS?
 - NGF
 - LIF
 - NT-3
 - CNTF
 - GMCSF
- Which is the correct explanation for “onion bulb” formation in CIDP?
 - Macrophages phagocytose myelin debris
 - Abundant T-cell apoptosis
 - Repetitive De- and Remyelination with Schwann cell proliferation*
 - Nitric oxide release
 - Genetic myelin deficiency
- Which of the following infectious agents is linked to axonal damage and GBS?
 - C. jejuni* Lior O4
 - Neisseria meningitidis*
 - Listeria monocytogenes*
 - Proteus mirabilis*
 - C. jejuni* serotype Penner 19
- The influence of the immune system on disease progression in genetic myelin disorders...
 - Has been excluded
 - Has been clearly shown in experimental models*
 - Has immediate therapeutic implications
 - Is merely speculative
 - Has been confirmed only in the Lewis rat

24.6 Answers

- Key Points: GBS is a monophasic disease of the PNS, mostly with preceding infection, 3% mortality, no response to steroids alone, fair to good response to plasmapheresis or polyvalent normal immunoglobulin G from healthy donors; CIDP is mostly chronic progressive or relapsing progressive, often responsive to corticosteroids alone, to plasmapheresis, immunoglobulin G from healthy donors, and to immunosuppressive drugs.
- EAN of Lewis rat—monophasic, predominantly demyelination upon immunization with crude myelin (dose-dependent), demyelination and axonal damage with cell transfer (cell-number dependent)—both types mimicking GBS; EAN of DA rat—relapsing remitting. EAN in SJL mice: mild disease, better mimicking relapsing CIDP.
- Multiple factors including cytokines like TNF alpha, free oxygen radicals, nitric oxide (NO); cytotoxic CD8 positive T cells, autoantibodies with complement.
- Autoimmunization with myelin proteins, genetic elements, epigenetic factors
- Autoantibodies (against P0 and other nerve antigens), Nitric oxide (NO)

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Abstract

Autoimmunity is implicated in a small but important group of peripheral nerve diseases. These include the acute inflammatory neuropathies referred to as the Guillain-Barré (GBS) and Fisher syndromes. Early recognition and appropriate management of these neuropathies can prevent significant morbidity and mortality. Substantial evidence exists for an autoimmune pathogenesis in GBS and its subtypes. Although most current evidence supports an antibody driven pathogenesis triggered by infection, T-cell and other cellular immune components are crucial effectors of disease particularly in the demyelinating forms of GBS.

Keywords

Acute flaccid paralysis • Anti-ganglioside antibodies • *Campylobacter jejuni* • Experimental allergic/autoimmune neuritis (EAN) • Gangliosides • Immune neuropathies • Molecular mimicry • T-cells

25.1 Introduction

The term Guillain-Barré syndrome (GBS) is used to denote a group of clinically and pathophysiologically heterogeneous disorders of peripheral nerves that are characterized by acute onset, monophasic course, and potential for substantial recovery, which is expedited by two immunomodulatory therapies. After the near-eradication of polio, GBS is the commonest cause of acute flaccid paralysis worldwide. Current evidence supports the concept that GBS is likely of autoimmune origin. Postinfectious molecular mimicry (autoimmunity) is the currently favoured dominant theme in the pathogenesis of GBS. This concept implies antigens shared

between the infectious agents and peripheral nerves so that an infection results in an immune response to these cross-reactive antigens carried by the organism. The immune response triggered by infection then mediates injury to the peripheral nerves. The results from both clinical and experimental studies on some forms of GBS support this concept. This chapter outlines clinical and pathophysiological features of major variants of GBS and highlights the evidence that supports the hypothesis of postinfectious molecular mimicry in this group of disorders.

25.1.1 Historical Background

The history of Guillain Barré syndrome (GBS) is inseparably linked to the seminal paper of Georges Guillain (1876–1961), Jean-Alexandre Barré (1880–1967) and André Strohl (1887–1977) (Guillain et al. 1916). During the weekly meeting of the “*société médicale des hôpitaux de Paris*” in 1916, they presented the case history of two soldiers of the VI French army, who developed flaccid sensorimotor paralysis. Both patients, without any history of preceding infection, recovered

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completely after a few weeks. The most important discovery of Guillain, Barré and Strohl was the pathological cerebrospinal fluid (CSF) examination. They performed a lumbar puncture in both patients and found the typical “*dissociation cytoalbuminique*”. Previously, the pathological condition of raised CSF protein and normal cell count was only described in cases of syphilis and compression of the spinal cord, but was never been associated with acute ascending paralysis.

The presentation of Guillain, Barré and Strohl was not the first clinical description of the disease. Half a century before, in 1859, Jean Baptiste Octave Landry (1826–1865) described the case of an 43 years old man, who developed an acute ascending paralysis and died within a few days (Landry 1859).

Landry noted a preceding pulmonary infection in his patient. He brought attention to a disease, which he called “*paralysie ascendante aigue*” by further reviewing four own and five other cases in the contemporary literature. In contrast to the description of Guillain and Barré, two patients of Landry’s case series died because of the disease. Therefore Landry’s acute ascending paralysis had been associated with poor prognosis, whereas Guillain-Barré syndrome was considered to have a mild clinical course with almost complete recovery. Landry noticed preceding infections in two cases of his series, however, on autopsy of his own case he was not able to detect any pathological changes of the nervous system. In the same year the German Adolph Kussmaul (1822–1902) (Kussmaul 1859) reported two cases of a deadly ascending paraplegia. Like, Landry he did not find a toxic or anatomical explanation on autopsy. The affection of the peripheral nerve as underlying cause for the disease, was not recognized before 1864 when L. Duménil described a case of “*paralysie ascendante aigue*”, in which he found an atrophy of peripheral nerve roots in autoptical material (Schott 1982). In the following decades, German physicians referred variously to the disease. In 1871 M. Bernhardt (Bernhardt 1871) described a lethal case of acute ascending paralysis followed by Carl Westphal (1833–1890) in 1876, who presented a similar case. He called the disease “*akute tödliche Spinallähmung*” (acute deadly spinal paralysis) (Westphal 1876). One has to consider that Landry’s ascending paralysis was a pure clinical diagnosis. It is likely that the early descriptions of Landry and others cover a range of different entities, including atypical forms of polio and infectious neuropathies. Therefore, the application of the lumbar puncture technique by Guillain, Barré and Strohl was a landmark for classifying and diagnosis of the disease, since it helped to distinguish it from other entities. In 1927 the term Guillain-Barré syndrome was introduced for the first time and become the preferred eponym in the following years (Draganesco and Claudian 1927). The name of Strohl, who was a medical student in 1916, disappeared unfairly from the original work, although he contributed substantially by doing electrophysiological examinations of the two patients.

A fundamental step towards an understanding the pathogenesis of GBS was made by observations of inflammatory

infiltrates and demyelination in peripheral nerves. These pathological studies on autopsy material lead to the assumption of GBS being a single pathophysiological entity synonymous with acute inflammatory demyelinating polyradiculoneuropathy (AIDP) (Haymaker and Kernohan 1949; Asbury et al. 1969). In the same time Waksman and Adams described the induction of an allergic neuritis in rabbits by immunization with peripheral nervous tissue (Waksman and Adams 1955). In the following years this model of an experimental allergic neuritis (EAN) was further expanded to other species, and was used as in-vivo model for GBS. The Fisher syndrome (FS) is named after Charles Miller Fisher, a Canadian neurologist. In 1956, Miller Fisher described three patients with acute external ophthalmoplegia, absent tendon reflexes, and ataxia, who recovered spontaneously (Fisher 1956). Some cases start as FS but subsequently develop weakness. Finally, work over last two decades indicates that some forms of GBS lack features of demyelination and have pathophysiology that is consistent with axonal injury; these cases are termed axonal GBS.

25.1.2 Classification of GBS Variants

Clinical features and/or electrodiagnostic examination provide a framework for classifying GBS into different variants. GBS can be broadly divided into major and minor variants. The major variants typically have muscle weakness or motor neuropathy as the dominant manifestation; they are further divided into demyelinating and axonal variants on the basis of the predominant pathophysiologic process of nerve fiber injury as determined by electrodiagnostic testing, namely, primary demyelination or primary axonal degeneration. Axonal variants are further subclassified according to the fiber type affected. Minor variants are classified based on the constellation of clinical symptoms and not by electrodiagnostic findings. The constellation of symptoms in minor variants is taken to imply regional localization of the pathophysiologic process. On the basis of this schema a simple classification of GBS is proposed in Table 25.1.

Table 25.1 Classification of GBS

Major variants
Demyelinating
AIDP
Axonal
AMAN
AMSAN
AIDP with secondary axonal degeneration
Minor variants
Fisher syndrome
Sensory ataxic variant
Acute idiopathic autonomic neuropathy

The demyelinating form of the disease is termed acute inflammatory demyelinating polyneuropathy (AIDP). Two axonal forms of GBS (Feasby et al. 1986; Yuki et al. 1990; McKhann et al. 1993), however, are now widely recognized on the basis of nerve fiber type affected: acute motor axonal neuropathy (AMAN), the more common form of axonal GBS, is distinguished by nearly pure motor axonal injury; the less common variant is acute motor-sensory axonal neuropathy (AMSAN), characterized by degeneration of both motor and sensory axons. It has been postulated that AMAN and AMSAN represent a pathologic spectrum and that AMSAN actually represents a more severe form of AMAN (Griffin et al. 1996a). Fisher syndrome (FS) is a minor GBS variant characterized by gait disturbance (ataxia), areflexia, and ophthalmoplegia. Other rarer forms without significant motor weakness that may be included under the term GBS include a predominantly sensory variant and acute idiopathic autonomic neuropathy or acute pandysautonomia.

25.1.3 Epidemiology

The incidence of GBS 0.35–1.34 per 100,000 is surprisingly similar throughout the world, despite different infection rates in various geographical regions (McGrogan et al. 2009; Sejvar et al. 2011; Hauck et al. 2008; Lehmann et al. 2007b; Govoni and Granieri 2001). Men are slightly more affected than women (Hughes and Cornblath 2005) and incidence rates increase with age (Hauck et al. 2008). The occurrence of GBS after influenza vaccination has increased the interest in establishing accurate background incidence rates for GBS in countries in which immunisation with seasonal influenza is recommended by health authorities (Destefano and Tokars 2010; Black et al. 2009; Adverse Effects of Vaccines 2011). Those age and gender specific background rates for GBS have been reported for the countries Brazil, Finland, UK and USA (Black et al. 2009).

Demyelinating forms of the disease are most prevalent in the United States, Europe (Hadden et al. 1998; A prospective study on the incidence and prognosis of Guillain-Barré syndrome in Emilia-Romagna region, Italy (1992–1993). Emilia-Romagna Study Group on Clinical and Epidemiological Problems in Neurology 1997), and most of the developed world, accounting for over 90% of patients. Compared to western world, the incidence of axonal forms of GBS is higher in northern China, Japan, Mexico, Bangladesh and other developing countries but are also, less frequently, seen in northern America and other developed countries (McKhann et al. 1993; Ramos-Alvarez et al. 1969; Ogawara et al. 2000; Islam et al. 2010). Notably, in northern China there is a clear seasonal pattern, with peak incidence in summer months, and a predilection of disease in children and villages (McKhann et al. 1991, 1993). The Fisher variant probably represents 5% of cases of GBS and has a similar incidence worldwide.

25.1.4 Clinical Features

The diagnosis of GBS is primarily clinical. In the AIDP variant the majority of cases have some sensory symptoms or paresthesias at the onset of the disease; however, abnormalities on sensory examination are less frequent. Pain, particularly low back, buttock, or thigh pain, is an early symptom in approximately 50% of patients (Ruts et al. 2007, 2008; van Doorn et al. 2008). Subsequently the clinical picture is dominated by weakness often progressing to paralysis. Muscle weakness may begin in the lower limbs and ascend upwards, characteristically involving both proximal and distal muscles. Respiratory muscles can be involved in up to one-third of the hospitalized patients. Complete or partial loss of reflexes is seen in almost all patients. Cranial nerve involvement is seen in two thirds of cases, most commonly causing facial weakness and difficulties of eye closure, ophthalmoplegia, difficulty swallowing or altered taste. Autonomic manifestations include reduced sinus arrhythmia, sinus tachycardia, arrhythmias, labile blood pressure, orthostatic hypotension, abnormal sweating, and pupillary abnormalities. Respiratory and bulbar weakness and autonomic instability are the major cause of morbidity and mortality in GBS.

Clinically, it is difficult to distinguish between axonal and demyelinating forms of GBS. Electrodiagnostic testing is essential to differentiate these variants. AMAN has exclusively motor findings, with weakness typically beginning in the legs, but in some individuals affecting arms or cranial muscles initially (McKhann et al. 1993). Tendon reflexes are preserved until weakness is severe enough to preclude phasic muscle contraction. This probably reflects sparing of muscle afferent fibers. The incidence of dysautonomia has not been systematically examined in axonal cases but it was seen in a small proportion of cases.

Minor variants without significant motor weakness include FS characterized by ataxia, areflexia, and internal and external ophthalmoplegia. Other rarer forms of the disease without significant muscle weakness include a pure sensory variant and acute autonomic neuropathy.

Differential diagnoses include structural lesions, such as myelopathy and infections including HIV, Lyme disease, *Cytomegalovirus* (CMV), rarely paralytic rabies (Sheikh et al. 2005), and, in endemic areas, polio. In children botulism should be considered. Toxic and metabolic conditions such as tick bite and porphyria can also mimic GBS (Derksen et al. 2014). In the intensive care setting, critical illness neuropathy and quadriplegic myopathy may be clinically indistinguishable from GBS.

25.1.4.1 Investigations

The main aim of the investigations is to exclude other conditions that can mimic GBS and to confirm the diagnosis. Electrodiagnostic testing is the most critical investigation in

the evaluation of patients with GBS; it can potentially provide support for the clinical diagnosis and useful prognostic information. Nerve conduction studies (NCS) are abnormal to some extent in most patients with GBS, but normal studies in the first week do not exclude this diagnosis. Gordon and Wilbourn reported the NCS changes seen in the first week (Gordon and Wilbourn 2001). Changes in F wave latencies are probably the most common abnormality early in disease. In AIDP, typical electrophysiological features of demyelination such as slow motor conduction velocities, prolonged distal motor latencies, and partial motor conduction block may not be present until the second or third week. Reduced or absent sensory nerve action potentials (SNAPs) and slowing of sensory conduction velocity are common. The pattern of preserved sural SNAP and abnormal ulnar/median SNAP (“sural nerve sparing pattern”) is more specific for AIDP than for GBS mimics at hospital admission (Derksen et al. 2014). In contrast to AIDP, conduction times like distal motor latencies and motor conduction velocities are relatively preserved in the axonal forms, but reduced compound motor action potential (CMAP) amplitudes are characteristic. Inexcitable motor nerves can be seen in more severe cases and in patients with the AMSAN variant. Sensory conduction studies are usually normal in AMAN, but SNAPs are decreased or absent in the AMSAN variant. Examination of CSF is most useful in excluding other differential diagnoses, particularly infections. Typically, CSF protein is increased in 80–90 % of GBS cases without significant pleocytosis. A mild increase in mononuclear cells can be seen in up to 10 % of patients. Significant pleocytosis raises the possibility of infection such as Lyme disease or HIV. Serological studies for anti-ganglioside antibodies can be considered for the diagnosis in incomplete forms and unusual variants of GBS, particularly when nerve conduction studies and CSF are normal. The role of anti-ganglioside serology in routine diagnosis and clinical decision-making remains to be established. Table 25.2 summarizes the common anti-glycolipid antibodies reported in association with various forms of GBS.

25.1.4.2 Clinical Course

GBS may progress up to 4 weeks with a nadir being reached within 2–3 weeks in a majority of patients. Recovery usually begins within 2–4 weeks of this nadir, but can be delayed for several months. About one-half of patients become chair- or

bed-bound, one-third require intensive care admission, and one-quarter mechanical ventilation (Winer et al. 1988; Rees 1998; Hughes and Cornblath 2005). Functional recovery is a rule and occurs in a majority of patients over 6–12 months; however 20–30 % of patients are left with significant disability and about 10 % require assistance with walking. Recent studies reported mortality rates for GBS between 2.58 and 3.8 % within 12 months, which is lower than those reported a decade earlier (van den Berg et al. 2013; Alshekhlee et al. 2008). Most deaths are attributed to cardiac arrest due to autonomic disturbance, respiratory failure or infection, or pulmonary embolism.

25.1.4.3 Prognosis

The extent and location of axonal injury are the two most important determinants of prognosis after an episode of GBS. Residual disability almost always indicates axonal degeneration. Axon regeneration is required for restoration of function. In GBS, there is characteristically significant pathology in spinal roots and proximal nerves and as peripheral axon regeneration advances at a rate of approximately 1 in./month, recovery is slow and often incomplete. Although peripheral axons have the capacity to regenerate, experimental evidence indicates that: (a) the efficiency of axonal regeneration decreases over time after injury (Fu and Gordon 1995a); (b) the denervated distal segment of peripheral nerves can optimally support axon regeneration only for a limited time (Fu and Gordon 1995b); and (c) the efficiency of reinnervation of original pathways and targets (pathfinding) decreases with advanced age (Le et al. 2001). Poor recovery after axonal GBS is not always the rule; exceptions include children with electrical features of acute denervation, cases with distal axonal degeneration where regeneration is needed over only a short distance, or patients with reversible axonal conduction failure. Poor prognostic factors include advanced age, ventilator dependence, preceding gastrointestinal infection, rapid progression from onset to nadir, severe motor involvement, and electrodiagnostic evidence of extensive axonal injury.

25.1.5 Pathology

Pathology of demyelinating and axonal forms presented below is well established. Pathological changes in minor variants of GBS are not well-characterized.

AIDP: The pathological changes in AIDP have been extensively characterized in postmortem studies. The most prominent feature in AIDP is marked segmental demyelination, which can be found throughout the length of all peripheral nerves including the mixed spinal roots and even the distal terminal nerves (Hall et al. 1992; Massaro et al. 1998). In areas of severe demyelination, signs of secondary axonal degeneration

Table 25.2 Anti-glycolipid antibodies in different GBS variants

Variants	Anti-glycolipid antibody
Fisher syndrome	GQ1b/GT1a
AMAN	GD1a, GM1, GM1b, GalNAc-GD1a
AIDP	GM1, Asialo-GM1, GD1b, GM2, LM1, GD2, GalC, Forssman antigen
Sensory ataxic variants	GD1b and structurally related gangliosides

can be observed. Another pathological hallmark is the presence of inflammatory infiltrates, especially in the spinal roots and proximal nerves, which contain T-lymphocytes and macrophages (Asbury et al. 1969; Prineas 1981). Macrophage-mediated myelin stripping (ingestion/breakdown) is characteristic. Lymphocytic infiltration can be minimal, however, sometimes it may not occur; moreover, localization may differ markedly (Cornblath et al. 1990; Honavar et al. 1991). Inflammatory infiltrates have also been detected in the spinal cord of GBS patients, indicating that there may be subclinical inflammation of CNS structures (Muller et al. 2003). In some cases of AIDP there is breakdown of blood-nerve barrier with deposition of activated complement products on Schwann cells, suggesting a role for antibody-mediated immune injury (Hafer-Macko et al. 1996b). These pathological observations implicate T-cells or autoimmune antibodies in inducing peripheral nerve demyelination in individual cases. Further, T-cell and antibody-mediated immune injury can predominate or act synergistically in an individual case.

Axonal variants: Unlike AIDP, axonal variants of GBS show primary axonal injury without substantial T-cell inflammation or demyelination (Griffin et al. 1995, 1996a). In AMAN, axonal degeneration predominantly involves the motor axons, whereas in AMSAN sensory axons are also involved. It has been postulated that AMAN and AMSAN represent a pathologic spectrum and that AMSAN actually represents a more severe form of AMAN.

In AMAN, deposition of IgG and complement can be detected on the nodal and internodal axolemma (Hafer-Macko et al. 1996a). Macrophages assume close association with the axons at the nodes and these cells then extend into inter-nodal regions where they surround axons, without disturbing/disrupting the overlying myelin, eventually leading to axon degeneration (Griffin et al. 1995, 1996a). The findings of IgG deposition on axolemma and periaxonal location of macrophages strongly imply that the potential target antigen(s) are expressed on the surface of (motor) axons. Current pathogenetic concepts assume that antibody binding to motor axolemma leads to activation of complement, recruitment of macrophages, and subsequent degeneration of axons.

Pathologic examination of early cases has shown that at the onset the pathological alterations in AMAN are mainly restricted to the nodes of Ranvier of motor fibers in the ventral roots (Griffin et al. 1996b). In these cases nerve fibers still appear normal except that the nodal gap is lengthened. It is believed that this nodal lengthening is sufficient to cause failure of transmission of nerve impulses and profound clinical weakness. Motor nerve terminals are another site susceptible to injury in AMAN, as indicated by degeneration of intramuscular nerves on muscle biopsy (Ho et al. 1997). These observations suggest that nodes of Ranvier and motor nerve terminals are two sites that are susceptible to injury in AMAN.

In summary, the pathological and immunopathological findings in AMAN suggest that antibody-mediated injury directed against axonal antigens plays a prominent role in the pathogenesis of this disorder.

25.1.6 Treatment

- (a) *Supportive care* is the mainstay of medical management in patients with GBS, despite the availability of immunomodulating therapies like plasma exchange and (PE) and intravenous immunoglobulins (IVIg). All patients with GBS should be admitted to a hospital with an ICU experienced in GBS care. The major risks are complications arising from weakness of respiratory and bulbar muscles and autonomic instability. The principles of supportive management include respiratory support in patients with respiratory failure, monitoring and management of dysautonomia, measures to prevent nosocomial infections and complications of immobility, and pain management.
- (b) Immunomodulatory therapies controlled clinical trials have shown that IVIg and PE are beneficial immunomodulatory treatments in GBS in populations where AIDP is the predominant form of the disease (Hughes et al. 2012; Hughes and van Doorn 2012). These trials indicate that treatment with PE or IVIg should be considered for all nonambulatory adult patients with AIDP. IVIg is now the first line of treatment for most patients because it can be administered easily and patient acceptance is high. Immunomodulatory treatments should begin as soon as possible after the onset of symptoms to obtain maximal benefit and limit the extent of nerve injury. There are no controlled studies of immunomodulatory therapy in the FS and primary axonal variants of GBS, but anecdotal reports indicate that both PE and IVIg are beneficial. There is no evidence that corticosteroids are beneficial for treatment of GBS (Hughes and van Doorn 2012). The current data indicate that the use of multiple immunomodulatory treatment modalities (PE followed by IVIg) is not superior to single therapy (PE or IVIg) for the treatment of GBS.

The precise mechanism(s) of action of PE and IVIg in GBS remain uncertain. PE removes a number of potentially pathogenic circulating factors including autoimmune antibodies, cytokines and complement, and can alter lymphocyte activation (Lehmann and Hartung 2011). IVIg is purported to neutralize and inhibit production of autoantibodies, suppress antibody dependent cellular cytotoxicity, decrease natural killer cell function, down-regulate proinflammatory cytokines, and interfere with complement activation (Dalakas 2002; Ritter et al. 2014). It is also proposed

that IVIg induces increased catabolism of immunoglobulins including autoantibodies by saturating FcRn transport receptors (Berger et al. 2013).

25.2 Acute Inflammatory Demyelinating Polyradiculoneuropathy (AIDP)

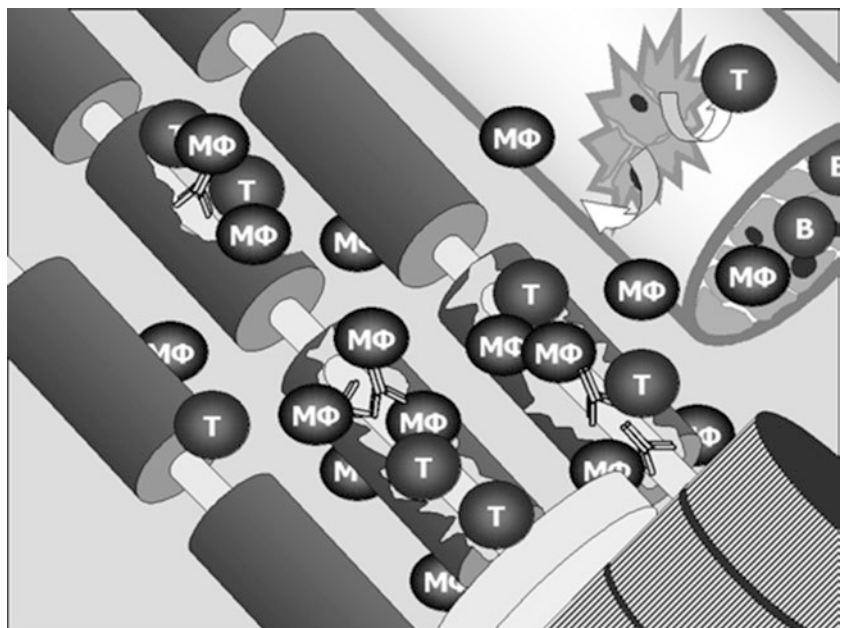
Most of our understandings about the cellular mechanisms in the pathogenesis of AIDP have been gained by studies of the experimental allergic/autoimmune neuritis (EAN). In particular, the important role of T-cells in the pathogenesis of EAN has paved the way for the development of hypothetical models for human AIDP (Fig. 25.1). EAN can be induced in different species with whole peripheral nerve tissues or neuritogenic epitopes of peripheral nerve proteins P0, P2, and PMP22 (Hughes et al. 1999). A modified model is the adoptive transfer EAN, where specific T-cell lines against P0 or P2 are passively transferred to the animals induce neuropathy. EAN is a monophasic disease; its severity is associated with cell or antigen dosage (Hartung et al. 1996b). Depletion of T-cells by thymectomy or antibodies against T-cells can protect against EAN, indicating that disease induction is dependent on the presence of T-cells (Holmdahl et al. 1985). Further, a normal function of T cells (Holmdahl et al. 1985; Hartung et al. 1987; Jung et al. 1992) and the presence of costimulatory molecules B7.1/CD80 or B7.2/CD86 and CTLA4/CD28 (Kiefer et al. 2000; Zhu et al. 2001a, b) are required for inducing and supporting EAN, which emphasizes the important role of T-cells as mediators for experimental neuritis.

T cell activation in EAN is accompanied by expression of inflammatory cytokines such as IFN- γ , TNF- α , TNF- β , IL-6 and IL-12 (Zhu et al. 1994, 1997). In sera and CSF of GBS, inflammatory cytokines detected include IFN- γ , TNF- α and IL-2, which are increased in patients and partially correlate with disease activity (Taylor and Hughes 1989; Hartung and Toyka 1990; Hartung et al. 1991; Exley et al. 1994; Creange et al. 1996; Sainaghi et al. 2010). In contrast, levels of TGF- β 1, a pleiotrophic cytokine, are lower in GBS patients than in controls (Creange et al. 1998).

Systemic immune activation in AIDP is reflected by an increased number of activated T-cells circulating in the peripheral blood during the early disease course (Taylor and Hughes 1989). Many studies to identify the putative antigen of a specific T cell response in AIDP provided conflicting results. Some studies postulated that peptides from P0 and P2 might be the epitope of specific T-cell lines in GBS, but most of them failed to obtain disease specific T-cell responses against neural antigens (Makowska et al. 2008; Csurhes et al. 2005; Pette et al. 1994). The CD4/CD8 ratios of T-cell populations detected in the peripheral nerves in AIDP closely resemble those in the peripheral blood. Of those, $\alpha\beta$ T-cells constitutes most of the nerve infiltrates (Cornblath et al. 1990). The usage of V β 15 T-cell receptor gene suggest the specific T-cell activation by a common antigen or superantigen (Khalili-Shirazi et al. 1997).

In the normal human immune repertoire there are far fewer T-cells that express the $\gamma\delta$ T-cell receptors than those expressing $\alpha\beta$ T-cell receptors. Unlike $\alpha\beta$ T-cells they do not recognize antigenic peptides in the context of MHC molecules and these cells are mainly localized in epithelial barrier

Fig. 25.1 Hypothetical scheme of the immune response in acute inflammatory demyelinating neuropathy (AIDP): Inflammatory cells migrate from the systemic immune compartment through the damaged blood-nerve barrier into the endoneurium. Inflammatory infiltrates, which contain T-lymphocytes and macrophages cause marked segmental demyelination and secondary axonal degeneration (*B* B-cell, *T* T-cell, *M ϕ* macrophage)



tissue. Interestingly, $\gamma\delta$ T-cells were isolated from sural nerve biopsies of GBS patients (Winer et al. 2002). A specific role of those $\gamma\delta$ T-cells has been postulated, as they may recognize non-protein antigens. Therefore, they could initiate an immune response to potential carbohydrate and glycolipid antigens (Winer et al. 2002). The proliferation of $\gamma\delta$ T-cells in response to *C. jejuni* lysates has been shown. Nonetheless, $\alpha\beta$ T-cells or stimulatory cytokines such as IL2 are required for expansion of $\gamma\delta$ T-cells (Van Rhijn et al. 2003). These studies suggest that T-cell subtypes with specificity for peptide and glycolipid antigens can invade the nerves in AIDP.

A crucial step during the process of inflammation is the migration of activated T-cells from the systemic immune compartment to the sites of inflammation. Like the CNS, the peripheral nerve represents an immunologically privileged environment, protected by an intact blood-nerve barrier. In EAN, the breakdown of the blood-nerve barrier is one of the earliest pathological changes that can be found. The upregulation of specific endothelium-binding molecules on T-cells and their specific ligands on vascular endothelium precedes the migration of T-cells through the blood-nerve barrier. E-Selectins binding sialyl Lewis antigens (Hartung et al. 2002) and VCAM-1/ICAM-1, are both upregulated in GBS and EAN (Enders et al. 1998; Creange et al. 2001). EAN can be partially inhibited by blocking VCAM-1 or its ligand VLA-4/a4b1 integrin (Enders et al. 1998). E-selectins are released into the blood and can be found in elevated levels in GBS patients. Chemokines, which exert chemotactic effects on leucocytes by binding to their specific receptor, contribute to the migration of inflammatory cells (Baggiolini 1998; Campbell et al. 1998). The differential expression of chemokines and their receptors has been studied in detail in sural nerves of patients with AIDP (Kieseier et al. 2002). Of those, specific upregulation of CCR-2 and CCR-4 were detected in invading T-cells, whereas endothelial cells expressing the chemokine IP-10 were identified (Kieseier et al. 2002).

Matrix metalloproteinases (MMPs), a heterogeneous group of zinc-dependent endopeptidases, are assumed to contribute to the structural breakdown of the blood-brain barrier, extravasation of leukocytes, and direct demyelination. In EAN, MMP9 and MMP7 are upregulated during early disease course and correlate with disease severity (Kieseier et al. 1998). Further, the administration of MMP inhibitors may attenuate the disease (Redford et al. 1997b). Human CSF samples from patients with GBS show increased gelatinase B activity (Creange et al. 1999). In sural nerve biopsies from GBS patients, augmented proteolytic activity for gelatinase B as well as increased mRNA expression for gelatinase B and matrilysin were detected (Kieseier et al. 1998). MMPs in the inflamed peripheral nervous system (PNS) appear not only to promote inflammation, but may also play a role in nerve repair. For example, in EAN, matrilysin remained upregulated throughout the clinical recovery

phase, implicating a possible role of this metalloproteinase in restoring the integrity of the PNS (Hughes et al. 1998). Schwann cell derived MMP-2 can also be found in the CSF of GBS patients and has been shown to promote myelination in vitro (Lehmann et al. 2009).

Because macrophages represent the major cell population in infiltrates of affected nerves, they are considered to be key mediators of injury to myelin, Schwann cells, and axons. This hypothesis is reinforced by experimental studies, where depletion of macrophages prevents the development of EAN. Although endoneurial macrophages are present in low frequency in the normal peripheral nerve, most of them migrate through the blood-nerve barrier during the process of inflammation (Hartung et al. 2002). Macrophages may exert their pathological effects by release of inflammatory mediators. Of those, MMPs, TNF α , nitric oxide, and others have been postulated to mediate neurotoxicity (Redford et al. 1997a, b; Hartung et al. 2002; Lehmann et al. 2007a). Besides direct harmful effects to nerve fibres, macrophages also perpetuate the inflammatory process by antigen-presenting to T-cells. It is assumed that macrophages also attack Schwann cells in AIDP (Prineas 1981; Hartung et al. 1996a; Hartung and Kieseier 1999) by antibody-mediated cytotoxicity and activation of complement (Hafer-Macko et al. 1996b). Another mechanism for Schwann cell injury/demyelination is via CD8+ T-cells, which are cytolytic T-cells that mediate cytotoxic effects by release of perforin and granzymes. CD8+ T-cells can be detected in postmortem tissue of GBS patients with prolonged disease course (Wanschitz et al. 2003). These results point to an additional direct role of T cell-mediated cytotoxicity in AIDP.

There is a growing interest in the role of humoral factor induced demyelination in AIDP. In demyelinating cases from China, complement activation markers were found on the abaxonal Schwann cell surface. They were associated with vesicular demyelination (Hafer-Macko et al. 1996b) and closely resemble the experimental nerve fiber demyelination induced by anti-galactocerebroside (a glycosphingolipid enriched in myelin) antibody in the presence of complement (Saida et al. 1979). It is likely that in these cases, the antibody and complement are directly involved in targeting the Schwann cell and myelin, and the role of T-cells may be to open the blood-nerve barrier (Spies et al. 1995a, b). This concept is supported by the observations that disease severity in models of adoptive transfer-EAN is enhanced by transferring antibodies that recognise myelin or Schwann cell epitopes (Spies et al. 1995a; Hahn et al. 1993). Several clinical and experimental observations also support a role for antibody-mediated mechanisms, including the response to plasmapheresis, the presence of anti-myelin and anti-glycoconjugate antibodies (reviewed in (Hughes et al. 1999)), and the ability of AIDP sera to induce demyelination after intraneural injection. Anti-ganglioside antibodies of

various specificities have been described in AIDP but their pathogenic role is not accepted because of lack of experimental models demonstrating their demyelinating activity. Antibodies against GM1, GD1b, asialo-GM1, the Gal(β 1–3)GalNAc epitope, GM2, LM2, and GT1b (reviewed in (Hughes et al. 1999; Willison and Yuki 2002) have been described, but antibodies to these individual gangliosides are not routinely detected. The observation that adjacent gangliosides in the plasma membrane can form conformational epitopes that are recognized by autoantibodies (anti ganglioside complex antibodies) has raised the possibility that newly formed complex epitopes may be targeted in AIDP as well. By use of glycoarrays the Willison lab could demonstrate that antibodies against various glycolipid complexes are present in more than 60 % of patients with AIDP (Rinaldi et al. 2013).

More recently antibodies against nodal proteins have been implicated to play a role in the pathogenesis of AIDP. In EAN, antibodies against two nodal proteins, gliomedin and neurofascin can be detected, supporting a role for those proteins as immune targets in this model (Lonigro and Devaux 2009). In about 5 % of patients with AIDP antibodies against neurofascin can be detected in the serum and monoclonal anti-neurofascin antibodies aggravate the disease course of EAN when passively transferred (Ng et al. 2012). Those studies testify substantial progress that has been made in understanding of the cellular and humoral mechanisms that might underlie nerve fibre injury in AIDP; however, definition of target antigens and how the undesirable processes of inflammation, myelin degradation, and subsequent axonal damage are initiated remain unclear.

25.2.1 Acute Motor Axonal Neuropathy (AMAN)

Unlike for AIDP there are almost no clinical or experimental data that have systematically examined the role of cellular immunity in AMAN. Clinical studies over the last 15 years show that patients with AMAN have specific antibodies against two major gangliosides, GM1 and GD1a, and two minor gangliosides, GalNAc-GD1a and GM1b in the peripheral nerves (Figs. 25.2 and 25.3a) (Rees et al. 1995a; Jacobs et al. 1996; Hadden et al. 1998; Ho et al. 1999; Ogawara et al. 2000; Yuki et al. 1993b; Kusunoki et al. 1994, 1996). Most of the data originates from Japan and northern China, where the AMAN form of the disease and preceding *C. jejuni* infections are frequent. Anti-GM1 antibodies have been reported in up to 50 % of Japanese patients with AMAN (Ogawara et al. 2000). Anti-GD1a antibodies are present in up to 60 % of patients with AMAN compared to 4 % in AIDP in northern China (Ho et al. 1999). The frequency of anti-GalNAc-GD1a and -GM1b in motor-predominant GBS is

about 10–15 % (Kusunoki et al. 1994, 1996; Yuki et al. 1999). Anti-ganglioside antibodies in AMAN are mostly IgG isotype and are commonly IgG1 and IgG3 (Willison and Veitch 1994; Ogino et al. 1995), which are complement-fixing subtypes in humans. These anti-ganglioside antibodies are oligoclonal or polyclonal and can have a broad range of crossreactivity. In AMAN, the differences of antibody specificity in different populations are not well understood. There are some data to suggest differences in immunogenetic repertoire, and geography may affect the specificity and isotype distribution of anti-ganglioside antibodies in various populations (Ogawara et al. 2000; Ang et al. 2001).

Apart from antibodies against individual gangliosides there is also evidence that in some GBS patients, antibodies are present that recognize conformational epitopes that are formed by two neighbouring gangliosides (ganglioside complexes). Around 8–17 % of GBS patients have antibodies that target epitopes on GD1a/GD1b, GM1/GD1a, GD1b/GT1b, GM1/GT1b, or GM1/GD1b complexes. Those patients can have predominant axonal or demyelinating damage but tend to have more often antecedent gastrointestinal infection and poorer prognosis than patients without those antibodies (Kusunoki and Kaida 2011; Kaida et al. 2004, 2007).

A brief discussion of peripheral nerve gangliosides is necessary for understanding the pathogenetic effects of anti-ganglioside antibodies. Gangliosides, the target antigens of anti-ganglioside antibodies, are sialic acid-containing glycolipids enriched in the mammalian nervous system. They contain one or more sialic acids linked to an oligosaccharide chain of variable length and complexity, which is attached to ceramide lipid anchor (Kolter et al. 2002). The ceramide portion of gangliosides anchors them in plasma membranes and glycan moieties are expressed on cellular/axonal surfaces. This organization allows anti-ganglioside antibodies to bind to glycan moieties on cell surfaces. The most abundant gangliosides in the adult mammalian nervous system are GM1, GD1a, GD1b, and GT1b; in peripheral nerves LM1 ganglioside is also enriched, particularly in myelin (Yu and Saito 1989; Svennerholm et al. 1992, 1994; Ogawa-Goto et al. 1990). Complex gangliosides are more concentrated in axolemmal fractions; GM1 is enriched in both axons and myelin (Yu and Saito 1989). Immunolocalization studies indicate that in normal rodents and humans, all complex gangliosides including GM1 and GD1a reside in axons, and GM1 is also found in paranodal Schwann cells, but compact myelin in internodal segments is difficult to stain with anti-ganglioside antibodies or toxins (Ganser et al. 1983; Sheikh et al. 1999; Gong et al. 2002). Minor gangliosides GalNAc-GD1a and GM1b are expressed in peripheral nerves and one study suggests that GalNAc-GD1a may only be expressed by motor neurons and nerve fibers (Ilyas et al. 1988; Yoshino 1997). In summary, it is important to emphasize that GM1 and GD1a gangliosides are present at the nodes of Ranvier and motor

Fig. 25.2 Proposed pathogenesis in acute motor axonal neuropathy (AMAN): In AMAN there is primary axonal injury without T-cell inflammation and demyelination. Deposition of autoantibodies and complement on the axolemma is followed by structural axonal injury or alteration of axon conduction. Macrophages within the periaxonal space contribute to the axonal damage (*B* B-cell, *Mφ* macrophage, *C5b-9* complement factors)

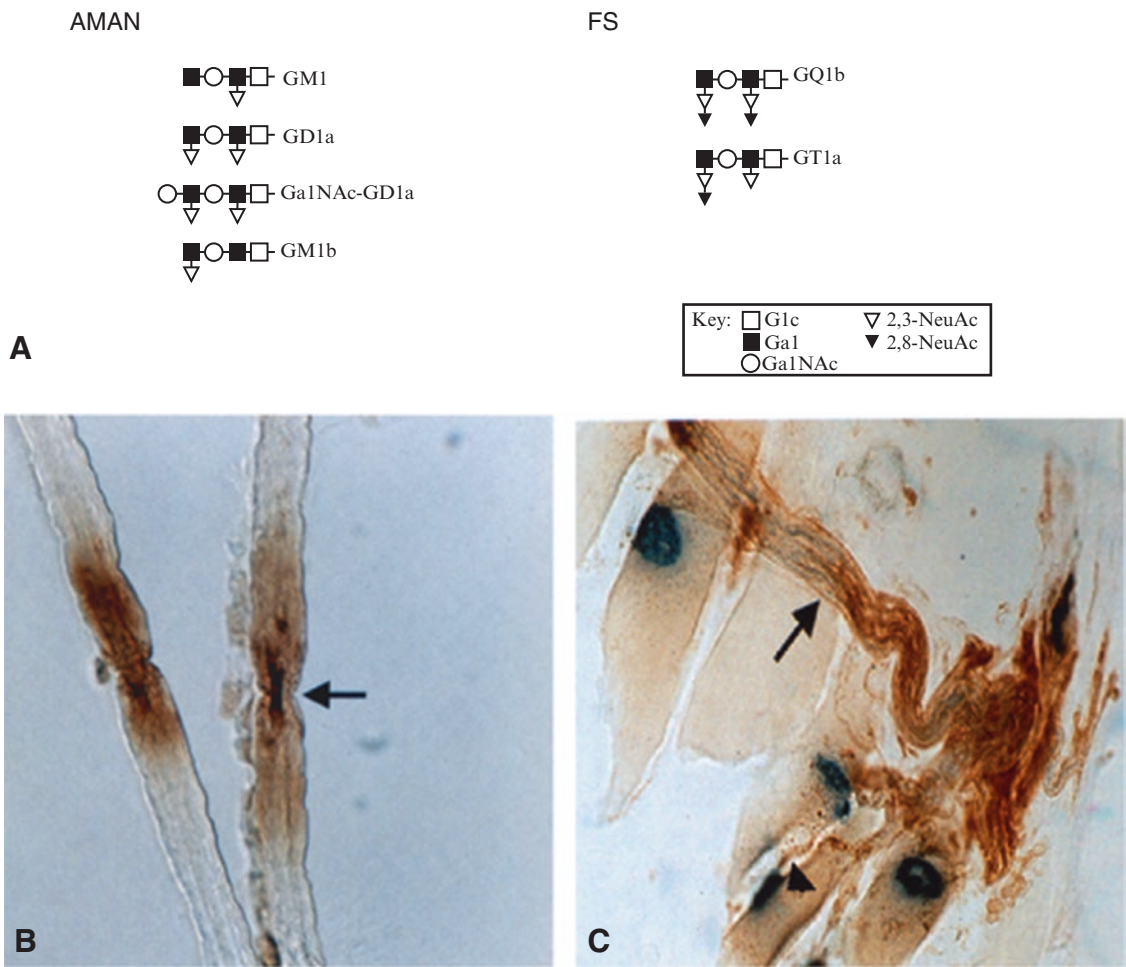
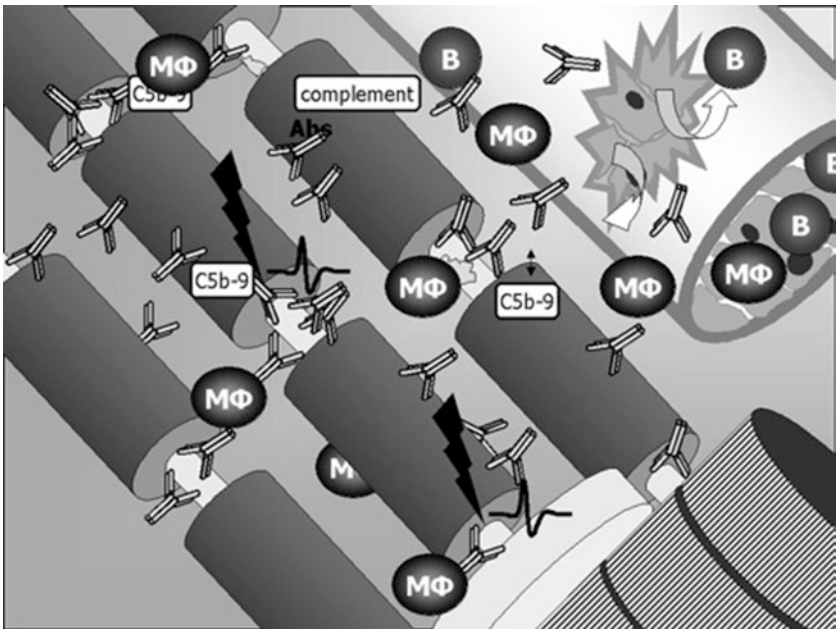


Fig. 25.3 (a) Schematic diagrams showing the glycan structures of gangliosides implicated as target antigens in AMAN and FS. (b) Teased nerve fibers showing GM1 staining at node of Ranvier (arrow) and paranodal Schwann cells. (c) GM1 is also localized to intramuscular

nerve (arrow) and motor nerve terminal (arrowhead); blue staining marks motor end plates (adapted with permission from Sheikh et al. 1999) (See color plates)

nerve terminals: two sites along the motor nerve fibres reported to be involved in patients with AMAN. Distribution of GM1 gangliosides at nodes of Ranvier and motor nerve terminals is shown as an example in Fig. 25.3b, c.

Several anatomical and physiological features make nodes of Ranvier and motor nerve terminals susceptible to antibody-mediated nerve injury: (1) most complex gangliosides are concentrated at the nodes and motor nerve terminals; (2) in myelinated fibers, axonal target antigens are exposed at these sites; (3) sodium and potassium ion channels are clustered at the nodes and disruption of their function can disrupt impulse conduction; (4) structural integrity of the node of Ranvier is critical for nerve fiber conduction; (5) motor nerve terminals are enriched in sodium and calcium channels and disruption of their functions can affect distal impulse conduction or neurotransmitter release at the neuromuscular junctions, respectively. Further, multiple studies indicate that gangliosides are concentrated in microdomains called lipid rafts that are specialized for cell signaling and that modulation of these lipid raft gangliosides can modulate receptor function, including ion channel function.

With this background information, we outline the experimental data that supports the notion that anti-ganglioside antibodies can induce nerve fiber injury in animal models and briefly review the effects of these antibodies on nodes of Ranvier and motor nerve terminals. There are several animal models that provide 'proof of concept' that anti-ganglioside antibodies with -GM1 and -GD1a specificities can induce neuropathy. First, Yuki and colleagues used GM1 ganglioside or *C. jejuni* lipopolysaccharide (LPS) with a mixture of keyhole limpet hemocyanin and complete Freund's adjuvant for repeated immunizations in rabbits to induce high titres of anti-GM1 antibodies, clinical paralysis, and electrophysiological and pathological evidence of motor axon injury (Yuki et al. 2001, 2004). The pathological and immunopathological studies in these animals showed features similar to that of human disease, i.e., deposition of IgG on motor axons and the presence of periaxonal macrophages. Further, analysis of animals shortly after the onset of clinical weakness showed only lengthening of the nodes of Ranvier without morphological features of axon degeneration (Susuki et al. 2003). We took an alternative approach to induce neuropathy in mice with anti-GD1a antibodies. This included generation of monoclonal anti-GD1a antibodies (Lunn et al. 2000) and implantation of antibody-secreting hybridoma in mice (Sheikh et al. 2004). Animals implanted with hybridoma develop high titres of anti-GD1a antibodies and axonal neuropathy in peripheral nerves and motor nerve terminals. Our studies showed that hybridoma implantation in transgenic mice lacking complex gangliosides (including GD1a) did not induce neuropathy despite high circulating levels of antibodies, confirming that gangliosides are indeed the target antigens of anti-ganglioside antibodies in this model (Sheikh

et al. 2004). Critical for anti-ganglioside antibody mediated injury to peripheral nerve fibers is the breakdown of the blood-nerve-barrier. In a recent study it was demonstrated that mechanical disruption of the blood-nerve-barrier by lumbar (L5) spinal nerve transection allowed sufficient access of systemically transferred anti-ganglioside antibodies to peripheral nerve endoneurial compartment (He et al. 2015). Moreover, transgenic mice that lack components of the innate immunity such as complement or FcγRs were used in this study to show that anti-ganglioside antibody-mediated injury to intact nerve fibers requires formation of immune complexes on the nerves and this then interact with activating FcγRs on macrophages to cause node of Ranvier and axonal injury (He et al. 2015).

Apart from direct nerve injury, antibodies against GD1a can also interfere with the regeneration of injured nerve fibres. Monoclonal mouse and polyclonal human anti-GD1a-antibodies were found to inhibit axonal regrowth after injury in vivo and in vitro, thereby interfering with the RhoA pathway (Lehmann et al. 2007c; Lopez et al. 2010; Zhang et al. 2011).

The pathophysiologic effects of anti-ganglioside antibodies on nodes of Ranvier have been examined both in vitro and in animal models. Most of the studies have used anti-GM1 antibodies derived from patients with acute and chronic immune neuropathies or produced experimentally. It has been reported that intraneural injections of sera containing anti-GM1 can induce acute conduction block (Santoro et al. 1992; Uncini et al. 1993). The immunization of rabbits with anti-GM1-antibodies can induce marked changes in the molecular structure of the nodes of Ranvier that include disruption of Na(v) channels clusters (Susuki et al. 2007). Deposition of IgG and complement suggest antibody mediated damage not only at the nodes itself, but also at the paranodal axoglial junctions and Schwann cell microvilli which are required for stabilization of Na(v) channel clusters (Susuki et al. 2007).

Takigawa and colleagues examined the effects of anti-GM1 sera on ion channel function at the nodes of Ranvier by using a voltage clamp technique on isolated myelinated fibers. They found that in the presence of complement these antibodies induced irreversible decreases in sodium currents and eventual blockade of the channels (Takigawa et al. 1995, 2000). Nonetheless, other investigators have been unable to reproduce these findings (Harvey et al. 1995; Hirota et al. 1997). Possible explanations include differences in experimental paradigms, including sources of antibodies, and affinity and fine specificity of these antibodies. Overall, these studies support the concept that anti-ganglioside antibodies can alter the nodal architecture and directly modulate ion channel function at the nodes of Ranvier. The effects of anti-ganglioside antibody-mediated injury on motor nerve terminals have been extensively examined by in vitro phrenic nerve-diaphragm preparations. Current concepts indicate that

anti-ganglioside antibodies can exert immunopharmacological effects on motor nerve terminal physiology and that they can also induce complement-dependent cytotoxic injury to motor nerve terminals. We have previously examined the effects of IgG anti-GM1 and -GD1a antibodies on motor nerve terminals by a perfused macro-patch clamp model with Buchwald's group. Our studies indicate that anti-GM1 and -GD1a antibodies depressed the evoked quantal release (Buchwald et al. 2007). This blockade was reversible or partially reversible after washout of these antibodies and did not require complement. Since calcium channels are critical in evoked quantal release, we examined the effects of these antibodies on depolarization-induced calcium influx by calcium imaging. These studies indicate that anti-GM1 and -GD1a antibodies significantly decrease depolarization-induced calcium influx, suggesting that antibody binding to gangliosides in the presynaptic motor terminals alters calcium channel function (Buchwald et al. 2007). Work done by others has characterized the complement-dependent pathophysiologic effects of anti-GD1a antibodies on motor nerve terminals in ex vivo hemidiaphragm preparations (Goodfellow et al. 2005). They showed that dense antibody and complement deposits develop over presynaptic motor axons, accompanied by severe ultrastructural damage and electrophysiological blockade of motor nerve terminal function. This pathophysiologic effect, however, required high density expression of GD1a ganglioside (Goodfellow et al. 2005).

In summary, experimental data indicate that active immunization with GM1 ganglioside or *C. jejuni* LPS can reproduce pathophysiological features of AMAN in a rabbit model. Passive transfer with an anti-GD1a monoclonal antibody (by hybridoma implantation) also reproduced axon and motor nerve terminal degeneration in a mouse model. Anti-GM1 and -GD1 antibodies can alter the nodal and motor nerve terminal function and architecture. Tissue and cell culture studies indicate that sodium and calcium ion channel function can be modulated by these antibodies at nodes of Ranvier and presynaptic motor nerve terminals, respectively. These antibodies have both complement-independent immunopharmacologic and complement-dependent cytotoxic effects. The detailed subcellular and molecular effects of these antibodies on motor axons remain to be elucidated. Preferential susceptibility of motor axons to anti-GM1 and -GD1a antibody-mediated injury in AMAN is another fundamental issue that remains unresolved, because biochemical studies indicate that both motor and sensory nerve fibres have similar levels of ganglioside expression (Gong et al. 2002). Despite these and other gaps in our knowledge about the pathogenetic sequence of this disorder, current data support the hypothesis that anti-ganglioside antibodies can induce pathophysiological effects on intact motor nerve fibres.

25.2.2 Fisher Syndrome

Several studies have indicated that anti-GQ1b antibodies are present in more than 80 % of the cases with FS (Chiba et al. 1992; Willison et al. 1993; Yuki et al. 1993a). Preceding *C. jejuni* infection is not uncommon in this disease. Anti-GQ1b antibodies in patients with FS can be IgA, IgM, or IgG isotype but the IgG response is most robust and persistent. These IgG antibodies are of complement-fixing isotypes, similar to those in AMAN. Studies indicate that anti-GQ1b antibodies commonly cross-react with a structurally related ganglioside GT1a (Fig. 25.3a) (Chiba et al. 1993; Ilyas et al. 1998). Biochemical and immunolocalization studies have mapped the distribution of GQ1b ganglioside in the peripheral nerves: anti-GQ1b antibodies bind to paranodal myelin and nodes of Ranvier, and neuromuscular junctions (NMJs) in extraocular and somatic muscles, and it has been shown that the GQ1b is twice as frequently expressed in the extraocular cranial nerves than in other cranial and somatic peripheral nerves (Chiba et al. 1993, 1997). Pathogenetic studies indicate that anti-GQ1b antibodies bind at the nodes of Ranvier but are unable to induce acute conduction failure (Paparonas et al. 1999). This experimental result has led to the notion that antibody-mediated conduction failure at the levels of the nodes of Ranvier may be less important in the pathogenesis of FS. Because extraocular muscles are paralyzed in FS, anti-GQ1b antibodies do not affect nodal function, and anti-GQ1b antibodies bind to NMJs, experimental approaches have focused on pathophysiologic effects of anti-GQ1b antibodies on NMJs to model ophthalmoplegia seen in FS. In this regard phrenic nerve hemi-diaphragm preparations have been used extensively to study the pathological effects of anti-GQ1b antibodies.

Investigators have examined the effects of FS sera, FS IgG, or human anti-GQ1b antibodies on NMJs and showed that these antibodies bind to NMJs, cause massive quantal release of acetylcholine from nerve terminals and eventually block neuromuscular transmission, primarily through pre-synaptic mechanisms. These pathophysiologic effects were complement-dependent and resembled the effects of paralytic neurotoxin alpha-latrotoxin (Plomp et al. 1999). Willison and colleagues have shown that these antibodies induce degeneration of preterminal motor axons both by direct cytotoxicity and indirectly through damage to peri-synaptic Schwann cells at NMJs (Halstead et al. 2004, 2005; O'Hanlon et al. 2001). In contrast, Buchwald et al. (1995, 1998, 2001), reported complement-independent immunopharmacological effects of IgG fractions from both GQ1b-positive and -negative FS on NMJs. They showed that these IgG fractions blocked release of evoked acetylcholine and depressed the amplitude of postsynaptic potentials, implicating both a pre- and postsynaptic blocking effect. This effect was reversible. That the antibody-mediated pathophysiological effects at

NMJs may be relevant to clinical disease is supported by an electrophysiological study suggesting that this site is affected in some cases with FS (Uncini and Lugaresi 1999). In a mouse model, the intraperitoneal injection of anti-GQ1b antibodies can cause complement-dependent respiratory paralysis due to transmission block at diaphragm NMJs. This effect can be reversed by application of complement-inhibiting compounds, including eculizumab (Halstead et al. 2008a, b).

25.3 Molecular Mimicry Hypothesis

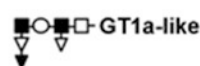
In this section we consider microbial agents that commonly cause infections preceding GBS and are reported to carry antigens crossreactive with peripheral nerves. The demonstration of peripheral nerve-crossreactive epitopes in these microbes is the basis for the hypothesis of post-infectious molecular mimicry in GBS.

In this regard *Campylobacter jejuni* (*C. jejuni*) is the best characterized, and stringent data exist that demonstrate the presence of ganglioside-like antigens in these organisms. Recent studies have also implicated *Haemophilus influenzae* (*H. influenzae*), *Mycoplasma pneumoniae* (*M. pneumoniae*), and *Cytomegalovirus* (CMV) as microbes carrying peripheral nerve-crossreactive epitopes. A common theme with these microbes is that they express carbohydrate epitopes that mimic glycolipid antigens in peripheral nerves, and autoimmunity against these epitopes is manifested as anti-carbohydrate antibodies. Most of the discussion highlights molecular mimicry in *C. jejuni*. A brief discussion on *H. influenzae*, *M. pneumoniae*, and CMV is also included. Glycans mimicking peripheral nerve glycolipids expressed by these microbes are shown in Fig. 25.4.

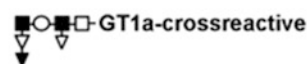
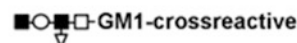
25.3.1 *Campylobacter jejuni*

C. jejuni is a gram-negative rod that is one of the most common causes of bacterial gastroenteritis worldwide (Hughes and Rees 1997; Friedman et al. 2000; Oberhelman and Taylor 2000). *Campylobacter* infections in US are mostly sporadic and are associated with ingestion of improperly handled or cooked food, particularly poultry products. In northern China contaminated well water was reported as a mode of transmission (McKhann et al. 1993). *C. jejuni* gastroenteritis is reported to be the most frequently recognized event preceding AMAN and other variants of GBS (reviewed in (Hughes and Rees 1997)). In GBS cases, both stool culture and serologic methods are used to diagnose *Campylobacter* infection, because by the time neurological symptoms develop, the yield of *C. jejuni* from stool culture is relatively low (Nachamkin 1997). Association of GBS with preceding *C. jejuni* infection was noted in early 1980s

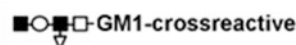
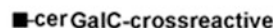
Campylobacter jejuni



Haemophilus Influenzae



Mycoplasma pneumoniae



CMV

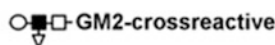


Fig. 25.4 Glycan structures (mimics) expressed by different microbes that are invoked in molecular mimicry. The term “like” is used when glycan structure has been determined by biochemical/mass spectrometry studies and term “crossreactive” is used when evidence for glycan structure is indirect and based on antibody inhibition or binding studies

(Rhodes and Tattersfield 1982). Studies indicate that in patients with GBS, the incidence of preceding *C. jejuni* infection varies widely, ranging from 4% in North America to 74% in northern China (Hughes and Rees 1997; Ho et al. 1999), with an overall prevalence estimated around 30% (reviewed in (Moran et al. 2002)).

Ganglioside-like moieties in *Campylobacter* are contained in its lipopolysaccharide (LPS). *C. jejuni* LPS consists of three components: lipid A, the hydrophobic region inserted into the membrane, an oligosaccharide core divided into inner and outer parts, and capsular polysaccharides also called O-chains. Lipooligosaccharide (LOS) is differentiated from LPS by lack of O-chains. In *C. jejuni* it is the oligosaccharide core region that carries ganglioside-like moieties. Since 1990 a large number of studies have characterized the

core regions of LPS/LOS of GBS- and enteritis-associated *C. jejuni* strains. These studies demonstrate that different strains or serotypes of *C. jejuni* LPS/LOS contain several ganglioside-like molecules, including GM1-, GD1a-, GalNAc-GD1a-, GM1b-, GT1a-, GD2-, GD3-, and GM2-like structures (Yuki et al. 1992; Aspinall et al. 1993, 1994a, b; Sheikh et al. 1998; Nachamkin et al. 2002). Biochemical/structural studies with mass spectrometry have failed to demonstrate a GQ1b-like structure, target antigen for FS, but antibody binding assays with human or murine monoclonal antibodies did show the presence of GQ1b- and GT1a-cross-reactive moieties in *C. jejuni* LPS/LOS (Jacobs et al. 1995, 1997; Yuki et al. 1994). Figure 25.4 shows the ganglioside-like moieties in *C. jejuni* LOS that are implicated in AMAN and FS. Recent studies show that *Campylobacter* has genetic machinery for LOS biosynthesis with relevant genes clustering in a locus. It has been reported that specific polymorphisms in a sialyltransferase gene are associated with preferential expression of GM1- and GD1a- or GQ1b-like moieties. Based on this observation it has been postulated that these genetic polymorphisms determine the specificity of anti-ganglioside antibody response (anti-GM1 and -GD1a or -GQ1b) and clinical phenotype (AMAN or FS).

Despite the relationship of preceding *Campylobacter* infection, we emphasize that GBS is a very rare complication after *C. jejuni* infection: it is estimated that 1 in 1000 cases of *Campylobacter* infection is complicated by GBS (Allos 1997). Because GBS follows very rarely after *C. jejuni* infection, investigators have examined *Campylobacter* and host-factors that may lead to this complication. Thus, *Campylobacter* strains isolated from patients with GBS and enteritis have been characterized for ganglioside mimicry. It is known that both GBS- and diarrhea-associated isolates carry ganglioside-like moieties and associated synthetic genes, but GBS-related organisms are more likely to do so (Nachamkin et al. 1999, 2002). Notably, studies indicate that expression of ganglioside-like structures in LOS, even when accounting for increased probability of synthetic machinery for ganglioside-like moieties in GBS-associated isolates, is not sufficient by itself to explain the calculated rate of GBS after *Campylobacter* infection (1 in 1000). Some studies indicate that post-*Campylobacter* GBS cases preferentially associate with certain HLA alleles, the significance of these findings remains unclear because of lack of confirmatory studies (Yuki et al. 1991; Rees et al. 1995b). The host properties that could confer susceptibility to GBS after *C. jejuni* infection are not well established.

Clinical studies showing that GBS sera or purified anti-ganglioside antibodies from GBS sera bind to ganglioside-like moieties in the LPS/LOS (Wirguin et al. 1994; Oomes et al. 1995; Sheikh et al. 1998) promulgated the hypothesis that cross-reactive carbohydrate moieties in LOS incite production of these antibodies. This hypothesis has been

examined experimentally by immunization of laboratory animals with *C. jejuni* LPS/LOS to induce anti-ganglioside antibodies (Wirguin et al. 1997; Goodyear et al. 1999; Ang et al. 2000a). There is a high level of tolerance to self-gangliosides, and immunizations in experimental animals have generally induced low affinity non-T cell-dependent antibodies of IgM and IgG3 type (non-complement-fixing isotype in mice), despite the use of adjuvants to recruit T cell help (Wirguin et al. 1997; Goodyear et al. 1999; Bowes et al. 2002).

In contrast, serological studies in AMAN and FS indicate class switching to IgG and subclass restriction to IgG1 and IgG3 (complement-fixing isotypes in humans), both usually features of T-cell help. Transgenic mice lacking complex gangliosides are immune naïve to complex gangliosides such as GM1, GD1a, and GQ1b, and immunization with gangliosides or *C. jejuni* LPSs in these animals induces IgG anti-ganglioside antibodies with complement-fixing subclass isotypes, indicating recruitment of T cell help (Bowes et al. 2002; Lunn et al. 2000). These experimental observations suggest that break-down of tolerance to self-gangliosides might be critical in the pathogenesis of post-*Campylobacter* GBS. Despite these experimental advances the mechanism(s) of anti-ganglioside antibody induction in patients with *Campylobacter* infection remains unclear.

25.3.2 Haemophilus Influenzae

H. influenzae is a gram-negative bacterium that causes human respiratory tract infections. In GBS, preceding infection with *H. influenzae* is rare, ranging between 1 % in western Europe to 13 % in Japan (Jacobs et al. 1998; Mori et al. 2000; Koga et al. 2005b). In comparison to post-*Campylobacter* GBS, preceding infection with *H. influenzae* is associated with faster recovery, less cranial nerve involvement, and milder disease course (Kuwabara et al. 2001; Jacobs et al. 2008). Based on GBS or FS sera (antibody) binding to LOS/LPS it has been postulated that *H. influenzae* may carry ganglioside-like moieties such as GM1 and GT1a (Koga et al. 2001, 2005a; Mori et al. 1999). Structural/mass spectrometry studies demonstrating ganglioside-like moieties on *H. influenzae* are not yet available. Isolation of *H. influenzae* does not necessarily indicate that this infection is the trigger for induction of anti-ganglioside antibodies because a considerable number (15 %) of GBS patients with *H. influenzae* isolated by culture were seronegative for this bacterium, but seropositive for *Campylobacter*. Further, anti-ganglioside antibodies of seronegative patients did not show any crossreactivity with *H. influenzae* LOS (Koga et al. 2005b). Whether immunization with *H. influenzae* triggers induction of anti-ganglioside antibodies in experimental animals remains to be determined.

25.3.3 *Mycoplasma pneumoniae*

Antecedent infection with *M. pneumoniae* can be found in 2–12 % of patients with GBS (Hao et al. 1998; Jacobs et al. 1998; Ogawara et al. 2000). Studies indicate that some cases of demyelinating form of GBS are associated with *M. pneumoniae* (Hao et al. 1998; Ang et al. 2002). This infection is reported to trigger antibodies against galactocerebroside (GalC), a major glycolipid antigen in myelin, in some patients with GBS. Anti-GalC antibodies have been shown to induce demyelination in experimental animal models (Saida et al. 1979, 1981; Hahn et al. 1993). The hypothesis of molecular mimicry is supported by the findings that anti-Gal-C reactivity in GBS sera following *M. pneumoniae* infection was specifically inhibited by adding *M. pneumoniae* reagent (Kusunoki et al. 2001). According to a recent study GBS associated with anti-GalC antibodies have more frequent sensory deficits, autonomic involvements, and antecedent *M. pneumoniae* infection (Samukawa et al. 2014). One study has reported the AMAN form of GBS in association with anti-GM1 antibodies and preceding *M. pneumoniae* infections (Susuki et al. 2004). These investigators report that anti-GM1 antibodies bind to lipids extracted from *M. pneumoniae*, suggesting that GM1-like structures are also expressed by this microbe.

25.3.4 Cytomegalovirus (CMV)

In GBS, serological evidence for preceding infection with CMV ranges between 8 and 15 % (Dowling and Cook 1981; Visser et al. 1996; Ogawara et al. 2000; Hadden et al. 2001). Patients with CMV-associated GBS appear to have a different clinical phenotype: significantly younger patients are affected, the disease course is more severe, there is prominent cranial and sensory nerve involvement, and functional recovery is incomplete (Visser et al. 1996). With the availability of modern techniques, polymerase chain reaction (PCR) is more appropriate to demonstrate infection with CMV. A previous study failed to detect CMV genome in sural nerve biopsies from GBS patients (Hughes et al. 1992) but a recent study demonstrated the presence of CMV DNA in the CSF of about 30 % of GBS patients with positive CMV serology (Steininger et al. 2004). Several studies have reported an association of CMV infection with GM2 antibodies in patients with GBS (Irie et al. 1996; Yuki and Tagawa 1998; Khalili-Shirazi et al. 1999). It could be demonstrated that anti-GM2 reactivity in GBS sera was abrogated after incubation with fibroblasts infected with a GBS-associated CMV strain, indicating that CMV-infected fibroblasts express GM2-like epitopes recognized by anti-GM2 antibodies (Ang et al. 2000b). This intriguing finding raises the possibility that either CMV itself expresses GM2-like epitopes or it induces the expression of this ganglioside

in the host cells which renders them immunogenic. The later possibility would suggest that direct molecular mimicry by the infecting agent and target tissue antigens may not be necessary. Besides GM2, antibodies against GalNAc-GD1a and GM1 have also been reported in the setting of CMV infection and GBS (Khalili-Shirazi et al. 1999; Kaida et al. 2001).

25.3.5 Gaps in Molecular Mimicry Hypothesis

Despite the accumulation of significant data supporting the hypothesis of post-infectious molecular mimicry in GBS, clearly several fundamental questions remain unresolved: (1) Infectious agents invoked as triggers for anti-glycolipid antibodies in patients with GBS are microbes that induce gastrointestinal and upper respiratory tract infections in a large number of people but only rare individuals develop GBS after these common infections. (2) What are the mechanism(s) of anti-glycolipid antibody induction in patients with post-infectious GBS? The induction of these antibodies in GBS patients is likely to be substantially different from experimental approaches that involve repeated use of infectious organism and adjuvants or immune naïve animals to produce disease-associated anti-ganglioside antibodies. (3) Antibodies against GM1, GM2, and GalC can be seen in other neurological disorders or sometimes in normal controls. What are the antibody-related properties that can distinguish between disease-associated and non-disease-associated antibodies? These and other unresolved issues need further research to increase our understanding of post-infectious autoimmune disorders.

25.4 Review Questions

1. What is meant by the term molecular mimicry?
2. What is the basis of hypothesis of molecular mimicry in the pathogenesis of GBS?
3. What are the potential mechanisms of antibody-mediated peripheral nerve dysfunction/injury?
4. What are the potential mechanisms of T-cell mediated peripheral nerve dysfunction/injury?

25.5 Answers

1. The term molecular mimicry describes a mechanism whereby epitopes incidentally shared by microbial antigens and nerve structures elicit an autoreactive T- or B-cell response in the wake of an infective illness.
2. In GBS there is clinical and experimental evidence that molecular mimicry is an important mechanism how an autoimmune response against peripheral nerve tissue is induced. Several observations support this hypothesis: Epidemiological studies have established a relationship

between *Campylobacter jejuni* and the occurrence of Guillain-Barré Syndrome. GBS-patients with antecedent *C. jejuni* infection frequently associate with an axonal subtype of GBS called acute axonal motor neuropathy (AMAN). Cases of AMAN are significantly associated with the presence of antibodies against the gangliosides GD1a and GM1. *C. jejuni* strains isolated from patients with AMAN bear lipo-oligosaccharides on the cell-surface which share identical chemical structures like the tetrasaccharide structure of GD1a and GM1. The strongest evidence arises from animal models of AMAN in which immunization with relevant gangliosides or *C. jejuni* LPS reproduces clinical and pathological features of AMAN.

3. Experimental evidence suggests that a variety of effects are produced by antibodies on peripheral nerve. For example studies indicate that anti-GM1 antibodies may block or alter channel function. Alternatively, Willison and colleagues demonstrated on ex vivo phrenic nerve-diaphragm preparation, that anti-GQ1b and anti-GD1a antibodies bind to nerve terminals, cause complement-dependent quantal acetylcholine (ACh) release, which results in neuromuscular blockade. In patch-clamp experiments IgG GQ1b, GD1a, GD1b and GM1 antibodies have been shown to cause reversible complement independent pre- and post-synaptic blockade depending upon the antibody used.
4. Potential mechanisms of T-cell mediated peripheral nerve injury include direct mechanisms like release of toxic cytokines such as interferon-gamma and tumor necrosis factor-alpha. Both have proinflammatory effects and mediate myelin damage through activation of macrophages. Furthermore, blood-derived neural antigen-specific T-cells become reactivated in the PNS, expand clonally and release cytokines to orchestrate and perpetuate the immune response. The crucial role of T-cells is proven by the adoptive-transfer EAN, which means the transfer from lymph node cells from animals that had been immunized with myelin induces demyelinating peripheral nerve injury.

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Abstract

The dogma of the immunological privilege of central nervous system (CNS) has been increasingly challenged by compelling evidence showing reciprocal communication pathways between the CNS and the immune system. Brain antigens may drain to the cervical lymph nodes and induce antibody production and T lymphocyte homing to the CNS. It is now clear that, besides classical autoimmune diseases of the CNS such as multiple sclerosis, many CNS diseases which were previously considered as purely neurodegenerative include inflammatory and autoimmune components. The present chapter critically summarizes available evidence concerning autoimmune neuroinflammation in classical neurodegenerative disease such as Alzheimer's disease and Parkinson's disease, and in amyotrophic lateral sclerosis. Attention will be paid also to the possible autoimmune basis of some forms of epilepsies, and the possible neuroprotective role of autoimmune T lymphocytes (neuroprotective autoimmunity) will be finally discussed.

Keywords

Alzheimer's disease • Amyloid β -protein peptide • Parkinson's disease • α -synuclein • SNCA1 • Amyotrophic lateral sclerosis • Autoimmune epilepsy • Rasmussen's encephalitis • Protective autoimmunity • CD4+CD25+ regulatory T cells

26.1 Introduction

An autoimmune response implies the immune response of an organism towards its own tissues and organs, possibly resulting in an autoimmune disease. Classical autoimmune diseases affecting the nervous system include central nervous system (CNS) demyelinating diseases, multiple sclerosis being the most prominent, and autoimmune diseases of the peripheral nervous system, ranging from acute inflammatory demyelinating polyneuropathy to chronic forms (Kieseier et al. 2012; Kamm and Zettl 2012).

The CNS has been considered for long an immunological privileged site (Streilein 1993), however compelling evidence exists for afferent and efferent pathways of communication between the CNS and the immune system (Weller et al. 1996). In particular, antigens may drain from the brain to induce antibody production in the cervical lymph nodes in the presence of activated lymphocytes. T lymphocytes in turn home to the CNS and are mainly recently activated/memory lymphocytes (Weller et al. 1996). Alpha 4-integrin among others plays a key role in lymphocyte recruitment across the blood-brain barrier, as demonstrated by the efficacy as well as by the side effects in the treatment of multiple sclerosis of natalizumab, a humanised monoclonal antibody that blocks alpha4beta1 integrin-mediated leukocyte migration (Rudick and Sandrock 2004).

Awareness of immune surveillance in the CNS paved the way to the identification and characterization of peculiar inflammatory responses in many CNS diseases which were previously considered as purely neurodegenerative

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(Matyszak 1998). The CNS itself has an innate immune system which includes astrocytes and microglia regulating the initiation and progression of immune responses and interacting with inflammatory (e.g. Th1/Th17) and anti-inflammatory (e.g. Th2/Treg) subsets of peripheral T cells which in turn might regulate microglial fate towards neurodegenerative or neuroprotective phenotypes (González and Pacheco 2014).

Hereafter, evidence for autoimmune-mediated neuroinflammation will be discussed in classical neurodegenerative disease such as Alzheimer's disease and Parkinson's disease, and in amiotrophic lateral sclerosis, which etiopathogenesis remains so far controversial. Evidence for an autoimmune basis of some forms of epilepsies will be then discussed. Finally, the possibility that autoimmune T lymphocytes can contribute to repair, following an injury to the CNS (neuroprotective autoimmunity) will be examined.

The present chapter will not consider the multiple evidence supporting an association between schizophrenia and perturbations of autoimmunity (Goldsmith and Rogers 2008), since it is so far unclear whether autoimmunity actually contributes to abnormal signaling in multiple neurotransmitter systems and brain regions, or it is a consequence of the central and peripheral alterations which occur during such devastating disease. Anyway, the areas stringly calls for in-depth investigation of the neuroimmunological mechanisms actually involved.

26.2 Alzheimer's Disease

Alzheimer's disease (AD) accounts for the majority of cases of dementia. It has been estimated that AD affected between 21 and 35 million people worldwide in 2010. AD usually begins over 65 years of age, although 4–5 % of cases are early-onset AD which begin before this, and affects about 6 % of people 65 years and older (Querfurth and LaFerla 2010). Besides neurofibrillary tangles, dystrophic neurites and significant neuronal loss, a hallmark of AD is the presence of extracellular aggregates of the amyloid β -protein ($A\beta$) peptide, called $A\beta$ plaques, which seem to have proinflammatory properties. For instance, in the APPPS1 AD mouse model deposition of $A\beta$ peptide activates the NLRP3 inflammasome in microglia (Heneka et al. 2013), and it has been suggested that persistent $A\beta$ -driven release of p40 by microglia, could binds the IL-12R β 1 receptor in neighboring astrocytes and microglia, impairing their ability to attenuate AD pathology (Vom Berg et al. 2012).

The occurrence of chronic neuroinflammation in AD is now well established and it has been the subject of several reviews (Monsonogo et al. 2013; Heneka et al. 2015). It is however less certain to what extent autoimmune responses may contribute to AD. Immunoaging (i.e. immune changes occurring with age) often include increased inflammation and autoimmunity associated with a decline of T cell func-

tion and a decreased ration naive/memory T cells (Sardi et al. 2011). In search of putative autoantigens in AD, the hypothesis that novel macromolecules synthesized in the brain during the process of memory consolidation might be recognized by the immune system as “non-self” antigens has been also put forward (Arshavsky 2006). In 3xTg-AD mice, a well-established murine AD model, manifestations of systemic autoimmunity and inflammation, including splenomegaly, hepatomegaly, elevated serum levels of anti-nuclear/anti-dsDNA antibodies, low hematocrit, and increased number of double-negative T splenocytes, develop early, in parallel with behavioral deficits, in agreement with a causal association between autoimmunity and aberrant behavior (Marchese et al. 2014). Another murine AD model, the 5XFAD AD mice, develop an age-dependent increase in anti-ceramide antibodies, and ceramide administration may lead to increased amyloid plaque formation (Dinkins et al. 2015). The significance of autoantibodies in AD is however debated, as autoantibodies against $A\beta$ are found naturally in aging humans, however their pathological significance is far from clear (discussed in Kapadia and Sakic 2011).

It must be considered that, nearly two decades ago, AD was the first disease attempted to be cured through the elicitation of an immune response to a self peptide (Monsonogo et al. 2013). Based on evidence from animal models, the idea was that triggering an adaptive immune responses towards the $A\beta$ peptide would have considerably reduced its accumulation in the brain. A clinical trial of active $A\beta$ vaccine was however stopped because in the brains of some vaccinated AD patients developed severe inflammatory reactions, possibly attributed to the use of the full length of the $A\beta$ peptide together with a very strong adjuvant, thus leading to the induction of pathogenic T cells. Although itself a failure, the clinical trial had the merit to attract the attention on the occurrence of $A\beta$ -reactive T cells in AD as well as in healthy subjects. Stronger $A\beta$ -reactive T cell responses may occur in elderly healthy subjects and in AD patients in comparison to middle-aged subjects, and the immunodominant $A\beta$ epitopes in humans seem to reside in amino acids 16–33 (Monsonogo et al. 2003). The current hypothesis, depicted in Fig. 26.1, is that $A\beta$ -reactive T cells can promote pathogenic autoimmunity, but can also be used to enhance neuronal repair mechanisms (Monsonogo et al. 2013). See also below, 26.6 Neuroprotective autoimmunity.

26.3 Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder, with prevalence raising from 0.3 % of the whole population up to 1 % in those over 60 years of age and up to 4 % over 80, thus imposing an increasing social and economic burden on societies with increased aging of populations (de Lau and Breteler 2006). The pathogenesis of PD

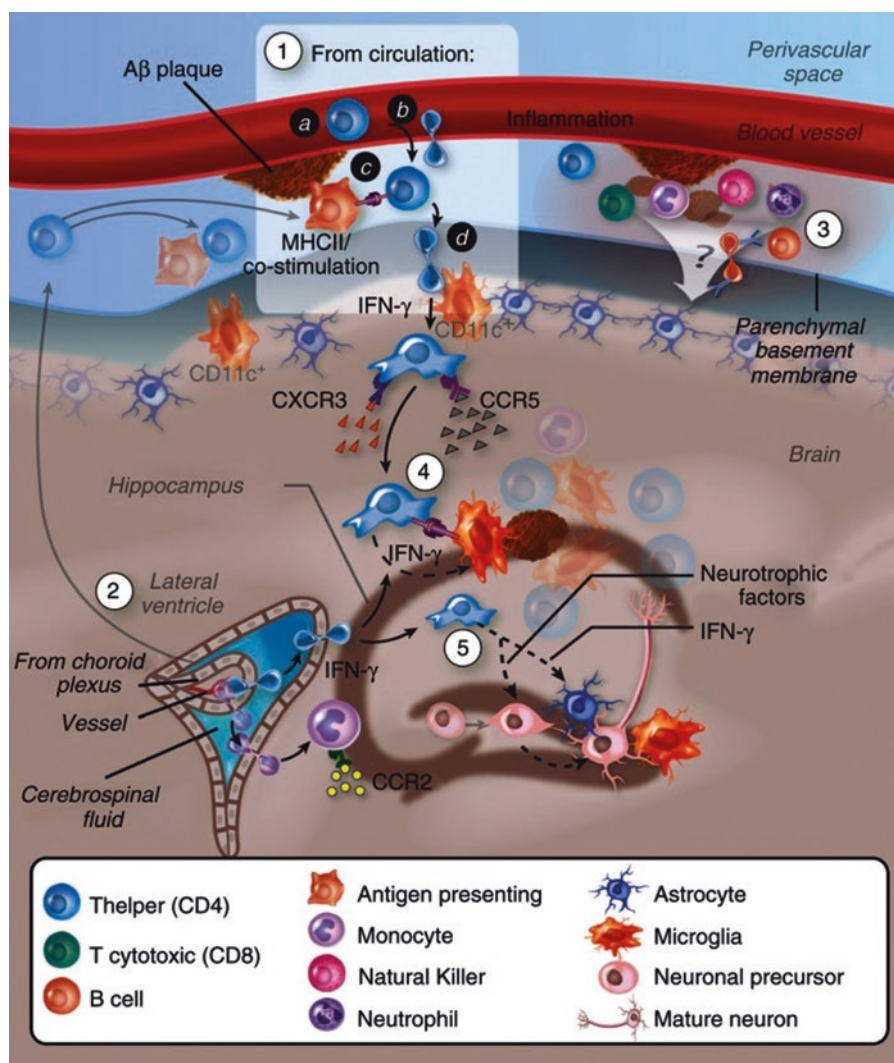


Fig. 26.1 Migration of Aβ-specific T cells towards Aβ plaques in the brain parenchyma. (1) T cells may undergo activation following Aβ immunization or following drainage of Aβ or antigen-presenting cells (APCs) that carry Aβ to peripheral lymph nodes. Aβ-reactive helper T (Th) cells adhere and transmigrate into the perivascular space of Aβ-deposited vasculature in the brain (a, b). To cross the glia limitans, Th cells need to be re-stimulated by dendritic cells or, possibly, by other competent APCs located at the perivascular space and/or juxtavascular with processes sent into the perivascular space. Similar T-cell infiltration processes may occur at the choroid plexus (2), and/or the leptomeninges followed by their dissemination in the central nervous system subarachnoid space. Low levels of interferon-γ (IFN-γ) promote the infiltration process. Adhesion molecules such as P-selectin, vascular cell adhesion molecule-1 or intercellular adhesion molecule 1 (interacting with P-selectin glycoprotein ligand-1, integrin α4 and lymphocyte

function-associated antigen-1, respectively, on the T cells) and chemokine signalling (such as via CCR5 and CXCR3) play a key role in mediating the extravasation of the T cells through the blood-brain barrier (BBB) or the blood-cerebrospinal fluid barrier. (3) Leucocytes accumulate at the subarachnoid and perivascular spaces and may impact on the overall inflammatory reaction at both the vasculature and parenchyma. (4) Once Aβ Th cells cross the glia limitans they migrate and accumulate around Aβ plaques, possibly interacting with APCs (i.e. microglia, or peripheral monocytes or dendritic cells recruited towards CCL2) that present Aβ T-cell epitopes. Cytokines such as IFN-γ are secreted by the T cells and facilitate Aβ clearance either by brain endogenous microglia or by infiltrating microglia-like cells. (5) T cells secreting IFN-γ and/or neurotrophic factors stimulate neural precursor cell proliferation and differentiation (reproduced with permission from Monsonego et al. 2013)

remains elusive, and the mechanisms underlying the selective dopaminergic cell loss in the brain are still far from being understood. Mitochondrial dysfunction, oxidative stress, and protein mishandling have a central role in PD pathogenesis, however more than 90% of PD cases are sporadic, and it is likely that all these processes are induced by non-genetic factors, probably in interaction with susceptibility genes.

Neuroinflammation and the adaptive immune system in PD are increasingly seen as key players in triggering and maintaining the pathogenic processes leading to neurodegeneration, although these processes are still poorly understood. Microglial cells are main contributors to chronic inflammation in PD brains. Their toxicity towards dopaminergic neurons occurs in vitro and in animal models of PD

(McGeer and McGeer 2008). Reactive microglia expressing human leukocyte antigen (HLA)-DR and CD11b can be found in the substantia nigra of PD patients (McGeer et al. 1988). Less is known about the role of astrocytes, but they are known to secrete both inflammatory and anti-inflammatory molecules and may play a role in modulating microglial activity (McGeer and McGeer 2008). Besides microglial activation, several lines of evidence suggest the occurrence of dysregulated peripheral immunity in PD patients. Baba and coworkers (2005) showed in PD patients reduction of total numbers of lymphocytes, of CD4+ T cells, as well as of the ratio CD4+/CD8+ T cells, while the ratio IFN- γ /IL-4 producing cells was increased. Saunders and coworkers (2012) showed increased percentages of CD45RO+ and FAS+ cells, while the percentages of CD45RA+ and CD31+ were decreased. In addition they reported decreased α 4 β 7+CD4+ T cells and increased α 4 β 1+CD4+ T cells. Low expression of α 4 β 7 and high expression of α 4 β 1 are characteristic of T cells that are in charge for immune surveillance of the brain (Denucci et al. 2009).

CD4+ T cells are attracting increasing attention as key players in PD. CD4+ T lymphocytes infiltrate postmortem human PD specimens and mediate dopaminergic toxicity in murine models of PD (Benner et al. 2008; Brochard et al. 2009). In recent years, evidence accumulated regarding a dysregulation of the CD4+ T cell compartment in PD. Baba et al. (2005) already reported the occurrence of decreased CD4+/CD8+ T-cell ratios, fewer CD4+CD25+ T cells and significantly increased ratios of IFN- γ -producing to IL-4-producing T cells in peripheral blood of PD subjects. In murine models, CD4+ T cells isolated from lymph nodes and spleens of animals protect the nigrostriatal system from neurodegeneration (Laurie et al. 2007); in particular, adoptive transfer of CD3-activated CD4+CD25+ regulatory T cells to MPTP-intoxicated mice provides greater than 90% protection of the nigrostriatal system (Reynolds et al. 2007). Recently, CD4+CD25+ regulatory T cells were shown to suppress microglial activation. In contrast, CD4+CD25- effector T cells exacerbated microglial inflammation and induced putative neurotoxic responses (Reynolds et al. 2009). In human peripheral blood, CD4+ T cells include CD4+CD25- effector T cells as well as CD4+CD25+ regulatory T lymphocytes (Treg), the latter being a specialized subset of T cells which are crucial for the control of immune homeostasis. Besides PD, Treg seem to be key neuroprotective immunomodulators in acute experimental stroke (Liesz et al. 2009) possibly through modulation of microglial oxidative stress and inflammation (Reynolds et al. 2007) and preliminary evidence in patients with neurodegenerative disease indicates the occurrence of specific functional alterations affecting the Treg subset (Rosenkranz et al. 2007). A recently published report also shows that in PD patients, peripheral CD4+ T cells have an increased susceptibility to apoptosis with Fas involve-

ment, which may explain the decreases number of CD4+ T-cell subsets in PD patients, possibly in relationship with the neurodegenerative process (Calopa et al. 2010).

Several lines of evidence suggest that α -synuclein (α -syn) may represent the connection between central microglia, the peripheral immune system and neuroinflammation (Sanchez-Guajardo et al. 2013). Alpha-syn is a protein which is abundant in the human brain but which can be found also in periphery. Although its function is not well understood, it may help regulate the release of several neurotransmitters such as dopamine. Alpha-syn is crucial in PD pathogenesis: mutations in SNCA1, the gene encoding α -syn, cause autosomal dominant forms of PD, while aggregated and post-translationally modified forms of α -syn are present in Lewy bodies in both sporadic and familial PD. Accumulation of abnormal forms of α -syn is a trigger for PD, and recent evidence suggests that much of the downstream neurodegeneration may result from inflammatory responses which raise from an interplay between central microglia and peripheral lymphocytes which migrate to the brain following peripheral activation (Allen Reish and Standaert 2015). Three missense mutations, A53T, A30P and E46K have been linked so far to familial PD, as well as multiple copies of the wild-type (wt) α -syn gene to a sporadic form of PD (Kruger et al. 1998; Polymeropoulos et al. 1997; Zarranz et al. 2004). Of potential relevance for PD pathogenesis, α -syn can self-assemble to form ordered fibrillar aggregates, characterized by a cross β -sheet structure (Chiti and Dobson 2006), and it has been hypothesized that aggregated α -syn may become (neuro) toxic (Bennett 2005). While typically considered as an intracellular protein, α -syn is also found in human CSF and plasma. Indeed, in PD patients α -syn levels may be elevated in plasma (Lee et al. 2010), and decreased in CSF (Tokuda et al. 2006). Plasma α -syn in PD could be predominantly oligomeric (El-Agnaf et al. 2006).

Abnormal species of α -syn are linked to microglial activation, oxidative stress, neuroinflammation, and hence loss of dopaminergic neurons in affected brain regions (Zhang et al. 2005; Reynolds et al. 2008), however various α -syn forms occur also in periphery. Nitrated (N)- α -syn, has been found in cervical lymph nodes of MPTP-treated mice (Benner et al. 2008), and modified forms of α -syn are also present in gut tissue of PD patients (Lebouvier et al. 2010; Forsyth et al. 2011; Hilton et al. 2014). Aggregated α -syn is one of the main proteins recognized by autoantibodies in PD patients (Benner et al. 2008; Spillantini et al. 1998), and 90% of patients with familial PD are positive for antibodies against α -syn (Papachroni et al. 2007).

It has been proposed that α -syn could stimulate TLRs resulting in microglial activation. Thereby, inflammatory cytokines produced by M1-like microglia would promote an immunogenic microenvironment for the presentation of neoantigens by APCs, thus triggering an inflammatory T-cell

response against cells expressing α -syn (Ohmori and Kanayama 2005). In particular, N- α -syn induces a neurotoxic microglial phenotype (M1) (Thomas et al. 2007; Zhang et al. 2005; Zhang et al. 2007; Zhou et al. 2005), while extracellular, non-aggregated α -syn produces a moderate proinflammatory response in glia, and a moderate stimulation of Th1 chemokine secretion (Roodveldt et al. 2008).

Also circulating lymphocytes express α -syn, which is increased in cells from idiopathic PD patients, and its expression is correlated to glucocorticoid-sensitive apoptosis, possibly caused by the enhanced expression of glucocorticoid receptor, caspase activation, CD95 (Fas) up-regulation, and ROS production (Kim et al. 2004). Expression of α -syn also occurs in human macrophages and its levels are increased by stimulation with IL-1 β or LPS (Tanji et al. 2002). Antibodies against all the three forms of α -syn (monomeric, oligomeric, and fibrillar) have been shown in the serum of 65 % of all PD patients (Papachroni et al. 2007), apparently reaching the highest values after 5 years of disease duration, and decreasing after 10-year duration (Gruden et al. 2011).

Several α -syn-targeting active vaccine are currently going to be introduced into the phase of clinical development, on the hypothesis that a reduction of α -syn in the brain has the potential to modify the pathophysiology underlying the disease. Companies developing immunotherapies against α -synuclein include, besides Prothena and AFFiRiS, AstraZeneca, BioArctic, Biogen, Genentech, and Lundbeck (Anonymous 2015).

26.4 Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease and Charcot disease, or motor neuron disease, has a global incidence about 1–2 per 100,000 and risk of ALS is estimated to be 1/600–1/2000. ALS is characterized by muscle stiffness and twitching and muscle wasting due to the death of spinal motor neurons (Eisen 2009). Besides 5–10 % familial cases due to Superoxide Dismutase I mutations, the pathogenesis of sporadic ALS is still a matter of debate, and etiopathogenic hypotheses include autoimmunity, neurofilament, and glutamate toxicity (Silani et al. 2001).

Preliminary evidence obtained mainly in animal models strongly suggests that immune-mediated neuroprotection may be relevant in ALS (Schwartz and Ziv 2008) (see also below: 2.3.1.5. Neuroprotective autoimmunity). For instance, in the (SOD1)(G93A) transgenic mouse model of ALS, T cells seem to play an endogenous neuroprotective role by modulating a beneficial inflammatory response to neuronal injury (Chiu et al. 2008; Banerjee et al. 2008). The involvement of immune mechanisms was recently suggested on the basis of decreased CD4+ T cells and increased CD8+ T cells, as well as diminished CD45RA/CD45RO (naive/memory)

ratio among CD4+ T cells, due to reduction in CD45RA+ cells and concomitant increase in CD45RO+ cells (Banerjee et al. 2008). The increase in CD8+ cytotoxic T cells in ALS was recently confirmed and findings were extended to increased natural killer (NK) T cells reduced regulatory T lymphocytes. Interestingly, regulatory T cells were also negatively correlated with progression of the disease (Rentzos et al. 2012). Significantly reduced regulatory T cells have been reported in ALS patients at a less severe stage of disease, suggesting their early recruitment towards the CNS area of primary neurodegeneration (Mantovani et al. 2009). Available evidence thus suggests a systemic immune activation in ALS patients, possibly to some so far undetected endogenous proteins or viruses. A dysfunction of regulatory T cells likely contributes to disease development and progression (He and Balling 2013).

26.5 Autoimmune Epilepsy

Humoral and cell-mediated immune responses against the glutamate receptor subunit GluR3 was first identified less than 20 years ago in Rasmussen's encephalitis, a rare progressive pediatric epileptic syndrome (Andrews and McNamara 1996). Soon data began to accrue suggesting the involvement of immune mechanisms in the pathogenesis of intractable childhood epilepsies. For instance, epilepsy is more common in patients with systemic lupus erythematosus with antiphospholipid antibodies, raising the possibility that such antibodies are also responsible of immune-mediated cortical damage (Aarli 2000). Besides antibodies to glutamate receptors, over the years antibodies to voltage-gated calcium and potassium channels have been detected in serum and liquor of patients with ataxia, limbic encephalitis and certain forms of epilepsy, and some patients also have antibodies to glutamic acid decarboxylase or specific ribonuclear proteins. Some patients respond to immunotherapies, although the pathogenicity of such antibodies has been questioned and in only a few cases IgG derived from patients produced pathogenic effects in vivo or in vitro (Lang et al. 2003; Billiau et al. 2005).

In 2007, Dalmau et al. reported a series of 12 patients with teratoma of the ovary (11) and of mediastinum (1) who developed prominent psychiatric symptoms, amnesia, seizures, frequent dyskinesias, autonomic dysfunction, and decreased level of consciousness often requiring ventilatory support (Dalmau et al. 2007). Psychosis may be linked to both abnormalities in autoimmunity and glutamatergic signaling in the context of an anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis. The syndrome was originally described in young women but it is now established that also children and males can be affected, either as a paraneoplastic manifestation of a teratoma or without a tumor. Reversible internalization of NMDAR due to binding of IgG antibodies

is thought to be the main cellular mechanisms (Hughes et al. 2010). Current knowledge about anti-NMDAR encephalitis has been recently revised (Kayser and Dalmau 2014).

From a general point of view, neuroinflammation may occur in epilepsy, and brain inflammatory reactions may result in enhanced neuronal excitability, impairing cell survival, and increasing blood-brain barrier permeability, while some antiinflammatory treatments reduce seizures in experimental models and, in some instances, in clinical cases of epilepsy (Vezzani and Granata 2005). In 2002, for the first time a session with the “autoimmune epilepsy” concept took place in at the International Congress of Autoimmunity in Geneva, Switzerland (Levite 2002). Levite and Ganor (2008) reported AMPA GluR3 autoantibodies in 27% of patients with different epilepsies, and NMDA NR2A or NR2B autoantibodies in 30% of systemic lupus erythematosus patients, with or without neuropsychiatric impairments. NR2 autoantibodies were also found in patients with epilepsy (33%), encephalitis and stroke. Glutamate receptor autoantibodies can damage the brain in both human and animal models. GluR3 autoantibodies cause neuronal death either by receptor activation and excitotoxicity or by complement fixation independent of receptor activation, resulting in multiple brain damage and neurobehavioral/cognitive impairments (Levite and Ganor 2008). Auto-antibodies which have been found over the years in patients with seizures include antibodies against to the NMDA, GABAB and AMPA receptors and to LGI1, CASPR2 and Contactin-2, components of the voltage-gated potassium channel complex, which are all important elements controlling the brain electrical activity. Patients with temporal lobe epilepsy may have antibodies to glutamic acid decarboxylase (GAD) (Errichiello et al. 2011), while around 90% of patients with the newly described faciobrachial dystonic seizures syndrome may have antibodies to LGI1. All of these antibodies bind to the extracellular domains of their targets and their pathogenicity is very often supported by the effectiveness of immunotherapies (Irani et al. 2011).

Besides auto-antibodies, evidence from several types of epilepsy (including Rasmussen’s encephalitis, West syndrome, Landau-Kleffner syndrome and continuous spikewaves during sleep, limbic encephalitis, hemiconvulsion–hemiplegia syndrome, Batten disease), suggest the contribution of both adaptive and innate immunity (Granata et al. 2011). For instance, in Rasmussen’s encephalitis is brain inflammation dominated by cytotoxic CD8+ T cells and microglial activation, followed by neuronal loss and astrogliosis restricted to the affected hemisphere (Granata et al. 2011). Data supporting the involvement of IL-1 β , TNF- α and toll-like receptor 4 in seizure generation and the process of epileptogenesis have also been recently summarized (Friedman and Dingledine 2011).

Immune mechanisms may also be a factor in a number of epilepsies such as Rasmussen’s encephalitis, Lennox-Gastaut syndrome, Landau-Kleffner syndrome, and tempo-

ral lobe epilepsy. Immunologic abnormalities are found in routine epilepsy surgical specimens, suggesting a broader role of immunopathology in the etiology of epilepsy. The prevalence and impact of immunopathology in epilepsy syndromes remains to be determined by future research (Najjar et al. 2008). In any case, the identification of autoimmune encephalitis has changed the diagnosis and management of several syndromes that occur with seizures and status epilepticus previously attributed to viral or idiopathic etiologies (Davis and Dalmau 2013). Awareness of autoimmune encephalitis in addition is bringing to increased recognition of autoimmunity as a cause of neurologic presentations in the pediatric intensive care unit (ICU) setting. Autoimmunity is now routinely suspected in *N*-Methyl-D-aspartate (NMDA) receptor encephalitis, CNS vasculitis, demyelinating disorders, neurologic involvement of systemic autoimmune disorders, febrile infection-related epilepsy syndrome (FIRES) and rapid-onset obesity, hypoventilation, hypothalamic dysfunction, and autonomic dysregulation (ROHHAD) syndrome (Benson et al. 2014).

The contribution of immune mechanisms in epileptic disorders of unknown etiology has opened up the way for new immunosuppressive and immunomodulatory treatments. The benefits of corticosteroids in certain epileptic encephalopathies, such as West syndrome and continuous spike waves during sleep (CSWS), suggest a role of inflammation in these syndromes. This hypothesis is further supported by the efficacy of ketogenic diet, which also has an antiinflammatory effect, in some epileptic encephalopathies and in severe epilepsies (Nabbout 2012).

Finally, immunological mechanisms (possibly leading to ischemia and neuronal damage) may also underlie the occurrence of seizures in well-established autoimmune disorders, including multiple sclerosis (MS), diabetes mellitus, celiac disease, thyroid disease, and systemic lupus erythematosus (SLE) (Vincent and Crino 2011).

26.6 Neuroprotective Autoimmunity

Neuroprotective autoimmunity is a condition in which cells of the adaptive immune system promote or restore the functional integrity of the nervous system, or facilitate its repair following any damage. According to the theory of “protective autoimmunity”, CNS-specific T cells, along with circulating and local innate immune cells, can enhance CNS healing processes following non-infectious injuries, or any deviation from homeostasis, including chronic pathological conditions (reviewed in Schwartz and Raposo 2014; Graber and Dhib-Jalbut 2009). Brain-specific T cells have been shown to play beneficial roles in several experimental models of disease of the nervous system, including brain injury (Hauben et al. 2000; Moalem et al. 1999), amyotrophic lateral sclerosis

Table 26.1 Diseases, animal models and therapies with evidence for protective immunity (modified with permission from Graber and Dhib-Jalbut 2009)

Disease	Model	Therapeutics
MS	EAE	Oral myelin, statins, vaccination, hormonal therapy, IVIg
	Theiler's virus	Anti-CD154
	Lysolecithin	GA
	Cuprizone	IFN-beta
Stroke	Ischemic infarct	Vaccination, IFN-beta, FK506, minocycline, poly-YE
CNS trauma	Optic nerve injury	Vaccination
	Spinal cord injury	IFN-beta, Anti-Nogo-A, MMP-3
	Head trauma	GA
Parkinson's disease	MPTP rat	GA
	6-OHDA rat	VIP
ALS	SOD1 mouse	GA
HIV encephalitis	HIV encephalitis	GA
Peripheral nerve injury	Facial nerve injury	GA
	Nerve root avulsion	GA

(Holmoy 2008) and stroke (Frenkel et al. 2003) (Table 26.1). For example, in a rodent model of neurodegeneration, neuronal loss caused by intraocular injection of aggregated A β was significantly greater in immunodeficient mice than in normal mice, neurodegeneration was respectively attenuated or augmented by elimination or addition of CD4+CD25+ regulatory T cells, and in hippocampal slices, microglia encountering activated T cells overcame the cytotoxicity of aggregated A β (Avidan et al. 2004). Such kind of results were interpreted suggesting that locally activated T cells induce a microglial phenotype that helps neurons withstand the insult irrespective of toxicity type, concluding that possibly both acute and chronic neural damage might be arrested or retarded by immune strategies that increase T cell effector activity and/or weaken regulatory T cell-dependent immunosuppression.

According to the theory of “protective autoimmunity”, autoimmune mechanisms help maintaining life-long plasticity of the adult CNS and loss of immunity to self contributes to the emergence of chronic neurodegenerative conditions (Schwartz et al. 2009). As a prominent example, Ziv and co-workers (2006) showed that in mice hippocampal neurogenesis is associated with T cell recruitment and microglia activation, while in immune-deficient mice hippocampal neurogenesis was markedly impaired (Fig. 26.2), but was restored by T cells recognizing myelin basic protein. Spatial learning and memory and the expression of brain-derived neurotrophic factor in the dentate gyrus required the presence of CNS-specific T cells (Ziv et al. 2006). Other types of immune cells can contribute to CNS neurogenesis, as in the case of macrophages in the olfactory bulb (Borders et al. 2007). Likely mechanisms for immune cell-induced CNS plasticity are the production of growth factors like BDNF and IGF-I, the ability to locally buffer excess glutamate, and to activate the local microglial response in manners that result

in benefits to the CNS (reviewed in Schwartz et al. 2009). Despite the impressive amount of evidence in animal models, translation of “protective autoimmunity” to the clinics still requires the identification of adequate conditions, which results in augmentation of autoimmunity, without the risk of induction of an autoimmune disease (Fig. 26.3).

26.7 Other Neurologic Disease with an Autoimmune Component

Obsessive-compulsive disorder (OCD) and related conditions are chronic, relapsing disorders of unknown etiology associated with impairment and disability, which affect as many as 0.3–3 % of the pediatric population. They include Tourette syndrome (also called Tourette's syndrome), a neuropsychiatric disorder with onset in childhood, characterized by multiple physical (motor) and vocal (phonic) tics. Immune contribution in the development of such movement disorders began to be considered following the description of Sydenham chorea (SC), a classical poststreptococcal movement and psychiatric disorder, often associated with other features of rheumatic fever. When sudden onset of neuropsychiatric symptoms is temporally associated with Group A Streptococcus infection, the presentation is termed pediatric autoimmune neuropsychiatric disorders associated with Streptococcus (PANDAS). The immunobiology of such movement disorders remains unestablished, however the predominating theory, based on the clinical evidence of the presence of several types of antineuronal antibodies, is molecular mimicry whereby antibodies intended to target Group A Streptococcus target brain proteins instead. Preliminary evidence exists also for a possible role of T and B lymphocytes (Murphy et al. 2010; Dale 2013; Williams and Swedo 2014).

Fig. 26.2 Impaired neurogenesis in T cell-deficient mice. (a) Quantification of BrdU+ cells and BrdU+ DCX+ cells in the dentate gyrus of nude and wild-type mice 7 days after the first BrdU injection. *** $P < 0.001$, t -test; $n = 4$ per group. (b) Number of BrdU+ DCX+ cells as a percentage of the total number of BrdU+ cells in the dentate gyrus. *** $P < 0.001$, t -test; $n = 4$ per group. (c) Dendritic arborization examined by DCX staining in wild-type and nude mice. (d) Number of BrdU+ DCX+ cells as a percentage of the total number of BrdU+ cells in the dentate gyrus of wild-type, nude and splenocyte-replenished nude mice, 7 days after the first BrdU injection. * $P < 0.05$, ANOVA; $n = 5$ per group. (e) Total numbers of PCNA+ cells in the dentate gyrus of wild-type, nude and splenocyte-replenished nude mice. * $P < 0.05$, ANOVA; $n = 4$ per group. (f) T cells (CD3+) in the brain of a replenished nude mouse. (g) Higher magnification of the marked area in (f). (h) CD3+ cells lining the wall of the third ventricle in the brain of a wild-type mouse. (i) CD3+ cells in the parenchyma adjacent to a blood vessel in the brain of a replenished nude mouse. Data are expressed as mean plusminus s.e.m. LV, lateral ventricle; CA3, CA3 of the hippocampus. Scale bar 20 μm in (c), 50 μm in (g) and 25 μm in (h) and (i) (reproduced with permission from Ziv et al. 2006)

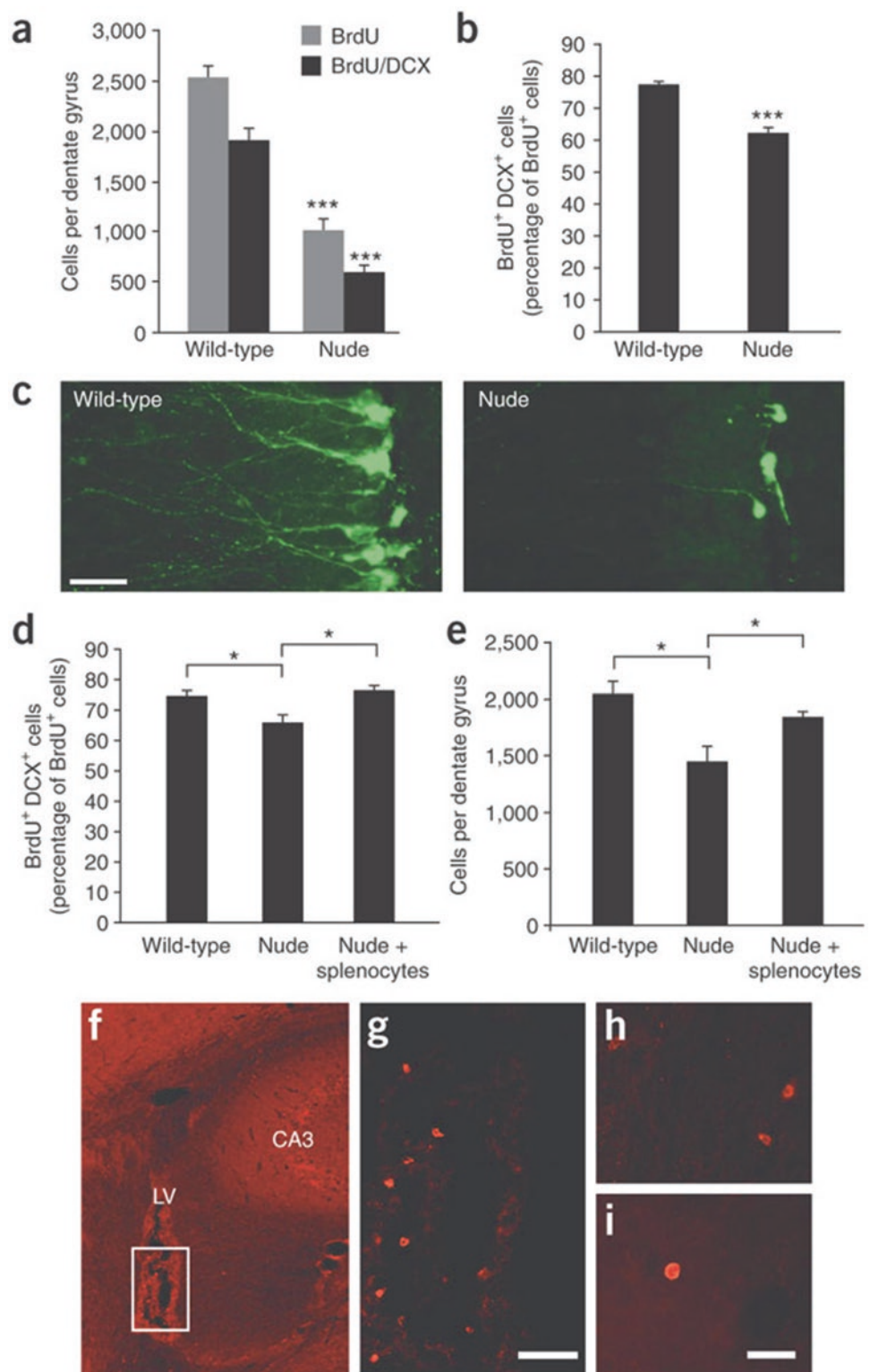
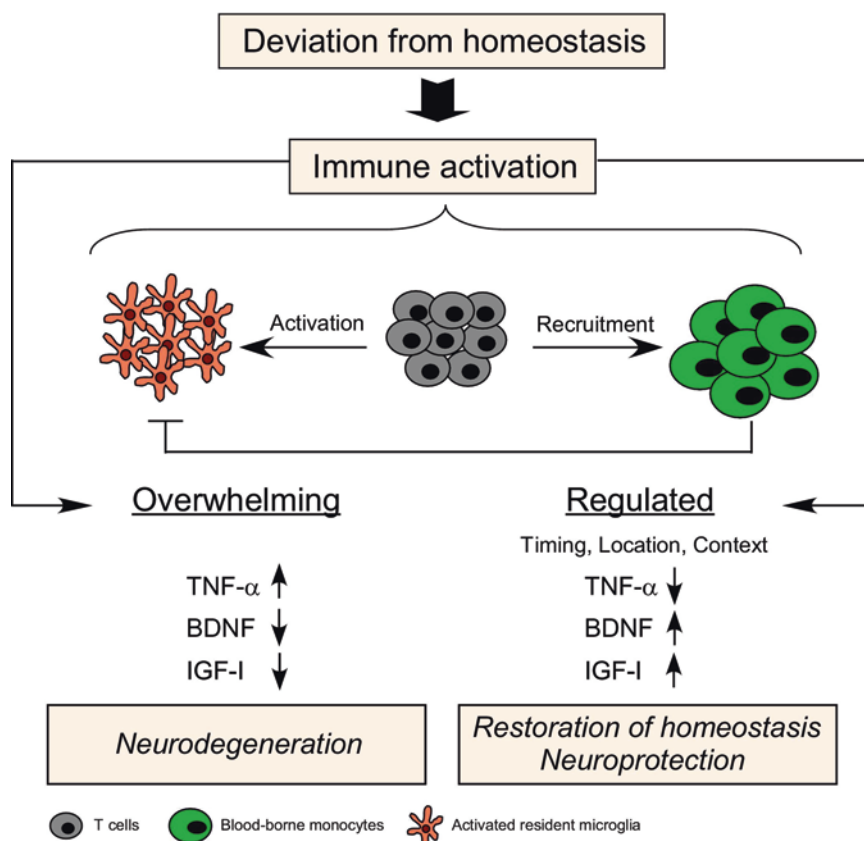


Fig. 26.3 Adaptive immunity, in concert with innate local and peripheral immunity, contributes to CNS maintenance and repair.

Under healthy conditions the resident microglia are capable of containing small deviations from homeostasis, assisted by peripheral immunity, that presumably act at the perivascular spaces. Under injurious conditions or chronic diseases, the assistance of the T cells is local and is manifested in part by the recruitment of blood-borne monocytes to the damaged parenchyma. The T cells together with the monocytes provide a mechanism of amplifying a regulated immune response, which alleviates the risk of cytotoxic factors (e.g. TNF- α) and provides the growth factors that are needed for survival, repair and regrowth (IGF-1, BDNF) (reproduced with permission from Schwartz et al. 2009)



26.8 Review Questions

1. How does the immune surveillance awareness of the CNS pave the way to the identification and characterization of peculiar inflammatory responses?
2. List 4 hallmarks of Alzheimer's disease discussed within this chapter.
3. What was the hypothesized adaptive immune response used in animal models of Alzheimer's Disease discussed in this chapter?
4. Why are CD4+ T cells being used more and more in treatments of Parkinson's disease?
5. What evidence suggests that α -synuclein may represent the connection between central microglia, the peripheral immune system and neuroinflammation?
6. Why are immune mechanisms becoming a hypothesized factor in a number of epilepsies?

26.9 Answers

1. The CNS itself has an innate immune system which includes astrocytes and microglia regulating the initiation and progression of immune responses and interacting with inflammatory and anti-inflammatory subsets of peripheral T cells which in turn might regulate microglial fate towards neurodegenerative or neuroprotective phenotypes.
2. Neurofibrillary tangles, dystrophic neurites, significant neuronal loss, and the presence of extracellular aggregates of the amyloid β -protein (A β) peptide, called A β plaques.
3. By triggering an adaptive immune response towards the A β peptide, it would have considerably reduced its accumulation in the brain.
4. CD4+ T lymphocytes can infiltrate postmortem human Parkinson's disease specimens and mediate dopaminergic toxicity in murine models.
5. α -synuclein is crucial in PD pathogenesis: mutations in SNCA1, the gene encoding α -syn, cause autosomal dominant forms of PD, while aggregated and post-translationally modified forms of α -syn are present in Lewy bodies in both sporadic and familial PD. Accumulation of abnormal forms of α -syn is a trigger for PD, and recent evidence suggests that much of the downstream neurodegeneration may result from inflammatory responses which raise from an interplay between central microglia and peripheral lymphocytes which migrate to the brain following peripheral activation.
6. Immunologic abnormalities are found in routine epilepsy surgical specimens, suggesting a broader role of

immunopathology in the etiology of epilepsy. In any case, the identification of autoimmune encephalitis has changed the diagnosis and management of several syndromes that occur with seizures and status epilepticus previously attributed to viral or idiopathic etiologies.

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Abstract

HIV infection in the central nervous system (CNS) is responsible for a spectrum of neurocognitive and behavioral disorders. The wide use of appropriate combination antiretroviral therapy (cART) has decreased the severity of cognitive dysfunction. Viral loads are reduced and CD4+ T cell counts are increased. In addition, patients are living longer with improved quality of life. However, cART does not completely eliminate CNS dysfunction in HIV infected patients. There is an increase in milder forms of HIV-associated neurocognitive disorders (HAND), which are the result of a complex interplay between many factors including: aging, vascular disease, Alzheimer's disease, and psychiatric disease. The underlying biochemical and molecular changes that underlie these changes and the underlying mechanisms of HAND pathogenesis continue to be an area of ongoing research. New advances in imaging and biomarker discovery will continue to advance clinical diagnosis and management. Treatments that can perturb the viral reservoirs and decrease chronic inflammatory response, in addition to continuing cART, may give the clinician additional tools to prevent HAND.

Keywords

cART • HIV-associated neurocognitive dysfunction (HAND) • HIV pathogenesis

27.1 Introduction

Human Immunodeficiency virus (HIV), the cause of the acquired immunodeficiency syndrome (AIDS), was identified over 30 years ago and continues to infect, and affect, many. It is estimated that more than one million people are living with HIV infection in the United States with 15% unaware of their HIV status (CDC 2014). Worldwide, 35 million people are living with HIV (WHO 2014). HIV is a lentivirus that predominately infects CD4+ T cells and macrophages expressing CD4. In addition to CD4, HIV requires the expression of a co-receptor, utilizing the chemokine

receptors CCR5 and/or CXCR4 to target susceptible cells. The incidence of new HIV infection is approximately 50,000 per year in the U.S. and has been constant over the past several years, although the incidence has spiked in certain areas of the U.S. The wide use of combination antiretroviral therapy (cART, previously referred to as highly active antiretroviral therapy, HAART) began in the 1990s and has dramatically increased the life span of patients living with HIV. These therapies (including nucleoside and non-nucleoside reverse transcriptase inhibitors, protease inhibitors, integrase inhibitors, entry/fusion inhibitors) have decreased HIV related mortality by effectively reducing viral loads and improving immune cell function, which can be decimated by the infection. The use of cART, which prevents HIV-mediated loss of CD4+ T cells, (and leads to degrees of recovery in those with CD4+ T cell loss) has decreased the incidence of opportunistic infections, which afflicted those with immunodeficiencies. cART has turned HIV infection from a fatal diagnosis into a treatable, chronic condition.

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In addition to effects on the immune system, HIV infects and perturbs the brain. Before the onset of effective treatment 8–15 % of HIV infected individuals developed a severe cognitive disorder, HIV-associated dementia (HAD) (Janssen et al. 1992; McArthur et al. 1993). HAD, in the post-cART era, has now been reduced to 2 % (Heaton et al. 2010). Unfortunately, cART does not completely shield the brain from HIV. Effectively treated patients with undetectable plasma viral levels, continue to have chronic immune activation in the central nervous system (CNS), along with morbidities more commonly associated with aging including atherosclerosis, metabolic syndrome, cancer and neurodegenerative disease (Alzheimer's). Furthermore, many of the widely used antiretrovirals are poorly CNS penetrable, perhaps facilitating pathogenic effects as well as HIV reservoirs in the CNS. Therefore, despite a drastic decline in HAD, there continues to be a large number of patients with mild neurocognitive disorders (MND) and asymptomatic neurocognitive impairment. Nearly half of patients in the CNS HIV Antiretroviral Therapy Effects Research (CHARTER) study, which categorized neurocognitive impairment in over 1500 patients with HIV at six large research universities in the U.S., have either mild neurocognitive disorders or asymptomatic neurocognitive impairment (Heaton et al. 2010). Similar findings were reported in a study of 400 members of the ANRS (Agence Nationale de Recherche sur le Sida) CO3 Aquitaine cohort in France (Bonnet et al. 2013). Another study selected a cohort of 200 patients in Switzerland because of undetectable plasma HIV-1 RNA, no history of major opportunistic infections of the CNS in the past 3 years, no current use of intravenous drugs, and no major depression and they continued to have neurocognitive impairment (Simioni et al. 2010).

With the onset of cART, the clinical aspects of HIV-induced CNS disease evolved. The National Institute of Mental Health and the National Institute of Neurological Diseases and Stroke convened a panel of experts to design consensus criteria for HIV-associated neurocognitive disorders (HAND). These are outlined and discussed below. Now that neurocognitive impairment is less severe than in the pre-cART era many investigators have turned their attention to the milder forms of impairment which continue to lead to significant issues with memory, motor and behavioral abnormalities that may require clinical assessment.

27.2 Diagnostic Criteria

HAND encompasses a number of disorders previously referred to as HIV encephalitis (HIVE), HIV encephalopathy, minor cognitive motor disorder, and HIV or AIDS dementia complex (ADC). The 2007 HAND classifications were intended to revise and update the 1991 American

Academy of Neurology AIDS taskforce descriptions of HIV CNS manifestations (AAN 1991). The 1991 descriptions focused largely on HAD, differentiating between HAD with motor symptoms, with behavioral/psychosocial symptoms, and HAD with both motor and behavioral/psychosocial symptoms. There was also a less severe condition classified as minor cognitive motor disorder (MCMD). The 2007 classification, known as the Frascati criteria, describes three separate clinic entities of increasing severity under the collective umbrella of HAND (Antinori et al. 2007):

- **Asymptomatic neurocognitive impairment (ANI)** is the least severe form and does not interfere with normal daily functioning. It is defined as greater than one standard deviation deficit in at least two areas of standardized neuropsychological testing that includes language, concentration, abstraction, memory, mental speed, perception and motor skills.
- **Mild neurocognitive disorder (MND)** has the same neuropsychological testing criteria (>1 SD deficit in two areas) with impairment in daily functioning defined as patient or family reported changes in mental acuity, inefficiency at home or work, or social functioning.
- **HIV associated dementia (HAD)** is the most severe form of HAND demonstrated by impairment greater than two standard deviations in at least two of the same areas described above. In addition, this impairment causes marked dysfunction at work, home or social situations.

All three HAND diagnoses assume that there are no pre-existing causes for these disorders. If patients meet criteria for major depressive episodes or substance abuse disorders, HAND diagnoses are deferred. Another important caveat is to rule out delirium, which is especially prevalent in hospitalized patients (European Delirium Association and American Delirium Society 2014; Watkins and Treisman 2015). Unfortunately, this classification system does not extensively deal with psychiatric manifestations of HIV infection including depression, mania, or anxiety. Although this classification system has been clinically validated and widely used, it requires the need of multiple hours of testing by practitioners with expertise in clinical neuropsychology to identify at least five of the following abilities: attention/information processing, verbal/language, abstraction/executive functioning, memory including learning/recall, simple motor skills, complex perceptual motor skills, or sensory perceptual abilities. Several groups have developed screening tools that may be less clinically cumbersome (Cysique et al. 2006; Munoz-Moreno et al. 2013; Koski et al. 2011; Zipursky et al. 2013; Carey et al. 2004; Brouillette et al. 2015; Power et al. 1995; Morgan et al. 2008; Sacktor et al. 2005) and may be used for clinical monitoring (Kamminga et al. 2013). Even with these tools, a major complicating

factor is the lack of a linear relationship between ANI, MND and HAD and the unpredictable time course of HAND progression. Patients with ANI and MND treated with cART rarely progress to HAD; therefore, there is no predestined progression with neurocognitive decline as in other neurodegenerative diseases. To further confuse the situation, the course of neurocognitive decline can differ dramatically along the spectrum from decline to improvement (Heaton et al. 2015). Since neurocognitive status can change, the need to diagnose the high prevalence of ANI (although by definition it does not interfere with daily function) is extremely important because the presence of ANI carries a twofold to fivefold increased risk for development of symptomatic impairment at some point in time. Specific and accurate diagnosis of ANI enables optimal clinical and pharmacologic treatment (Grant et al. 2014).

27.3 Clinical Manifestations

As described, the clinical features of HAND impairment are variable. HAND has been classically characterized as subcortical impairment or dementia with the triad of cognitive, behavior and motor dysfunction (Ances and Ellis 2007). Generally, the cognitive changes are observed long before any identifiable motor dysfunction. While many of the abnormalities are attributed to subcortical processes, deficits in learning and executive function imply the likely presence of other regional brain abnormalities (Cysique and Brew 2011; Heaton et al. 2011). Other prominent clinical manifestations include mood disturbance, psychomotor retardation and impaired memory. Motor problems are generally mild and include weakness, bradykinesia and tremor. Severe cases of HAD display features such as incontinence, paraplegia and global dementia, although these cases are now extremely rare (Glass et al. 1993). As severe HAD cases have decreased, MND and ANI continue to affect the daily lives of patients and continue to be a clinical dilemma for healthcare providers (Joska et al. 2010). Rates of MND and ANI remain high even in patients with optimal cART treatment and low HIV viral loads (Cysique and Brew 2011; Robertson et al. 2007). In the post cART era, the observed neurocognitive dysfunction seems to be independent of current CD4+ T cell counts and history of HIV-related CNS disease (Cysique and Brew 2011). Overall, the incidence of any type of HAND has increased in the post cART era, with mild disorders now accounting for the vast majority of cases (Heaton et al. 2011). The overall increased incidence of HAND is likely due to both chronic HIV infection and an aging population with HIV.

The best predictor for future HAND symptoms is CD4+ T-cell nadir (Ellis et al. 2011). Patients who receive cART before their T-cell level fall are much less likely to develop HAND, emphasizing the need for early antiviral treatment

(Heaton et al. 2010). This also points to the importance of early detection of HIV infection. Unfortunately, many infected individuals are not being identified at an early stage and more than 30% of U.S. HIV patients present to their initial clinical evaluation with a CD4+ T cell count of less than 200/mm³ (Spudich and Ances 2012). This will improve as new clinical guidelines, which encourage routine HIV screening, are widely implemented. While cART treatment has lowered viral load and reduced immune abnormalities, it is likely that the extended lifespan in combination with low viral CNS levels and chronic immune activation is driving neurocognitive changes. The changes, although mild in comparison to HAD, continue to affect the lives of those with HIV in important areas such as medication adherence (Hinkin et al. 2002), driving (Marcotte et al. 1999), work disability (Albert et al. 1995) and risky decision making (Iudicello et al. 2013). Post-cART era patients have a higher prevalence of dysfunction in both executive function and learning/memory (Heaton et al. 2011), differing from the classic triad of cognitive, behavior and motor dysfunction that dominated the clinical picture before cART. Data suggest that optimal cART does not preclude HAND but does change the clinical picture of neuropsychiatric dysfunction. Fortunately, cART significantly delays the onset and lowers the incidence of HAD. In individuals taking and adhering to cART, HIV has now turned into a chronic disease; and infected individuals are aging with HIV. Differentiating between normal aging, HIV-mediated chronic inflammation, antiretroviral effects and medical comorbidities make causative characterization of cognitive impairment very difficult but for optimal patient management, accurate diagnosis is ultimately required.

27.4 Comorbidities

There is significant overlap between the risk factors for HIV infection and risk factors for other conditions that can result in neurocognitive impairment, most notably substance abuse and psychiatric illness. The prevalence of both substance abuse and mental illness are higher in HIV positive patients (Byrd et al. 2011). There are reports that HIV-infected drug abusers have more severe neurological disease, along with higher overall viral loads (Bell et al. 1998); however, this report was in the setting of HIV encephalitis before the use of cART. In addition, such studies are difficult in this population as the reliability of drug histories is often suspect and whether effective treatment for HIV is both given and taken appropriately is often unclear (Kim and Hill 2003). In animal models, the effects of cocaine, methamphetamine and opioids in the background of HIV infection have been widely studied (Gill and Kolson 2014; Chang et al. 2014; Dutta and Roy 2012; Buch et al. 2012; Persidsky et al. 2011; Nath 2010; Loftis and Janowsky 2014). In a 2011, a large well-controlled study

(from CHARTER) comparing rates of neurocognitive impairment in HIV-infected individuals (approximately 50% with HAND) with no substance abuse, nonsyndromic substance abuse, and syndromic substance abuse revealed no effect of substance abuse on neurocognitive impairment (Byrd et al. 2011). However, in this study, most subjects were currently abstinent, so the effects of ongoing substance abuse remain unclear. Further investigations in this area may be more ethically studied in animal models. The other confounding factor in substance abuse studies is the increased non-adherence to antiretroviral treatment (Reback et al. 2003; Moore et al. 2012; Hinkin et al. 2004), thus removing that protective effect against HAND. Co-infection with other viruses such as hepatitis C (HCV) has also been proposed as a risk factor for neurocognitive dysfunction (Forton et al. 2002). The prevalence of HCV coinfection is quite high in HIV positive patients with some studies suggesting up to 80% in high-risk intravenous drug users (Gupta 2013; Sulkowski 2007). Again, the 2015 CHARTER study found that, in the absence of substantial liver damage, HCV did not contribute to neurocognitive impairment (Clifford et al. 2015).

Among the psychiatric conditions, depression is frequent in HIV infected individuals, as it is in many patients living with chronic diseases. Patients with symptomatic HIV infection are at higher risk of depression than those with asymptomatic infection and uninfected patients (Atkinson et al. 2008); viral infection itself does not appear to predispose to depression. The presence of depression in combination with HIV does appear to increase mortality rates and the level of neurocognitive impairment (Ickovics et al. 2001; Gibbie et al. 2006). Therefore, it is important for clinicians to be prudent with screening for depression, schizophrenia and bipolar disorder, which can all predispose to difficulty with medication adherence especially without adequate social support (Durvasula and Hinkin 2006).

27.5 Pathophysiology of Hand

The pathophysiology of HAND is multifactorial with contributions from viral infection, inflammatory cytokines, neuronal dysfunction and comorbid factors. HIV penetrance into the CNS is an early event after HIV transmission. Both cerebrospinal fluid studies and brain imaging have demonstrated signs of inflammation in acute HIV infection as soon as eight days after HIV exposure (Valcour et al. 2012). It is speculated that the initial inflammatory and vascular insults may lead to lifelong residual changes even with effective and prompt initiation of cART. Interestingly, a 2015 study found evidence for different states of virus and inflammation within the cerebrospinal fluid (CSF) early after infection, including a subset of cases with compartmentalization of virus and/or episodes of inflammation (Sturdevant et al. 2015). Whether

this results in an increased risk for HAND is not known but is provocative. Most of the studies on pathophysiological mechanisms of CNS disease due to HIV infection arose from the changes observed in the pre-cART era and are highly relevant for HIVE and HAD. However, the causes of the more common mild manifestations of HAND in the setting of cART are currently unknown. The unknown causes are likely still dependent upon the interplay of the virus and the immune response, including other host and exogenous factors in the now chronic condition.

The virus is dependent on hematogenous spread to enter the CNS. This is achieved by viral interaction with the CNS vascular endothelium or by passage of virally infected immune cells beyond the blood-brain barrier (BBB) (Nottet et al. 1996; Persidsky et al. 1997). A number of studies have revealed that free virus can enter the CNS. One mechanism is through a transcytosis-mediated process utilizing the viral gp120 envelope glycoprotein (Banks et al. 2001); a second is through the virus binding to the mannose-6 phosphate receptor (Dohgu et al. 2012); and a third possibility is through endothelial surface proteoglycans acting as electrostatic contacts for HIV (Bobardt et al. 2004; Moses et al. 1993). Additionally, viral proteins such as gp120 and Tat directly increase BBB permeability (Strazza et al. 2011). Increased permeability of the BBB, caused by this HIV-induced inflammation and common comorbid conditions such as substance abuse, can also facilitate HIV entry to the CNS (Shiu et al. 2007; Banks et al. 2005; Davidson et al. 2012; Mahajan et al. 2008). For example, expression of tumor necrosis factor- α (TNF- α) after acute or chronic HIV infection has been shown to increase free viral passage into the CNS (Fiala et al. 1997). In addition, illicit drugs such as cocaine can promote an additional cytokine release from resident brain monocytes, further affecting the BBB (Zhang et al. 1998). Both initial inflammatory cytokine release and chronic inflammation may permanently disrupt the BBB easing future viral entry.

Although it is possible that free virus infects the CNS, the lentivirus "Trojan horse" hypothesis was put forth to explain how cell-associated virus can be responsible for HIV infection of the brain (Peluso et al. 1985; Haase 1986; Gendelman et al. 1985, 1986). Infected monocyte derived macrophages and CD4+ T cells can migrate into the brain from the peripheral circulation to initiate and propagate infection within the CNS (Haase 1986; Wiley et al. 1986; Fischer-Smith et al. 2004). In the brain, microglia and macrophages are infected; and these long-lived cells are thought to be the reservoir and source of chronic infection (Kaul et al. 2001). Interestingly, neurons are spared from direct damage from primary HIV infection; however, products of infected cells, including viral proteins and inflammatory mediators such as cytokines have been shown to lead to neuronal damage and death. Many molecules have been implicated and well-studied in neuropathogenesis and neurotoxicity including the HIV proteins

gp120 and Tat, numerous cytokines including TNF- α , chemokines, small molecules such as arachidonic acid, nitric oxide, glutamate, and various free radicals (Kaul et al. 2001; Gonzalez-Scarano and Martin-Garcia 2005; Kraft-Terry et al. 2009; Gendelman et al. 1994; Ellis et al. 2007). While many of these molecules can be produced by infected microglia/macrophages, non-infected macrophage/microglia as well as astrocytes are activated and can contribute to local CNS damage. Astrocytes may also be susceptible to HIV infection. Although they do not support efficient and productive replication, astrocytes can still express certain neurotoxic HIV proteins (Ranki et al. 1995; Gorry et al. 1999).

Brain macrophages and microglia are the key cells infected by the HIV in the brain. SIV (simian immunodeficiency virus) infected rhesus macaques have been key to elucidating the underlying pathophysiology of HIV virus. SIV models have shown peripherally infected monocytes entering the CNS to become perivascular macrophages. The virus is thought to specifically invade the CNS in CD14+CD16+ mature monocytes that are more susceptible to HIV infection and may have preferential access to the CNS (Ellery et al. 2007; Zhu et al. 2002; Buckner et al. 2011). This is in part due to the high expression of the CCR5 coreceptor on this subset of mature monocytes (Ellery et al. 2007). HIV strains found in the CNS also have particular tropism for CCR5 (He et al. 1997). These CNS macrophages then release virus and infect surrounding cells including resident microglia (Kim et al. 2006; Soulas et al. 2009). Infected monocytes are attracted to the CNS by chemokines including monocyte chemoattractant protein type 1 (MCP-1) (Monteiro de Almeida et al. 2005) and migrate through the BBB in parallel with increased endothelial expression of adhesion molecules for monocytes. In addition to serving as a reservoir for HIV in the brain, monocytes can lead directly to neuronal dysfunction and death. Interestingly, CD16+ circulating monocytes are higher in patients with HIV (Ellery and Crowe 2005); and they may be elevated in HAND (Fischer-Smith et al. 2001). This cell population is also specifically increased in other primarily inflammatory diseases (Aguilar-Ruiz et al. 2011). In vitro HIV infected monocytes and monocyte derived macrophages have decreased ability to emigrate from the brain back out into blood vessels suggesting that these cells are confined to the CNS acting as another reservoir (Westhorpe et al. 2009).

As noted above, astrocytes may also harbor HIV infection, while the number of infected cells is generally very low (Wiley et al. 1986; Tornatore et al. 1994; Takahashi et al. 1996), extensive infection has been reported in a study on HAD (Churchill et al. 2009). In the brain, astrocytes act as an integral part of the BBB and are in direct communication with the CNS vascular endothelial cells. HIV infected astrocytes disrupt the BBB and can cause endothelial cell death

even in low numbers facilitating increased monocyte and lymphocyte migration (Eugenin et al. 2011). Both macrophages and astrocytes are implicated in the release of neurotoxic factors including inflammatory cytokines and viral proteins, and they are both likely responsible for prolonged CNS inflammation. Furthermore, a role for CD8+ T cells in pathogenesis, indicated by earlier studies in HIV infected people (Jassey et al. 1993) and SIV infected monkeys (Marcondes et al. 2001, 2015), was found in cART treated individuals with HAND (Schrier et al. 2015).

While most studies on pathophysiology of HAND have focused on virally infected macrophages, activated glia, and the production of neuroinjurious molecules in the brain, non-CNS sources are also possible. Given the effects of HIV infection on the BBB, blood-borne neurotoxic molecules, including many of those mentioned above, may enter the brain through the BBB with increased permeability. In addition, the role of the gut in HIV pathogenesis has received considerable attention, including aspects relevant for CNS pathogenesis. Bacterial products from the gastrointestinal tract, specifically LPS (lipopolysaccharide), can activate the immune system and affect the brain. The gut barrier also becomes more permeable in HIV infection, and elevated levels of LPS can contribute to a cascade of effects correlated with HAND (Ancuta et al. 2008; Ryan et al. 2001). In addition, although not studied in relationship to HAND, the gut microbiome is significantly different in those with HIV infection and correlates with immune activation (Mutlu et al. 2014). Another potential source of neuropathogenesis is iatrogenic, in the form of the antiretrovirals themselves. While potent in inhibiting the virus, the long-term effects on the CNS and other end organs is unknown, and for some, such as efavirenz, both clinical and experimental data indicate significant effects on cognition and neurotoxicity (Funes et al. 2014; Ciccirelli et al. 2011; Tovar-y-Romo et al. 2012). While many studies of candidate pathogenic molecules demonstrate frank toxic effects on neurons, changes in biochemical and cell biologic pathways have been proposed to explain the pathogenesis of HAND. As opposed to neuronal death, these changes can be reversible, perhaps correlating with the fluctuating course of neurocognitive abnormalities in some individuals and the response to antiviral treatment. These mechanisms include alterations in sphingolipid metabolism (Bandaru et al. 2013), changes in the glutamergic system (Potter et al. 2013), deficiencies in detoxifying enzymes (Gill et al. 2014) and defects in the two systems mediating protein turnover, the ubiquitin-proteasome system and autophagy (Zhou et al. 2011; Alirezai et al. 2008; Nguyen et al. 2010). While beyond the scope of this chapter, the interested reader is encouraged to reference the review articles cited above in regards to these specific topics.

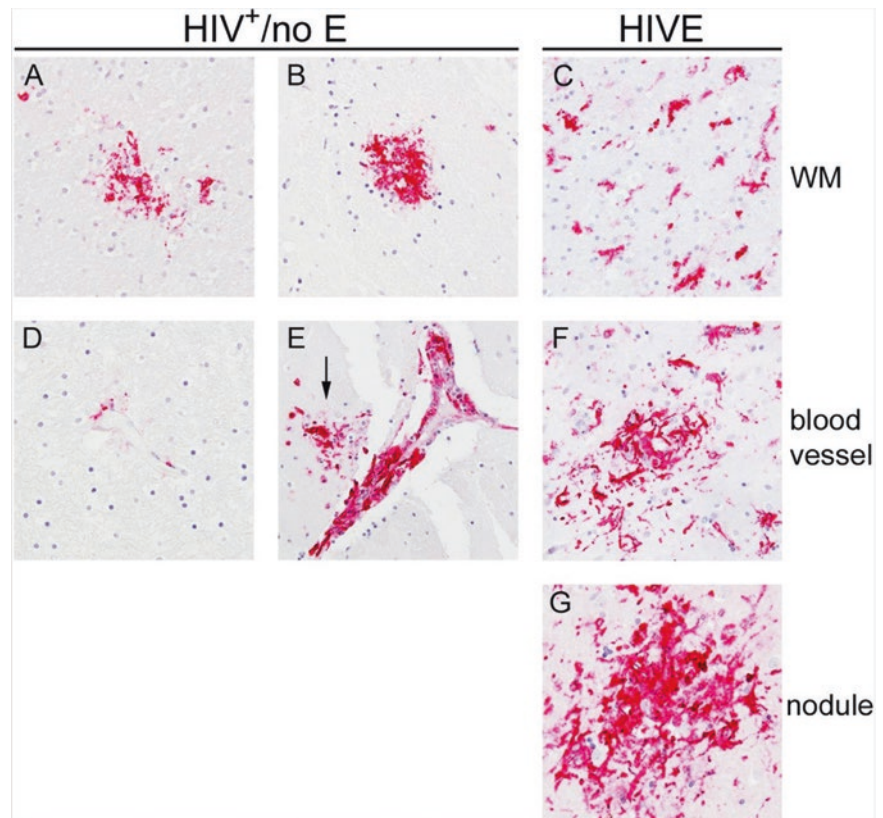
27.6 Study of HIV Induced Neuropathology

The combination of viral entry and infected macrophages in the CNS leads to the mounting of a defensive neuroinflammatory host response. This macrophage mediated inflammatory response leads to CNS disease. The hallmark neuropathologic findings in HIV infection are HIV infected macrophages (Koenig et al. 1986), which lead to microglial activation, formation of multinucleated giant cells, and diffuse astrogliosis (Michaels et al. 1988). This combination of factors contributes to the development of HIV encephalitis. Additional findings in the pre-cART era showed at the time of autopsy, the severity of HAD correlated with the total number of macrophages accumulated in the basal ganglia and the total number of perivascular macrophages (Glass et al. 1995; Fischer-Smith et al. 2001). HIV patient brains also have neuronal loss with both cortical and subcortical neurodegeneration (Moore et al. 2006; Everall et al. 1991).

Morbidity from HIV infection, including HAD, has declined with the use of cART, and studies directly on the brains of individuals with HAND are limited due to the reduced mortality and difficulty in procurement. To meet the need for research on the brain, the National NeuroAIDS Tissue Consortium (NNTC) was organized to obtain, properly store, and distribute samples of central and peripheral

nervous system tissue, other organs, biologic fluids such as CSF and plasma, from well-characterized HIV positive and negative patients, in order to support researchers studying HIV-related nervous system disorders (Morgello et al. 2001). This is ongoing at four sites in the U.S., which have already enrolled almost 3000 participants and has CNS materials from over 2000 individuals (NNTC 2015). These specimens have already been valuable, revealing the presence of synaptic and dendritic damage in HAND (Everall et al. 1999). NNTC specimens have revealed a range of brain pathology present in HAND, of which HIV related brain pathology was found to be associated with a low CD4+ T cell nadir and a higher plasma viral load within 6 months of death (Everall et al. 2009). Immunohistochemical analyses of brains from the NNTC brains indicated persistent signs of inflammation even with the use of cART and absence of overt HIVE, but the relationship between inflammation and the presence or absence of HAND was not determined (Fig. 27.1) (Tavazzi et al. 2014). Molecular studies on NNTC specimens have revealed additional possible causes of the damage in HAND, such as changes in the CNS neurovascular unit (Gelman 2015; Gelman et al. 2012). The effect of treatment was addressed in another study on NNTC specimens, with significant changes in brain gene expression found when comparing those with HAND who had a history of recent cART usage versus those naïve to treatment (Borjabad et al. 2011).

Fig. 27.1 HLA-DR expression in HIVE was seen by MΦs/microglia that accumulated perivascularly (*Panel F*) and within nodular lesions (*Panel G*). In the parenchyma, HLA-DR was expressed by activated microglia, with thickened and retracted processes, most often observed in areas of pathology (*Panel C*). In the CNS of subjects with HIV+/no E, HLA-DR was, for the most part, not expressed by cells in the parenchyma (not shown). Interestingly, when parenchymal HLA-DR was observed, it was seen on aggregates of cells, or “soft nodules” (*Panels A and B*). Rare HLA-DR expression was seen on perivascular MΦs in HIV+/no E (*Panel D*), with the exception of five subjects, who demonstrated some degree of HLA-DR+ MΦ accumulation perivascularly, that appeared to develop into cuffs in one subject (*Panel E*). A nodule also appears to have formed in the region of the cuff shown in *Panel E* (arrow). All panels shown at $\times 40$ magnification under oil (Figure 4 and legend from (Tavazzi et al. 2014) “Copyright 2014 Betham Sciences”)



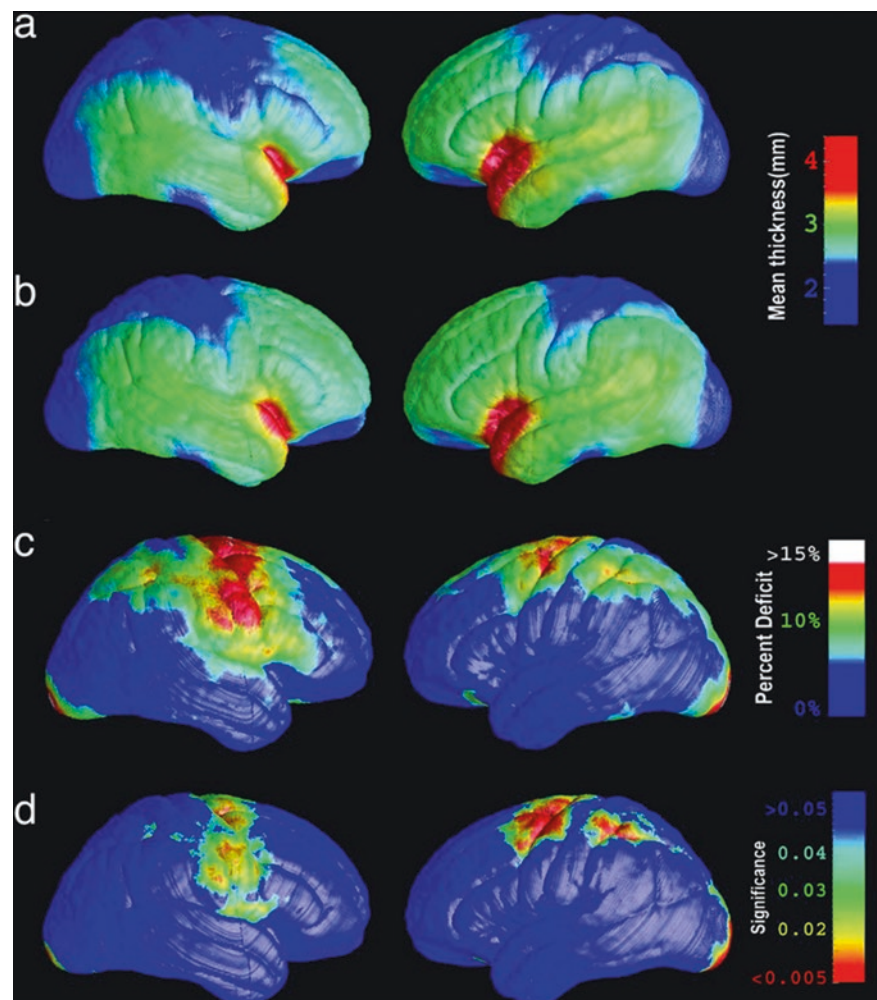
However, the exact history of antiretroviral usage, such as regimens, dosage, compliance, and how often/if they were taken in the time period before death, is often difficult to ascertain. This complicates the determination of the true nature of CNS pathology in individuals with HAND in the setting of cART.

27.7 Imaging and Biomarkers of Hand

Neuroimaging has had several recent advances that may aid in the diagnosis, treatment and investigations into the pathogenesis of HAND. Magnetic resonance spectroscopy (MRS) technology quantifies specific metabolites in regions of the brain and has revealed a number of changes within the brains of HIV infected individuals. Altered metabolites correlating with inflammation, gliosis, and injury or loss of neurons are associated with neurocognitive status (Harezlak et al. 2011; Mohamed et al. 2010). MRS alterations are still present with cART treatment (Harezlak et al. 2011; Cardenas et al. 2009). Treatment with cART can improve, but not totally reverse,

these changes (Chang et al. 1999). Magnetic resonance imaging (MRI) has revealed decrease in volume of brain structures, again most severe in HAD (Aylward et al. 1993; Stout et al. 1998). While studies on individuals before and after cART have not been reported, MRI abnormalities are still present in the post-cART era Fig. 27.2 (Ances et al. 2012; Thompson et al. 2005; Cardenas et al. 2009). Diffusion Tensor Imaging (DTI), examining fiber tracts through measurements of the diffusion process of water molecules, has revealed white matter abnormalities that correlate with HAD (Chen et al. 2009). Treatment with cART may be able to improve abnormalities found by DTI (Gongvatana et al. 2011; Wright et al. 2012). Finally, Positron Emission Tomography (PET) measures metabolic activity in the brain by monitoring glucose consumption (using fluorodeoxyglucose). Regions of altered metabolism have been identified in relationship to cognitive status specifically in HAD (von Giesen et al. 2000). In the cART era, hypometabolism is found in a high proportion of HIV infected individuals, but the link to HAND by PET is not clear (Towgood et al. 2013; Andersen et al. 2006). The role of imaging in the diagnosis

Fig. 27.2 Cortical thinning on the lateral brain surface in HIV/AIDS. (a) Average profile of cortical thickness in AIDS patients. Right hemisphere is on the left. (b) Mean cortical thickness for matched healthy controls. (c) Average percentage thinning of the cortex in AIDS relative to healthy controls. (d) Color-coded map that shows the significance of the group difference, at each cortical point (reds indicate significant cortical thinning). The band of thinner cortex encompasses the primary sensorimotor, premotor, and parietal cortices (Figure 1 and legend from (Thompson et al. 2005). Copyright (2005) National Academy of Sciences, U.S.A.)



of HAND is thus largely to rule out other disorders of the CNS and will have an ever-increasing role in studies unraveling the changes in the brain now occurring in chronic, treated HIV infection. Someday imaging may change the diagnostic criteria for HAND by better characterizing individual neurocognitive changes.

Much effort has also gone into investigating CSF and blood biomarkers that may aid in the diagnosis and study of HAND. As stated above, there have been a plethora of viral and host molecules associated with HAND; however, many were found associated with HIVE and HAD and not the less severe pathological and clinical states (MND and ASNI). While the presence of HIVE has declined, soluble markers of inflammation within the CSF persist even in treated individuals and correlate with imaging markers of CNS damage (Anderson et al. 2015). However while a number of markers in the CSF, including inflammatory molecules (neopterin, MCP-1), signs of neuronal injury (neurofilament) as well as viral escape (HIV in the CSF in the absence of detectable plasma HIV) have been studied, their utility as biomarkers is still unclear (Marcotte et al. 2013; Peluso et al. 2012; Hagberg et al. 2010; Canestri et al. 2010; Gisslen et al. 2007; Abdulle et al. 2007).

Peripheral markers would be a distinct clinical advantage, requiring a blood draw instead of neuroimaging or CSF. Before the advent of cART, an increased plasma viral load and decreased blood CD4+ T cell count correlated with the risk of HAND, similar to other HIV-induced pathologies; however, this is no longer the case (Robertson et al. 2007; Heaton et al. 2011). As indicated above, CD4+ T cell nadir, perhaps relating to the amount of maximal legacy damage HIV has caused, correlates both with risk of developing HAND and eventual neuropathology (Ellis et al. 2011; Everall et al. 2009). A 2014 study discovered that in patients with undetectable viral loads due to effective cART had an increase in CCR2 (the receptor for MCP-1, a frequently studied pathogenic marker attracting monocytes to the CSF) expression on CD14+CD16+ monocytes. Indeed this correlated with increased transmigration across an in vitro BBB model (Williams et al. 2014). Still, neuroimaging, CSF and plasma biomarkers remain in the research realm.

27.8 Treatment

The most effective treatment for any individual with HAND, as well as essential for HIV infection in general, is to suppress viral production through the use of cART. Many of the components of cART do not penetrate the brain to yield effective drug concentrations; thus, it is thought that the use of drugs with increased ability to reach the CNS and hence control virus in the brain would be useful. To accomplish this, a CNS penetration-effectiveness index (CPE) has been constructed (Letendre 2011; Letendre et al. 2008). However, it is unclear if use of a cART regimen with an increased CPE

is useful for preventing or treating HAND, due to issues inherent in the studies, characteristics of specific drugs used, differences in viral susceptibility, and possible neurotoxic effects of the drugs themselves (Libertone et al. 2014; Ciccarelli et al. 2013; Vassallo et al. 2014; Fabbiani et al. 2015; Smurzynski et al. 2011; Cysique et al. 2011). While it has been recommended that changing to a high CPE regimen may be useful in those with symptomatic HAND (Mind Exchange Working Group 2013), a 2014 randomized clinical trial found no benefit (Ellis et al. 2014).

Adjunctive (non-antiretroviral) treatments have also been studied, based on potential pathogenic mechanisms of neuronal damage. Valproic acid and lithium (targeting glycogen synthase kinase-3 β), rivastigmine (cholinesterase inhibitor), memantine (NMDA receptor antagonist), minocycline (anti-inflammatory antibiotic), thiocetic acid (anti-oxidant) deprenyl and selegiline (monoamine oxidase inhibitor), peptide T (a gp120-derived peptide), and nimodipine (calcium channel blocker) have all been studied clinically; however, no significant effects on HAND were found (Uthman and Abdulmalik 2008; Ances et al. 2008; Simioni et al. 2013; Zhao et al. 2010; Sacktor et al. 2011; Nakasujja et al. 2013; Dana Consortium 1998). Current clinical trials include studies on a number of antiretrovirals as well as paroxetine (selective serotonin reuptake inhibitor), fluconazole (antifungal), atorvastatin (HMG-CoA reductase inhibitor), and non-drug treatments (cognitive rehabilitation) (NIH 2015). While results of trials of non-drug treatments have not been reported for HAND, it is intriguing that exercise and fitness appear to be protective for HAND (Mapstone et al. 2013; Dufour et al. 2013; Fazeli et al. 2014). Indeed cognitive training and exercise have been frequently investigated for non-HIV mild cognitive impairment and early stage dementia. While some positive effects are found for such interventions, limitations to the studies make determination of benefits problematic (Rodakowski et al. 2015).

27.9 Review Questions

1. Since the wide use of cART the more severe forms of HAND are less prevalent, now the two most common clinical classifications of CNS HIV infection are
 - (a) Asymptomatic Neurocognitive Impairment and HIV-associated dementia
 - (b) Mild Neurocognitive Disorder and HIV-associated dementia
 - (c) HIV-associated dementia and HIV encephalitis
 - (d) *Asymptomatic Neurocognitive Impairment and Mild Neurocognitive Disorder*
2. The factors that may be responsible for development of HAND include
 - (a) Poor CNS penetrability of antiretrovirals
 - (b) HIV reservoirs in the CNS

- (c) Infection of neurons with HIV virus
 - (d) Production of inflammatory molecules by astrocytes
 - (e) *a, b, and d*
 - (f) All of the above
3. This is generally NOT considered one of the main clinical manifestations of HAND
- (a) Cognitive impairment
 - (b) Behavioral changes
 - (c) *Cardiovascular disease*
 - (d) Dysfunction in learning and memory
 - (e) Motor disturbances
4. Treatment for HAND
- (a) Requires treating virus in the brain with cART that penetrates the BBB
 - (b) Can be done with single drug regimens
 - (c) Requires neuroimaging confirmation and follow-up
 - (d) *Is basically the same as treatment of HIV infection*

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Neuroimmunomodulation of Human T-Lymphotropic Virus Type I/II Infection

28

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Abstract

Human T-Lymphotropic Virus type I (HTLV-1) is an oncogenic retrovirus. Infection is associated with a variety of human diseases including HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP). Large numbers of epidemiological, virological, immunological, and clinical studies on HTLV-1 and HTLV-1 associated diseases have been published, although the pathogenesis of HAM/TSP is not yet fully understood. In the last several years, researchers have shown that several key factors are important in HTLV-1 associated neurological diseases including high HTLV-1 proviral load (PVL) and a strong immune response to the virus. In this review, we summarize the pathophysiological findings on HAM/TSP and focus on the viral-host immune responses such as virus specific CD8+ T cell responses in infected individuals.

Keywords

Central nervous system • Cerebro spinal fluid • Cytotoxic T-cells • Human leukocyte antigen • Human T-lymphotropic virus type I/II—associated myelopathy/tropical spastic paraparesis • Peripheral blood mononuclear cells

28.1 Introduction

28.1.1 HTLV-1-Associated Myelopathy/Tropic Spastic Paraparesis

Human T-lymphotropic virus type 1 (HTLV-I) is the first human oncogenic retrovirus to be identified and infects approximately 10–20 million people worldwide (Poiesz

et al. 1980; de The and Bomford 1993). Endemic areas for HTLV-1 are known in the world such as southern Japan, the Caribbean, Central and South America, the Middle-East, Central and West Africa, and Melanesia. In addition, there are smaller foci in the aboriginal populations of Australia, Papua New Guinea and northern Japan (Gessain 1996). HTLV-1 has been demonstrated to be the etiological agent of both adult T cell leukemia (ATL) and a progressive neurological disease termed HTLV-1-associated myelopathy/tropic spastic paraparesis (HAM/TSP) (Hinuma et al. 1981; Gessain et al. 1985; Osame et al. 1986). 1985, Gessain first reported the high prevalence of anti-HTLV-1 antibodies in the sera of the patients with tropic spastic paraparesis (TSP) in Martinique of the French West Indies. Subsequently, Rogers-Johnson reported similar findings in Jamaica and Colombia. In 1986, Osame reported the association between HTLV-1 infection and spastic paraparesis in Kagoshima prefecture in southern Japan, where the climate is not tropical but temperate, and he proposed the diagnostic term HTLV-1-associated myelopathy (HAM). The official name of the

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disease, HTLV-1-associated myelopathy/tropic spastic paraparesis (HAM/TSP), and the clinical and laboratory guidelines for the diagnosis of HAM/TSP were formulated based on the World Health Organization (WHO) guidelines in 1987 (WHO 1989; Osame 1990). HAM/TSP is characterized by spastic paraparesis, persistent lower back pain, bladder dysfunction and mild sensory disturbances in the limbs. These features are typically symmetrical in most patients with HAM/TSP, while there are some patients whose disease progression and symptoms vary from the norm, and some patients present with atypical neurological feature like mononeuropathy (Leite et al. 2004), amyotrophic lateral sclerosis (Matsuzaki et al. 2000), or spinocerebellar degeneration (Kira et al. 1993). Interestingly, the majority of infected individuals remain lifelong asymptomatic carriers. In endemic areas, the seroprevalence varies from 1 to 20 %. Approximately 0.25 % (Kaplan et al. 1990) to 5 % of infected individuals develop HAM/TSP while 2–5 % develop ATL. The reason why only a subset of HTLV-1 infected individuals develop a clinical disease while the vast majority remain as asymptomatic carriers (ACs) are not known but it is believed to be related to genetic susceptibility associated with a deregulated immune response that results in neuropathology. Most people who go on to develop HAM/TSP have been infected with HTLV-1 for months, years or even longer.

28.1.2 Human T-Lymphotropic Virus Type-1

HTLV-1 is an exogenous, human retrovirus, which varies little in sequence. Thus is highly divergent in structure and function from the human immunodeficiency virus type-1 (HIV-1). It is classified in the genus deltaretrovirus of the subfamily Orthoretrovirinae. The HTLV-1 genome contains three open reading frames that encode four viral proteins. The three typical structural and enzymatic genes (Gag, Pol, and Env) are flanked by two long terminal repeats as seen in other retroviruses. In addition, a region called pX contains at least four partially overlapping reading frames (ORFs) encoding accessory proteins (p12, p13, p30), the post-transcriptional regulator REX (ORF3), and the TAX transactivator (ORF4).

Transmission of HTLV-1 occurs through three main routes that includes breast-feeding, sexual contact and blood transfusion. For all these routes, infected cells pass from infected individuals since HTLV-1 transmits by cell-cell contact. HTLV-1 infected cells produce and transiently store virions in extracellular adhesive structures rich in extracellular matrix components and linker proteins that are crucial for HTLV-1 cell-to-cell transmission (Pais-Correia et al. 2010). When an infected cell contacts an uninfected cell, a microtubule-organizing center is polarized at the cell-cell-

junction, and a virological synapse forms at the interface (Igakura et al. 2003). HTLV-1 integrates randomly into the host genome (Doi et al. 2005). Analyses of integration sites verify that the proliferation of HTLV-1-infected cells is clonal and persistent (Furukawa et al. 1992; Wattel et al. 1995; Etoh et al. 1997; Cavois et al. 1998). Unlike the amplification of HIV-1 proviral genomes that is associated with *de novo* infection of new cells (Miyazato et al. 2006; Taylor et al. 2006; Feuer and Chen 1992), clonal proliferation of HTLV-1 infected cells contribute to the increased number of infected cells in PBMCs.

28.2 HTLV-1 Immunity and Gene Expression in Infected Cell Populations

28.2.1 HTLV-1 in the CNS of HAM/TSP Patients

There is a strong antibody response to HTLV-1 with high titers of HTLV-1-specific IgM, IgA and IgG, which are correlated with proviral load (PVL) (Kira et al. 1992; Manns et al. 1999). Increased numbers of HTLV-1-specific HLA class I restricted CD8+ CTLs is also a noteworthy feature of HAM/TSP (Jacobson et al. 1990; Pique et al. 2000). These observations suggest that there must be persistent HTLV-1 proviral expression *in vivo*. Although HTLV-1 can infect a wide range of human and non-human cells *in vitro*, HTLV-1 has been thought preferentially to infect CD4+ cells (Richardson et al. 1990; Trejo and Ratner 2000). High HTLV-1 proviral loads are in PBMCs of patients with HAM/TSP and were higher than in asymptomatic carriers (Kubota et al. 1993; Hashimoto et al. 1998; Nagai et al. 1998), although HTLV-1 Tax-expressing cells were difficult to detect. HTLV-1 Tax encodes a transactivator protein for virus expression and plays important roles in activating cellular genes including inflammatory cytokines. Tax also has a dominant epitope recognized by HTLV-1-specific CD8+ cytotoxic T lymphocytes (Niewiesk et al. 1995). Therefore it has been suggested that strong HTLV-1 Tax expression, somewhere, is needed to drive these elevated CTL numbers. HTLV-1 proviral load and HTLV-1 Tax expression were found more frequently in the CSF than in the PBMCs of the patients with HAM/TSP (Nagai et al. 2001c; Moritoyo et al. 1999). The ratio of HTLV-1 PVL in the CSF cells to that in the PBMCs was significantly associated with clinically progressive disease and with recent onset of HAM/TSP (Takenouchi et al. 2003). In addition, HTLV-1-infected lymphocytes shared the same HTLV-1 integration site in the cellular DNA of both the CSF cells and PBMCs of the HAM/TSP patients (Cavois et al. 2000). These findings suggest that HTLV-1-infected lymphocytes migrate from the periphery into the CNS and that expression of HTLV-1 Tax in the

CNS may induce a significant immune-response prior to the development of HAM/TSP.

28.2.2 HAM/TSP Pathology

All patients with HAM/TSP have symptoms of a myelopathy but not meningitis; the brain is grossly unremarkable except for diffuse thickening of the leptomeninges (Izumo et al. 1992). The spinal cord shows mild to severe atrophy with a thickening of the leptomeninges. MRI/CT scans often detect atrophy of the spinal cord at thoracic lesions. Histochemically, chronic inflammation is mainly seen in the mid-to lower spinal cord, especially at the thoracic region and is consistent with clinical features of the patients with HAM/TSP. A number of lymphocytic infiltrates and foamy macrophages are seen in the thickened meninges, parenchyma and perivascular area of the mid-to lower spinal cords. Not only T cells but also B cells are sparse in the parenchyma and thickened meninges (Akizuki et al. 1989). NK cells are rarely observed in the lesion (Umehara et al. 1994a). Smaller numbers of infiltrating cells are also found in the midbrain, pons, medulla oblongata, cerebellum and cerebral white matter (Akizuki et al. 1988). MRI scans have detected abnormal signals of white matter in the cerebrum and these have been associated with inflammation (Kira et al. 1991). These lesions detected by MRI are usually small, less than 5 mm wide, and in the white matter. Almost all the patients with HAM/TSP do not show symptoms associated with those lesions in the cerebrum. The reasons why lesions are scattered in the CNS or why the thoracic spinal cord is mainly affected is not known. Importantly, the neuropathology of the spinal cord appears to change gradually during the progression of HAM/TSP (Umehara et al. 1993).

In cases with clinical history up to 3 years, parenchymal lesions with marked inflammation and those with degeneration rather than inflammatory changes co-exist in the spinal cord. Lymphocyte exudation occurs at random in both grey and white matter but is more frequently seen in the deeper portion of the cord than in the surface areas. Inflammation involves both the white and grey matter, but the white matter is preferentially degenerated in the lesions. Interestingly, the lateral column is commonly symmetrically and most extensively involved with inflammation, while the anterior and posterior columns are usually less affected. These lesions are associated with the presence of a number of CD4+ and CD8+ cells, foamy macrophages, and fibrillary gliosis. They are commonly seen along the course of the small vessels running from the grey matter to the white matter. B cells are also found in the lesions but mainly located in perivascular spaces of larger vessels (Iwasaki 1993). A number of infiltrating macrophages and microglia are activated with the expression of MRP14 and MRP8 (Abe et al. 1999). Neurons in the ante-

rior horn are relatively well preserved despite the presence of inflammatory cells. Immunoreactivity for HLA class I is present on endothelial cells and infiltrating mononuclear cells (Wu et al. 1993). Up-regulation of HLA class II expression is also found on endothelial cells, microglia, and infiltrating mononuclear cells in the lesions (Umehara et al. 1993). High expression of vascular cell adhesion molecule-1 (VCAM-1) on the endothelium has been demonstrated (Umehara et al. 1996). Expression of very late antigen-4 (VLA-4) and monocyte chemoattractant protein-1 (MCP-1) is also up-regulated in the infiltrating cells in active-chronic lesions of affected spinal cord. Intracellular adhesion molecules-1 (ICAM-1) and its counterpart molecule lymphocytes function-associated antigen-1 (LFA-1) are also suggested to be involved in the lymphocyte infiltration into the spinal cord (Cabre et al. 1999). Proinflammatory cytokines such as TNF- α , IFN- γ and IL-1 β were detected in perivascular infiltrating cells (Umehara et al. 1994a), while IL-1 β was predominantly expressed on the infiltrating macrophages and parenchymal astrocytes. IFN- γ is also expressed in glial cells.

In cases with disease duration of 4–6 years, smaller numbers of inflammatory cells are located in the meninges and perivascular spaces. CD8+ cell are predominantly seen in the parenchyma relative to CD4+ cells. Activated macrophages and microglia expressing MRP-8 are also present. The white matter is uniformly degenerated.

In HAM/TSP cases of longer disease duration show equal myelin and axon degeneration. Notably, tissue is largely replaced by glial scar formation with foamy cells, microglial cells and a small numbers of lymphocytes, mostly CD8+ cells with concomitant down-regulation of pro-inflammatory cytokine expression with the exception of IFN- γ (Umehara et al. 1993; Iwasaki 1990). Although a number of macrophages are detectable in the lesions, the markers of activated macrophages and microglia such as MRP-14 or 8 are down-regulated (Abe et al. 1999). Fibrous thickening of adventitia and hyalinized changes of the small vessels (hyalinous thickening of their wall) are conspicuous in both the grey and white matter in the lesion of the patients with long history. Myelin staining revealed symmetrical myelin pallor of the lateral column that is most conspicuous the entire length of the lateral pyramidal tract even cases with mild changes of the lateral column. Myelin pallor of the anterior or posterior funiculi is less involved and milder. Wallerian degeneration of ascending tracts in the posterior funiculi is less conspicuous than that of the pyramidal tract in the lumbar and sacral funiculi (Iwasaki 1990). In some cases, when the patient has had the disease for more than 20 years, a number of infiltrating CD8+ cells have been observed in the spinal cord (Iwasaki et al. 2004). The rate of progression of disease depends on the individual.

Aye et al. reported that perivascular inflammatory infiltration in HAM/TSP cerebrum was seen in deep white matter

and in the marginal area of cortex and white matter. The types of the infiltrating cells were similar both in the spinal cord and cerebrum. In addition to infiltrating cells, demyelination and axonal damage were also seen in the lesion. Therefore, they suggested that HAM/TSP inflammatory process progressed and diminished in the entire area of the CNS simultaneously and that the lesions had a correlation with the site of slow blood flow in the spinal cord of the brain (Aye et al. 2000).

28.2.3 Localization of CNS HTLV-1 and HTLV-I Tax Expression

Does HTLV-1 exist in the CNS? Pathological studies using in situ PCR techniques showed HTLV-1 DNA localized to inflammatory T lymphocyte infiltrates (UCHL-1: CD45RO positive cells) but not CD68+ cells in the affected area of spinal cords of HAM/TSP patients (Matsuoka et al. 1998). Another study using in situ hybridization showed that HTLV-1 Tax mRNA was found in infiltrating CD4+ T lymphocytes in active lesions of the affected areas of spinal cords (Moritoyo et al. 1996). Kubota et al. reported that quantitative PCR analysis showed that the amount of HTLV-1 DNA decreased concomitantly with the number of infiltrating CD4+ cells but with neither CD8+ cells nor macrophages in spinal cord lesions (Kubota et al. 1994). These findings suggest a preferential viral reservoir in the CNS in infiltrating CD4+ T lymphocytes that express HTLV-1 Tax. However, other cells may also harbor HTLV-1 in the CNS. For example, HTLV-1 RNA has been shown to localize to astrocytes (Lehky et al. 1995; Ozden et al. 2002). Recently, Afonso et al. reported the alterations in BBB function caused by HTLV-1-infected endothelial cells that may be associated with increased migration of inflammatory cells into the CNS (Afonso et al. 2008).

The proliferation and HTLV-1 Tax expression of HTLV-1 infected cells has not been determined in the CNS parenchyma and other tissues/organs of patients with HAM/TSP. In contrast, semi-quantitative studies on the distribution of HTLV-1 proviral DNA in situ suggested that strong HTLV-1 provirus signals could be detected by PCR in the spinal cord, peripheral nerves, muscles, lungs, and liver, and that weak HTLV-1 provirus signals were detected in the medulla oblongata, optic nerve, and lymph nodes (Sueyoshi et al. 1994).

28.2.4 HTLV-1 in PBMC

CD8+ cells including HTLV-1 specific CD8+ CTLs have been shown to be infected with HTLV-1 in PBMC (Hanon et al. 2000; Nagai et al. 2001a). Interestingly, HTLV-1 tax

expression in these infected CD8+ T cells rendered them susceptible to cytolysis mediated by autologous HTLV-1 specific CD8+ CTLs (Hanon et al. 2000). HTLV-1 specific CTLs may serve a dual function as both target and effector cells while HTLV-1 infected CD8+ cells have not been demonstrated in the CNS parenchyma of HAM/TSP patients.

Previous studies have shown that mononuclear phagocytes (MPs) including monocytes, macrophages and dendritic cells (DCs), are susceptible to HTLV in vivo, while the frequency of these infected MPs was determined to be 0.5–5% of the total MP population (Macatonia et al. 1992; Koyanagi et al. 1993; Makino et al. 1999). Although HTLV-1 PVL in DCs is positively correlated with that of PBMCs (Azakami et al. 2009), HTLV-1 tax expression of the MPs including CD14+ cells and macrophages was barely detectable compared with that of CD4+ or CD8+ cells in the PBMCs even after culture. More recent studies demonstrated that DCs can be efficiently infected by cell-free HTLV-1 virions in vitro (Jones et al. 2008) although this seems unlikely to contribute to maintaining the high HTLV-1 PVL in infected individuals since HTLV-1 cell-free virions have not been reported in the periphery and are known to be poorly infectious to CD4+ cells. Recently, CD14+ cells are reported to have cell surface expression of IL-15, inducing degranulation and IFN- γ production from CD8+ T cells in HAM/TSP patients. Also, it is reported that PVL in the CD14+ cells are higher in HAM/TSP patients than in ACs, suggesting HTLV-1 infection in CD14+ cells may be a major determinant of spontaneous degranulation in HAM/TSP patients (Enose-Akahata et al. 2008).

Yamano et al. has reported that CD4+ CD25+ cells are the predominant viral reservoir in HAM/TSP PBMCs and that the decreased Foxp3 expression in this subset was associated with a loss of suppressor function (Yamano et al. 2004; Oh et al. 2006; Michaelsson et al. 2008; Hayashi et al. 2008). It was suggested that this decrease of Foxp3 expression (loss of T-regulatory cell function) maybe involved in persistent T cell activation in lesions of HAM/TSP patients. In HAM/TSP patients, decreased demethylation in the FoxP3 Treg-specific demethylated region was also observed which may lead to reduced Treg function (Anderson et al. 2014). In contrast, Toulza et al. have reported that FoxP3+ cells in CD4+ T cell subset was increased in HAM/TSP patients (Toulza et al. 2008). In the report, the frequency of tax negative Foxp3+CD4+ cells was negatively correlated with the HTLV-1 specific CTL frequency, while the frequency of Tax-positive Foxp3+CD4+ cells was not. Yamano et al. reported the CD4+CD25+ CCR4+ T cell subset was increased in HAM/TSP patients and also highly infected with HTLV-1 compared with CD4+ CD25+ CCR4- cells (Yamano et al. 2009). The Foxp3-negative population in CD4+CD25+CCR4+ T-cells was significantly increased in HAM/TSP patients. In this study, purified CD4+ CD25+ CCR4+ T cells subset

proliferated independently, while CD4+CD25-CCR4- subset did not. Moreover, the HTLV-1 Tax protein increased expression of the T box transcription factor, the Th1 master regulator, and promote production of IFN- γ . Abundant CD4+CCR4+ T cells were observed to coexpress the Th1 marker CXCR3 and produce T-bet and IFN- γ in CSF and spinal cord lesions in HAM/TSP (Araya et al. 2014). In addition, immunostimulatory T cells (CD39+CD25-CD4+) were increased in HAM/TSP patients compared to those in ACs. Reduced number of Th17 cells and conversion of CD39+CD25+CD4+ T cells (suppressive phenotype) to those with increased IFN- γ , TNF- α , and IL-2 production is also demonstrated in HAM/TSP (Leal et al. 2013). Asquith et al. has also shown that CD4+ CD45RO+ T cell proliferation, which are also susceptible to HTLV-1 infection, was elevated in the PBMCs of HTLV-1-infected individuals. In this study, the increased proliferation *in vivo* was correlated with *ex vivo* HTLV-1 Tax expression (Asquith et al. 2007). Collectively, these observations suggest that the strong immune response to HTLV-1 seen in HTLV-1 infected individuals may be due to increased proliferation of HTLV-1-infected CD4+ cells and their HTLV-1 Tax expression.

28.3 Risk Factors for Acquiring HAM/TSP

Less than 5% of HTLV-1-infected individuals develop HAM/TSP while the majority remain lifelong asymptomatic carriers. It is therefore obvious that infection with HTLV-1 is necessary but not sufficient to cause HAM/TSP. The crucial risk factors that determine the outcome of an HTLV-1 infection is still unclear but include HTLV-1 proviral load, host genetic factors, variants of HTLV-1, age, and gender. Since the majority of HTLV-1 infected individuals remain lifelong asymptomatic carriers and each HAM/TSP patients greatly vary in progression, investigation of risk factors and prognosis biomarkers is important.

28.3.1 HTLV-1 Proviral Load (PVL) Is an Important Risk Factor for HAM/TSP

Higher HTLV-1 proviral loads (PVL) have been demonstrated in PBMCs of patients with HAM/TSP compared to asymptomatic carriers. Previous studies have shown that HTLV-1 PVL of HAM/TSP patients was about 5–16-fold higher than those of asymptomatic carriers (Kubota et al. 1993; Hashimoto et al. 1998; Nagai et al. 1998). The study in Kagoshima, southern Japan, is one of the largest endemic areas in the world for HTLV-1 showed that the prevalence of HAM/TSP rose steeply as the proviral load exceeded 1% of PBMCs (Nagai et al. 1998). Moreover, HTLV-1 PVL was also increased in the CSF of the patients with HAM/TSP

compared with that in the PBMCs (Nagai et al. 2001c). Importantly, the ratio of PVL in the CSF cells to that in the PBMCs was significantly associated with clinical progress and recent onset of HAM/TSP (Takenouchi et al. 2003). In a small prospective cohort study performed in the UK, Taylor et al. have validated these observations and suggests that high PVL predispose to a high risk of onset of HAM/TSP (Taylor et al. 1999). Collectively, these results demonstrate that an increased HTLV-1 PVL is a strong risk factor for HAM/TSP and is associated with clinical progression in this disorder.

28.3.2 HTLV-1 Integration and Proviral Load

HTLV-1 Tax expression in cultured PBMCs without CD8+ cells is more frequently detectable in HAM/TSP patients than in asymptomatic carriers at a given proviral load (Yamano et al. 2002; Asquith et al. 2005b). Asquith et al. reported that HTLV-1 Tax expressing rate in CD4+ cells after 18 h culture without CD8+ cells was positively correlated with HTLV-1 PVL in PBMCs (Asquith et al. 2005b). In this study, it was the rate of HTLV-1 Tax expression *ex vivo* in CD4+ cells that was thought to be a strong predictor for HAM/TSP independent of HTLV-1 PVL. The authors suggested the positive correlation between the HTLV-1 Tax expressing rate and HTLV-1 PVL was attributed to the infected cell proliferation by Tax-induced up-regulation of cellular genes involved in proliferation and deregulating cell cycle checkpoint (Bex and Gaynor 1998; Hollsberg 1999; Mesnard and Devaux 1999). They went on to demonstrate that CD4+CD45RO+ T lymphocyte proliferation was increased in HTLV-1-infected individuals *in vivo* and that the proliferation rate correlated with *ex vivo* HTLV-1 viral expression, suggesting that persistent viral gene expression *in vivo* was necessary for the maintenance of HTLV-1 PVL (Asquith et al. 2007). Recently, Meekings et al. reported that genomic integration in transcriptionally active genomic regions determines the rate of HTLV-1 proviral expression (Meekings et al. 2008). The genomic integration of HTLV-1 to certain regions may induce high HTLV-1 tax expression and subsequent Tax-induced proliferation that define HTLV-1 PVL and that may drive the expansion of HTLV-1-specific CTLs (Bangham et al. 2009). More recently, Melamed showed that HTLV-1 preferentially integrates within 1 kb of a host transcription start site and is strongly biased to specific transcription factor binding sites, in particular STAT1, p53, HDACs, and HATs. Relative position of the HTLV-1 integration site and the transcriptional orientation of the nearest host gene influence both clonal abundance and spontaneous Tax expression of the infected cells (Melamed et al. 2013). Furthermore, Niederer et al. demonstrated that AC clones

were more frequently integrated in transcriptionally active areas than clones from HAM/TSP patients or individuals who have HLA class I alleles that were able to effectively present HBZ peptides to CTL (Niederer et al. 2014).

28.3.3 Genetic Factors and HTLV-1 Tax Subtypes

While HTLV-1 PVL is an important risk factor for HAM/TSP, the range of HTLV-1 PVL seen in both groups of HAM/TSP patients and asymptomatic carriers is large with extensive overlap (Nagai et al. 1998). Of importance, HTLV-1 PVL in asymptomatic carriers of families with HAM/TSP patients was higher than those of unrelated asymptomatic carriers. In addition, since HTLV-1 varies little in sequence other than tax gene either within or between hosts (Daenke et al. 1990), the variation in proviral load among HTLV-1 infected individuals is thought to be caused by differences in the host rather than in the virus. There are wide variations (over 1000-fold) in PVL among infected individuals, although within an individual, the HTLV-1 PVL is stable over time (Takenouchi et al. 2003; Matsuzaki et al. 2001). These findings suggest that the genetic factors as well as HTLV-1 PVL and rates of HTLV-1 Tax expression in CD4⁺ cells play an important role in the development of HAM/TSP. Some HLA genotypes were found to influence HTLV-1 PVL and the risk for HAM/TSP. In a case-control studies, candidate gene association studies in the HTLV-1 endemic area of Kagoshima in Kyushu Island, Japan (Jeffery et al. 1999, 2000; Vine et al. 2002), it was reported that HLA-A*02 or Cw*08 were independently associated with a lower risk (absence or presence) for HAM/TSP. The association between these two class I alleles and PVL was found in only the group of asymptomatic carriers (Vine et al. 2002). HLA-B*5401 was also associated with higher HTLV-1 PVL and high risk for HAM/TSP. Furthermore, individuals who are heterozygous at all three HLA class I loci have a lower PVL than those who were homozygous at one or more loci (Jeffery et al. 2000). HLA class II alleles such as HLA-DRB1*0101 was also associated with high risk for HAM/TSP (Jeffery et al. 1999; Usuku et al. 1988). Other genetic factors not associated with HLA have also been reported to be associated with HAM/TSP. Vine et al. reported that a promoter polymorphism in the cytokine gene TNF- α -863A, stromal cell-derived factor-1 (SDF-1) and IL-15 also influenced the outcome of the HTLV-1 infection (Vine et al. 2002). Clearly, a wide range of genes both within and outside the HLA region will be shown to influence susceptibility to HTLV-1 and the clinical outcome of an HTLV-1 infection.

28.3.4 Cytokines and Chemokines as Risk Factors for HAM/TSP

Several cytokines and chemokines are known to be elevated in the peripheral blood and/or CSF of HAM/TSP patients, such as soluble interleukin-2 IL-1 β , granulocyte-macrophage colony-stimulating factor, IFN- γ , TNF- α , chemokine (C-X-C motif) ligand 9 (CXCL9), CXCL10, chemokine (C-C motif) ligand 5 (CCL5), and neopterin (Yamaguchi et al. 1989; Nomoto et al. 1991; Kuroda and Matsui 1993; Kuroda et al. 1993; Nakamura et al. 1993; Umehara et al. 1994a; Narikawa et al. 2005; Guerreiro et al. 2006; Tanaka et al. 2008; Tattermusch et al. 2012). In the spinal cords of HAM/TSP patients, astrocytes produce CXCL10 by IFN- γ secreted (Sato et al. 2013) by infected T cells. Subsequently, CXCL10 attracts CXC motif receptor 3⁺ T cells which include HTLV-1 infected T cells leading to further IFN- γ secretion in a positive feedback loop (Ando et al. 2013). Retrospective study demonstrated that elevated neopterin concentration, high levels of CXCL9 and CXCL10, were well-correlated with the disease progression over a 4-year-period and was better in predicting disease progression than HTLV-1 PVL in PBMCs (Sato et al. 2013).

28.4 Immune Response Against HTLV-1

28.4.1 Increased Tax-Specific CD8⁺ CTLs Have a Strong Association with the Pathogenesis of HAM/TSP

One of the most striking features of the cellular immune response in HTLV-1-infected individuals is the increased numbers of HTLV-1-specific HLA class I restricted CD8⁺ CTLs in PBL and CSF cells (Jacobson et al. 1990; Elovaara et al. 1993; Parker et al. 1994; Greten et al. 1998; Nagai et al. 2001b; Kubota et al. 2002). Cytotoxic T cells (CTLs) are an important component of the adaptive mammalian immune response to viruses and act by killing autologous cells that express viral antigen in association with MHC class-I and by suppressing viral replication by secretion of IFN- γ . While HTLV-1-specific CTLs are also detectable in PBMCs of asymptomatic carriers (Parker et al. 1992), the magnitude and frequency of these responses are clearly higher in patients with HAM/TSP, particularly in the CSF (Nagai et al. 2001c; Elovaara et al. 1993). In HAM/TSP, most of these cells express IFN- γ and TNF- α , were CD8⁺ cells and were HTLV-1-specific CTLs. The frequency of IFN- γ +CD8⁺ cells was significantly higher in the PBMCs of HAM/TSP patients than in that of asymptomatic carriers or healthy controls and was correlated with HTLV-1 PVL in PBMCs (Kubota et al. 1998, 2000), although positive correlations between CTLs frequency and HTLV-1 PVL was not always seen in other

studies. HTLV-1 PVLs were also increased in CSF cells compared with that in the PBMCs of HAM/TSP patients, and those were proportional to the frequency of HTLV-1-specific CTLs in the CSF (Nagai et al. 2001c). These observations coupled with pathological findings that infiltrating TIA-1+ cells (CTL) appear to correlate with the presence of apoptotic CD4+ T cells in inflammatory lesion in HAM/TSP (Umehara et al. 1994b), suggests that the increased HTLV-1 specific CTLs in the CNS are strongly associated with the pathogenesis of HAM/TSP.

Tax specific CD8+ CTL clones have also been demonstrated to secrete various inflammatory cytokines, chemokines and matrix metalloproteinase (MMP) such as IFN- γ , TNF- α , monocyte inflammatory protein (MIP)-1 α , MIP-1 β , interleukin(IL)-16, and MMP-9 (Biddison et al. 1997). TNF- α induces cytotoxic damage to endothelial cells, thus decreasing the integrity of the BBB. It can also directly injure oligodendrocytes. MIP-1 α and 1 β can enhance transendothelial migration of lymphocytes into the CNS. IL-16 is a chemoattractant for CD4+ cells, and CD4+ T cells are the major source of IL-2 that is required by IL-2 non-producer CD8+ cells for proliferation. Therefore, HTLV-1-specific CD8+ CTLs are an important source of proinflammatory soluble mediators that may contribute significantly to the pathogenesis of HAM/TSP.

28.4.2 Why Does the High Frequency of HTLV-1-Specific CTL Not Decrease HTLV-1 PVL?

HTLV-1 PVL and Tax mRNA positively correlated with the frequency of HTLV-1-specific CTLs in PBMCs (Yamano et al. 2002; Nagai et al. 2001b; Kubota et al. 2000). Why does the high frequency of HTLV-1-specific CTLs therefore not contribute to a decrease in HTLV-1 infected cells and PVL in the periphery of infected individuals? One possibility is that HTLV-1-specific CTLs are functionally dysregulated. Sabouri et al. reported that the frequency of intracellular perforin-positive CD8+ T cells was significantly lower in both HAM/TSP and ACs than in healthy controls (HCs) (Sabouri et al. 2008). In this paper, an inverse correlation between HTLV-1 PVL and the percent perforin-positive CD8+ T cells were observed only in HLA-A*02+ HCs but not in HAM/TSP patients. In this context, there may be differences in CTL function rather than CTL frequency. The CTL-mediated lysis effect (killing ability per CTL) of CTLs could be evaluated by measuring the effect of varying the frequency of CD8+ cells (not HTLV-1 Tax specific CTLs) on the rate of disappearance of HTLV-1 Tax-expressing cells ex-vivo (Asquith et al. 2005a). Asquith et al. reported that the CTL lysis effect of HAM/TSP patients was not different from that of ACs. In addition, the variation in HTLV-1 PVL

among HTLV-1 infected people was explained by differences in CTL lysis effect. The CTL lysis effect was negatively correlated with HTLV-1 PVL in both HAM/TSP patients and ACs groups. Interestingly, HTLV-1 PVL of HAM/TSP patients were significantly higher than ACs at any given lytic effect of CTLs. These results suggest that CTLs do contribute to decrease HTLV-1 PVL, and high HTLV-1 PVL in HAM/TSP patients is not a consequence of weak CTL lysis. Again, additional factors must also be associated with high level HTLV-1-specific CTLs that may be associated with the pathogenesis of HAM/TSP.

28.4.3 CD8+ CTL Activity and CD244-SAP Signaling

The ability of CD8+ T cells to degranulate (CD107 α) has been directly correlated with cytolytic activity of effector CD8+ T cell and has been used as a useful tool to characterize total CD8+ T cell lytic activity in various diseases (Rubio et al. 2003; Betts et al. 2003, 2006; Kozako et al. 2006; Gehring et al. 2007). Enose-Akahata et al. recently reported an increased spontaneous degranulation with IFN- γ expression (CD107 α /IFN- γ) in CD8+ cells of HAM/TSP patients but not in ACs (Enose-Akahata et al. 2008). These authors have extended these observations and have shown that the CD244/SAP pathway in CD8+ cell is involved in the active regulation of CTL of patients with HAM/TSP (Enose-Akahata et al. 2009). In this study, while the expression of CD244, a signaling lymphocyte activation molecule (SLAM) family receptor, was significantly higher on CD8+ T cells in both HAM/TSP patients and ACs than those on healthy normal donors (NDs), SLAM-related adapter protein (SAP) was over expressed only in HAM/TSP patients compared to ACs and NDs. Blockade of CD244 inhibited the degranulation and IFN- γ production (CD107 α /IFN- γ) and SAP knockdown by siRNA also inhibited IFN- γ production in CD8+ T cells of HAM/TSP patients. The results suggested that differential expression of SAP in the CD8+ cells results in a higher frequency of degranulation in HTLV-1 infected individuals, thereby contributing to the higher CTL activity observed in patients with HAM/TSP.

28.4.4 Interaction Between HTLV-1-Specific CD8+ CTL and Mononuclear Phagocytes (MPs)

While high SAP expression of CD8+ cells in HAM/TSP patients was shown to influence CTL activity, additional factors are also important. Recently, there has been a greater appreciation for the role of MPs including CD14+ cells, macrophages and dendritic cells. These populations have

been shown to play a role in the spontaneous cell proliferation that is seen in cultured PBMCs of HTLV-1 infected individuals and is inhibited by blocking with antibodies against IL-2, IL2R α , IL-15 and IL15R (Macatonia et al. 1992; Makino et al. 1999; Tendler et al. 1990; Ali et al. 1993; Azimi et al. 1999). Azimi et al. reported the role of MPs in proliferation of CTL in HAM/TSP patients (Azimi et al. 2001). Enose-Akahata et al. reported CTL degranulation to be mediated by HTLV-1 infection of MPs with the concomitant expression of IL-15 (Enose-Akahata et al. 2008). In this study, the frequency of CD107 α /IFN- γ cells in the CD8+ cells was reduced in purified CD8+ cells compared with total PBMCs in HAM/TSP patients and then restored by the addition of autologous CD14+ cells but not CD4+ cells of HAM/TSP patients. They showed that cell-to-cell interaction with CD14+ cells was necessary for degranulation and that the frequency of CD107 α /IFN- γ cells in CD8+ cells was increased by the addition of rhIL-15 or CD14+ cells and decreased by addition of both CD14+ cells and anti-IL-15 antibodies. Lastly, IL-15 expression on CD14+ cells was increased in PBMC of HAM/TSP patients compared with ACs. These findings suggested that MPs play an important role in exacerbating CTLs degranulation in HAM/TSP patients.

28.4.5 Tax-Specific CD8+ CTLs in the Brain

The phenotype of inflammatory cells in the CNS of HAM/TSP patients has been extensively studied. Significantly, later in disease, there is a predominance of CD8+ T cells in the lesions of affected spinal cord in areas that express HLA class I antigens and from which functional HTLV-1-specific CD8+ CTLs have been demonstrated (Levin et al. 1997). In addition, the infiltration of CD8+ CTLs in the affected spinal cord was characterized as TIA-1 positive (Umehara et al. 1994b; Anderson et al. 1990). TIA-1 is a monoclonal antibody that recognizes a 15-kDa granule-associated protein contained in CTLs and NK cells. Double staining for anti-CD8 and TIA revealed that 80 % of TIA-1+ infiltrating cells throughout the parenchyma and perivascular area were positive for CD8. TIA-1+ cells were scarcely observed in inactive-chronic lesions, though CD8+ cells dominated in the parenchyma and perivascular area. The number of TIA-1+ cells was clearly related to the amount of the proviral DNA in situ and the number of infiltrating CD8+ cells appeared to correlate with the presence of apoptotic cells. More recently, HTLV-1 Tax-specific CD8+ CTLs in parenchyma and meninges were directly detected in the spinal cord of the patients with HAM/TSP by using HLA-A2 HTLV-1 Tax peptide pentamers. Of importance, about 30 % of the infiltrating CD8+ cells were found to be

HTLV-1-specific CTLs (Matsuura et al. 2015). These observations continue to support a role for HTLV-1-specific CTLs as a major contributing factor in HTLV-1 associated neurologic disease.

28.4.6 Antibody Responses Against HTLV-1

As mentioned, HAM/TSP patients have significantly higher titers of anti-HTLV-1 antibodies (Abs) than ACs, suggesting the existence of a humoral immune response against HTLV-1 (Kira et al. 1992). Abs for all three HTLV-1 proteins (Gag, Env, and Tax) had higher immunoreactivity in HTLV-1 infected patients compared to NDs. Furthermore, HAM/TSP patients had higher Ab response for Gag and Env, but not for Tax, compared with ACs, but Ab responses for all 3 HTLV-1 proteins was higher compared with ATL patients. By using luciferase immunoprecipitation system, measurement of Ab response to HTLV-1 makes it possible to distinguish HAM/TSP patients from ACs at a positive rate of 85.42 % and from ATL patients at a positive rate of 75.00 % (Enose-Akahata et al. 2012). In addition, Enose-Akahata et al. reported that Abs against HBZ was detected in HTLV-1 infected individuals. Although this was not able to discriminate between AC, ATL patients, and HAM/TSP patients. Antibody responses against HBZ was also observed in CSF of HAM/TSP patients (Enose-Akahata et al. 2013). Further studies are needed to determine if these Ab responses will be of clinical use, to either predict disease risk of developing HAM/TSP, or to be able to monitor HAM/TSP progression.

28.5 Natural Killer (NK) Cell, NKT Cell and GDT Cell in Patients with HAM/ TSP

NK cell activity and frequency of this subset was reported to be significantly decreased in HAM/TSP compared with healthy controls (Azakami et al. 2009; Fujihara et al. 1991; Yu et al. 1991; Wu et al. 2000). Cytotoxic activity and antibody-dependent cell-mediated cytotoxicity (ADCC) were also lower in NK cells from HAM/TSP patients than those of controls (Yu et al. 1991). The study revealed NK cell activity was significantly increased after 4 weeks of oral administration of LcS preparation with improvements in spasticity (Modified Ashworth Scale Scores) and urinary symptoms, while HTLV-1 PVL was not changed (Matsuzaki et al. 2005). These results suggested that low NK activity may be an additional risk factor for HAM/TSP.

Human natural killer-cell receptors are expressed by NK cells and some T cells, primarily TCR+ CD8+ cytotoxic T lymphocytes. Inhibitory NK cell receptors (iNKR) can

down-regulate antigen-mediated T-cell effector functions including cytotoxic activity and cytokine release (Mingari et al. 1998; Biassoni et al. 2001). It is reported that CD8+ T cells that express the HLA-E ligand including an iNKR were significantly decreased in association with HAM/TSP but not in asymptomatic carriers (Saito et al. 2003). It was therefore suggested that the decrease in these iNKR cells in HAM/TSP patients contributes to the excessive antiviral immune response.

NKT subset was also significantly decreased in HAM/TSP compared with healthy controls (Azakami et al. 2009; Wu et al. 2000). iNKT cells are unique T cells that regulate the immune response to microbes, cancers, and autoimmunity. Frequency of iNKT, NK, and dendritic cells has been shown decreased in the peripheral blood of HAM/TSP and ATL patients (Azakami et al. 2009). In this study, an inverse correlation between the iNKT cell frequency and the HTLV-1 proviral load was found in the peripheral blood of infected individuals. In vitro stimulation of PBMCs with alpha-galactosylceramide increased iNKT cells and subsequently decreased HTLV-1-infected T cells in samples from asymptomatic carriers but not HAM/TSP or ATL patients. These authors suggested that iNKT cells contribute to the immune defense against HTLV-1 and iNKT cell depletion plays an important role in the pathogenesis of HAM/TSP and ATL.

28.5.1 Co-morbid Conditions

HTLV-1 has been known to be associated with not only HAM/TSP and ATL but also uveitis, alveolitis, myositis, arthritis, dermatitis, mononeuropathy, inclusion body myositis, hearing loss, Sjogren syndrome, Bechet disease, pseudohypoparathyroidism, and SLE (Leite et al. 2004; Morgan et al. 1989; Higuchi et al. 1992; Sugimoto et al. 1987; Vernant et al. 1988; Nakao et al. 1991; Nishioka et al. 1989; LaGrenade et al. 1990; Cupler et al. 1996; Ozden et al. 2001; Matsuura et al. 2008; Bakhshaei et al. 2015). Although HAM/TSP patients with HCV hepatitis are sometimes seen, there appears to be no epidemiological link (Taylor et al. 1999; Ijichi et al. 1993). Retrospective studies show stronger associations of HTLV-1 with HAM/TSP, uveitis, myositis and peripheral neuropathy (Gessain et al. 1985; Morgan et al. 1989; Higuchi et al. 1992; Nakao et al. 1991; Gilbert et al. 2001; Leite et al. 2003), but less clear associations are for alveolitis, arthritis and the other suggested diseases. While there is no epidemiological study for pulmonary diseases associated HTLV-1, some radiological studies suggest that there may be significantly higher radiological abnormalities in HTLV-1-infected subjects (Kohno et al. 1992; Okada et al. 2006). HTLV-1-associated myopathy, dermatitis and peripheral

neuropathy may be less frequent in southern regions of Japan than in the Caribbean and South America. Again, host genetic factors must clearly influence these conditions. Because monoclonal T cell expansion of HTLV-1 is not seen in these HTLV-1 related disorders except ATL, the immune responses in these conditions are thought to be similar to that of HAM/TSP. However, unlike HAM/TSP, there are much less reports on the pathogenesis of these other HTLV-1-associated diseases. In this context, HTLV-1 associated myositis has been studied. Pathological studies have demonstrated HTLV-1 proviral DNA and HTLV-1 Tax expression in the infiltrating cells in perimysium but not muscle fibers by in situ hybridization and in situ PCR, respectively (Higuchi et al. 1992, 1995). HTLV-1 DNA and Tax mRNA were also detected in the infiltrating cells in perimysium (Matsuura et al. 2008). These reports suggest that immunopathogenic CTLs may also be playing a similar role in these other conditions associated with HTLV-1 infection as has been suggested in HAM/TSP. Therefore, a better understanding the pathophysiology of HAM/TSP may lead to therapies that may ameliorate a number of disorders in which HTLV-1 is thought to play a role.

28.6 HAM/TSP Therapies

To date, various treatments have been used to HAM/TSP patient, such as corticosteroids (Nakagawa et al. 1996), and INF- α (Izumo et al. 1996). However, strong therapeutic efficacy of previous treatments has not been demonstrated. Although not the focus of this review, the following are select examples of treatment that are currently in clinical trials.

28.6.1 Mogamulizumab

As mentioned previously, CD4+ CD25+ CCR4+ T cell subset being a main reservoir in HAM/TSP patients (Yamano et al. 2009), Mogamulizumab, a defucosylated humanized anti-CCR4 immunoglobulin G1 monoclonal antibody, targets infected cells by marking CCR4+ T cells for elimination and reduce the number of infected cells. Using Mogamulizumab, Yamauchi et al. demonstrated the significant reduction of HTLV-1 proviral load in PBMCs from patient with HAM/TSP in vitro. As HTLV-1 is known to infect CD8+ cells as well in HAM/TSP patient (Nagai et al. 2001a), they also revealed that the majority of infected CD8+ cells are also CCR4+ (Yamauchi et al. 2015). The evidence suggests that Mogamulizumab would reduce HTLV-1 PVL by eliminating both CD4+ CCR4+ cells and CD8+ CCR4+ cells, subsequently improve chronic inflammation in HAM/TSP patients.

28.6.2 Raltegravir

Raltegravir, integrase inhibitor, have been in use for the treatment for human immunodeficiency virus type 1, same retrovirus as HTLV-1. Seegulam et al. found that Raltegravir also prevent integration of HTLV-1 in vitro for both cell-free and cell-to-cell modes of infection (Seegulam and Ratner 2011). In vivo study, HTLV-1 PVL showed transient decline in HAM/TSP patient, but it returned to a baseline level after the therapy, with no improvement in clinical manifestations. Also, there were no significant HTLV-1 PVL decreases observed in ACs (Trevino et al. 2012). However, larger cohorts are needed to assess the efficacy of this treatment.

28.6.3 Hu MiK β 1

IL-15 is one of the proinflammatory cytokines over expressed in PBMCs of HAM/TSP patients (Azimi et al. 1999), and IL-15 promoter activity have positive correlation with HTLV-1 Tax protein expression (Azimi et al. 1998). It is known that IL-15 plays a role in survival and function in HAM/TSP patients ex vivo (Azimi et al. 2001), suggesting a relation between the pathogenesis of HAM/TSP. Azimi et al. demonstrated that Antibodies against IL-15 or its receptor inhibit HAM/TSP PBMC spontaneous proliferation (Azimi et al. 1999). Therefore, Hu MiK β 1, a monoclonal antibody which block IL-15, is hypothesized to inhibit spontaneous proliferation and immune overreaction in HAM/TSP, leading to an improvement of chronic inflammation.

28.7 Concluding Remarks

In HAM/TSP, localization of HTLV-1 in CD4+ cells in the CNS may lead to the production of cytokines/chemokines that are thought to adversely effect resident cells in the CNS (bystander hypothesis) resulting in neurodegeneration of the long tract. Histological studies in HAM/TSP patients have demonstrated that although CD4+ cells are predominantly infected with HTLV-1, other cells may also harbor HTLV-1 including astrocytes and glia. All of these cells could potentially express HTLV-1 antigens (processed HTLV-1 peptides in association with HLA class I molecules) and be seen as targets by inflammatory, HTLV-1 specific CTLs. Recognition by CTLs could lead to a cascade of events that produce toxic products within the CNS associated with death or dysfunction of important neuronal components leading to clinical symptoms of HAM/TSP.

Although it has been known for over 30 years that HTLV-1 induces a chronic central nervous system dysfunction, the mechanisms associated with HTLV-1 infection still

remains unclear. New factors for the development of HAM/TSP have recently been demonstrated. Differences between HAM/TSP patients and ACs include host HLA, HTLV-1 genomic integration site, HTLV-1 Tax subtype, HTLV-1 PVL, HTLV-1 gene expression rate, adhesion or migration ability of infected CD4+ cells, and CTL activating factors (SAP in CD8+ cells and IL-15 in MPs). While high levels of HTLV-1 Tax expression has not been demonstrated to explain the strong virus-specific immune response seen in affected tissue, elevated proliferation of CD4+ and CD8+ cells in vivo may explain the continuous Tax expression that may serve to drive strong T cell responses. While HTLV-1-specific CTLs have been thought to play a role in decreasing HTLV-1 PVL, these same cells may be activated by MPs and cause immunopathology in the CNS. It is clear that further information regarding the frequency, distribution, activity and function of CTLs in the CNS of HAM/TSP as well as that of other HTLV-1 associated disorders will be needed to better understand the role that this virus plays in these diseases.

28.8 Review Questions

- Which level of spinal cord is predominantly affected in patients with HAM/TSP?
 - Cervical level
 - Thoracic level*
 - Lumbar level
- Which cells are infiltrated in the chronic phase of HAM/TSP?
 - CD4 positive T cells*
 - CD8 positive T cells
 - B cells
- Which part is mostly degenerated in spinal cord in patients with HAM/TSP?
 - Anterior column
 - Lateral column*
 - Posterior column
- Which is most unusual symptom in patients with HAM/TSP?
 - Spastic paralysis
 - Higher brain dysfunction*
 - Bladder and rectal disturbance
- Which is most endemic area of HTLV-I?
 - North America
 - Caribbean*
 - Europe
- Which is inappropriate infection route of the HTLV-I?
 - Transmission through breast milk
 - Sexual contact
 - Droplet infection*

7. Which inflammatory disease is shown to be associated with HAM/TSP?
 - (a) *Sjogren syndrome*
 - (b) Atopic dermatitis
 - (c) Crohn's disease
8. Which adhesion molecules is mostly detected in the spinal cord of HAM/TSP?
 - (a) ICAM-1
 - (b) VCAM-1
 - (c) *ICAM-1 and VCAM-1*
9. How much is the frequency of the development of HAM/TSP in HTLV-I carrier?
 - (a) *Less than 5 %*
 - (b) 10–20 %
 - (c) More than 50 %
10. Which cytokine is mostly detected at degenerated spinal cord in HAM/TSP?
 - (a) TNF-alpha
 - (b) IL-1 beta
 - (c) *IFN-gamma*

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Abstract

Viral encephalitis is a rare disorder by caused by numerous viruses. Herpes simplex virus 1 causes more cases of encephalitis than other viruses. Additional viruses that can cause encephalitis are Varicella zoster virus, human cytomegalovirus, and several RNA viruses, including West Nile virus. These viruses have novel properties with respect to epidemiology, pathogenesis, clinical features and therapeutic approaches. All viruses that cause encephalitis have the ability to induce inflammation in brain tissue (cerebral edema). Encephalitis, in general, destroys neurons, causes bleeding in the brain (intracerebral hemorrhage), and brain damage. This chapter discusses the clinical features, productive infection, latent infection, epidemiology, pathogenesis, and animal models used to examine encephalitis. Furthermore, therapeutic strategies used to treat encephalitis for many of the viruses that can cause encephalitis are discussed. The major antiviral agents used to treat herpes encephalitis are acyclovir, valacyclovir, ganciclovir, cidofovir and foscarnet. For each of these drugs, we discuss the pharmacology, mechanism of action and adverse effects.

Keywords

Arboviruses • Herpesviruses • Inflammation • Viral infection of brain

29.1 Introduction

Encephalitis is an acute inflammation of the brain that can be caused by several viruses. Members of the Herpesviridae family cause most cases of encephalitis: for example, herpes simplex virus 1, varicella zoster virus, human cytomegalovirus, human herpesvirus 6, and Epstein Barr virus. In addition, several arboviruses, including West Nile virus, Saint Louis encephalitis, Eastern encephalitic virus,

Western equine encephalitic virus, La Crosse virus, and Colorado Tick Fever can cause encephalitis. Arboviruses are RNA viruses transmitted by arthropod vectors: mosquitoes, ticks, and sandflies for example. Additional RNA viruses that are not arboviruses can cause encephalitis, and these include Mumps virus, Measles, Rubella, Henipah, and Enteroviruses. The only other DNA virus known to cause encephalitis is the JC virus, which belongs to the Polymavirinae subfamily. Although these viruses have very different biological properties, they can all enter the central nervous system, replicate in neurons, promote inflammation in the brain, and consequently cause encephalitis. In general, patients with encephalitis suffer from fever, headache, seizures, and photophobia. Less commonly, stiffness of the neck can occur with rare cases of patients also suffering from stiffness of the limbs, slowness in movement and clumsiness depending on which part of the brain is involved. Encephalitis is not a typical outcome of viral infections, but it is life threatening and

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difficult to treat. This chapter discusses the properties of viruses that cause encephalitis, how they cause clinical disease, and available therapeutic that are available.

29.2 Herpesvirus Mediated Encephalitis

The Herpesviridae family is divided into three subfamilies: α -herpesvirinae, β -herpesvirinae, and γ -herpesvirinae. Each subfamily member contains a human virus that can cause encephalitis and these viruses are discussed below.

29.2.1 Herpes Simplex Virus 1 (HSV-1) Mediated Encephalitis

29.2.1.1 Epidemiology of HSV-1

Approximately 90 % of the population is infected with herpes simplex virus 1 (HSV-1), and at least 10 % with HSV-2 (Whitley 1997; Nahmias and Roizman 1973). Humans are the only natural reservoir for this infection and no vector is needed for virus transmission (Stanberry 2004). HSV-1 primary infection occurs mainly in childhood and HSV-2 infection occurs predominantly in sexually active adolescents and young adults. HSV encephalitis (HSE) is the most common causative agent of encephalitis of the herpesviruses.

29.2.1.2 Pathogenesis and Clinical Symptoms of HSE

HSV is the most commonly identified cause of acute, sporadic viral encephalitis in the U.S. accounting for 10–20 % of all cases (Corey 2005). It is estimated that there are approximately 2000 new diagnosed HSE cases per year in the U.S. There are peaks at 5–30 years of age, and at more than 50 years of age. Since the 1940s, HSV-1 and HSV-2 have been implicated in the causation of acute necrotizing encephalitis in infants, children and adults. Encephalitis due to HSV 2 in newborn infants is a widespread disease in the brain and commonly involves a variety of other organs in the body including the skin, eyes and lungs (Stanberry et al. 2004). HSE is typically associated with necrotic cell death resulting from virus replication and inflammatory changes secondary to virus-induced immune response (DeBiasi et al. 2002). However, there is not a perfect correlation between virus burden in the brain and the severity of histological changes and neurological symptoms. Deficiency of a cellular gene, UNC-93B, was identified in a family that had a high incidence of encephalitis (Casrouge et al. 2006). The UNC-93B gene apparently plays a role in interferon signaling indicating the importance of controlling virus replication in the CNS of infected individuals.

Two recognizable groups of symptoms are seen in most patients. There are nonspecific clinical symptoms that include fever, headache, meningeal irritation, nausea, vomiting,

confusion, generalized seizures, and alteration of consciousness. The second group of changes is referred to as focal necrosis of the orbitofrontal and temporal cortexes and the limbic system, and includes anosmia, memory loss, peculiar behavior, speech defects, hallucinations (particularly olfactory and gustatory) and focal seizures. There is rapid progression in some cases with the appearance of reflex asymmetry, focal paralysis, hemiparesis and coma. Cerebral edema contributes to these symptoms and plays an important role in the outcome (Stanberry et al. 2004).

HSE is characterized by severe destruction of temporal and frontal lobe structures, including limbic mesocortexes, amygdala, and hippocampus. Without antiviral therapy, the mortality rate is as high as 70 %; but even after antiviral therapy 20 % of these patients die. Despite early treatment, chronic progressive tissue damage in magnetic resonance imaging (MRI) can be found up to 6 months following the onset of symptoms. Approximately 2/3 of HSE cases occur because of reactivation from latency (Yamada et al. 2002), which explains why there is high morbidity and long-term complications despite antiviral treatment (Lahat et al. 1999; McGrath et al. 1997; Skoldenberg 1991).

29.2.1.3 Summary of Productive Infection

HSV-1 and HSV-2 are double stranded DNA viruses with a genome size of 152 kb, and they encode at least 84 proteins. Binding and entry of HSV-1 into permissive cells is mediated by viral proteins interacting with cellular receptors (Spear 1993). After uncoating, the viral genome is present in the nucleus and viral gene expression ensues. HSV gene expression is temporally regulated in three distinct phases: immediate early (IE), early (E), or late (L) (Honess and Roizman 1974). IE gene expression does not require protein synthesis and is stimulated by VP16 (O'Hare 1993). In general, proteins encoded by IE genes regulate viral gene expression, and as such are important for productive infection. E gene expression is dependent on at least one IE protein, and generally E genes encode nonstructural proteins that control viral DNA synthesis. L gene expression is maximal after viral DNA replication, requires IE protein expression, and L proteins comprise the virion particle.

29.2.1.4 Summary of Latent Infection

Acute infection is typically initiated in mucosal epithelium, and then HSV-1 establishes latency in sensory neurons located in trigeminal ganglia (TG) or sacral dorsal root ganglia. Despite a vigorous immune response during acute infection, latency is established. As many as 20–30 % of sensory neurons are latently infected [reviewed in (Jones 2003; 1998)]. As a consequence of primary infection, HSV-1 genomic DNA is also present in the central nervous system (CNS) of a significant proportion of the adult human population (Fraser et al. 1981).

The latency associated transcript (LAT) is abundantly transcribed in latently infected neurons [reviewed in (Jones 2003; 1998)]. Mice, rabbits, or humans latently infected with HSV-1 express LAT. In productively infected cells or latently infected rabbits, an 8.3 kb transcript is expressed that has the same sense as LAT. Splicing of the 8.3 kb transcript yields an abundant 2 kb LAT and an unstable 6.3 kb LAT. The majority of LAT is not capped, is poly A-, appears to be circular, and is designated as a stable intron. In small animal models, LAT is important but not required for the latency-reactivation cycle [reviewed in (Jones 2003; 1998)]. The first 1.5 kb of LAT coding sequences are important for reactivation from latency. A study by (Umbach et al. 2008) concluded LAT is a micro-RNA (miRNA) precursor that encodes four miRNAs plus two within LAT promoter sequences. The various LAT encoded micro-RNAs inhibit expression of key viral transcriptional regulatory proteins (ICP4 and ICP0) and the neurovirulence protein ICP34.5 (Tang et al. 2008; 2009). The miRNAs that are abundantly expressed during latency do not appear to be essential for latency using a mouse model of infection (Kramer et al. 2011).

LAT interferes with apoptosis in transiently transfected cells, and in TG of infected rabbits or mice (Jones 2003). Inhibiting apoptosis may be the most important function of LAT because two anti-apoptosis genes, the bovine herpesvirus 1 LAT homologue (Mott et al. 2003; Perng et al. 2002) and the baculovirus IAP gene (Jin et al. 2005) restores wild-type levels of spontaneous reactivation to a LAT null mutant. Two additional small RNAs (s-RNAs) encoded within the first 1.5 kb of LAT coding sequences (Peng et al. 2008) inhibit apoptosis as well as productive infection (Shen et al. 2009). These LAT s-RNAs are not miRNAs because they lack Dicer cleavage sites and a mature miRNA band that migrates between 21–23 nucleotides is not detected.

29.2.1.5 Animal Models for Studying HSE

HSE occurs in a certain percentage of mice or rabbits following infection. The frequency of HSE in experimental infections is dependent on the pathogenic potential of HSV-1, and the mouse strain used for experimental infection. For HSE to occur after ocular infection, the virus must enter the TG, and then spread to the CNS, or the virus directly gains access into the brain via the optic nerve. Models have also been developed in which HSV is directly inoculated into the brain. In this model, transport from the peripheral tissue → peripheral nervous system → CNS is not important. Thus, viral genes necessary for neuronal transport and spread are not as important if the brain is inoculated.

Viral genes necessary for productive infection, inhibiting apoptosis, or inhibiting immune recognition play a significant role in the potential of HSV to initiate encephalitis. Innate immune responses play a significant role in lethal encephalitis because HSV-1 interactions with toll-like receptor 2 contributes

to HSE (Kurt-Jones et al. 2004). LAT, although not important for productive infection, enhances the frequency of encephalitis in male Balb/C mice (Jones et al. 2005). These studies add support to the concept that viral and host factors regulate the frequency of HSE.

29.2.2 Varicella Zoster Virus (VZV) Induced Encephalitis

29.2.2.1 Epidemiology of VZV

Humans are the only known reservoir for VZV, and VZV is a ubiquitous human pathogen. A serologic study of 1201 US military trainees indicated that 95.8 % of the population has been exposed to virus (Jerant et al. 1998). Chickenpox is common in childhood and affects both genders equally as well as people of all races. VSV is spread by droplet or airborne transmission and is highly contagious. Primary infection produces varicella (chickenpox), after which VZV becomes latent in neurons of TG, cranial nerve, dorsal root, and autonomic ganglia along the entire neuro-axis. Reactivation can occur decades later resulting in zoster (shingles). The details of latency and neuropathogenesis are presented below.

29.2.2.2 Clinical Features of VZV

Acute VZV infection leads to chickenpox, which results in an extensive vesicular rash (Abendorth and Arvin 2000). Chickenpox in the immunocompetent child is mostly benign and associated with lassitude and a temperature of 100–103 °F for 1–2 days. Other symptoms include malaise, itching, anorexia, weakness and exhaustion, which gradually resolve as the illness improves. The hallmark of chickenpox is the skin manifestations that consist of maculopapules, vesicles and scabs in varying stages. In general, immunocompromised children have more lesions. Although chickenpox is usually a mild disease, there are exceptions. For example, an epidemic of 292 cases of chickenpox occurred in rural India resulting in 3 deaths (Balraj and John 1994). In the United Kingdom, about 25 people die from chickenpox every year, in part because VZV vaccination is not mandatory (Rawson et al. 2001). VZV vaccine effectively prevents varicella; however breakthrough varicella and virus reactivation can still occur [reviewed by (Arvin and Gershon 1996)].

With chickenpox, the most frequent organ affected other than the skin is the CNS (Liesegang 1999). The neurologic abnormalities are often seen as acute cerebellar ataxia or encephalitis. Encephalitis is the most serious complication of chickenpox and it can be life threatening in adults. It occurs in 0.1–0.2 % of patients with chickenpox (Johnson and Milbourn 1970).

Herpes zoster is characterized by a unilateral vesicular eruption with a dermatome distribution and affects up to one mil-

lion people in the United States each year, reviewed in (Gilden et al. 2007). Since VZV is latent in most ganglia, herpes zoster can occur nearly anywhere on the body. The most common sites are the thoracic and in the cutaneous distribution of the ophthalmic branch of the trigeminal nerve. Postherpetic neuralgia (pain that persists more than 30 days after the onset of rash or after cutaneous healing) is the most serious complication in immunocompetent patients and generally occurs in patients more than 60 years old. Acute retinal necrosis caused by VZV occurs occasionally in immunocompetent patients although more recent reports have focused on ocular disease in HIV-infected patients. In HIV-infected patients, the lesions rapidly increase in size and coalesce. These lesions respond poorly to antiviral therapy and almost inevitably cause blindness in the involved eye. Retinitis is less aggressive in immunocompetent patients and can often be treated with antiviral therapy such as acyclovir. Neurologic complications associated with zoster are diverse, including motor neuropathies of the cranial and peripheral nervous system, encephalitis, meningoencephalitis, myelitis and Guillain-Barre syndrome (Liesegang 1999). Extracutaneous sites of involvement include the CNS as shown by meningoencephalitis or encephalitis. The clinical symptoms are similar to those of other viral infections of the brain. Involvement of the CNS with cutaneous herpes zoster is probably more common than recognized clinically. Classically, VZV infection involves dorsal root ganglia. Motor paralysis can occur as a consequence of the involvement of the anterior horn cells, in a manner similar to that encountered with polio. These patients may have severe pain. Herpes zoster in the immunocompromised patient is more severe than in the normal person but even in these patients disseminated herpes zoster is rarely fatal. Following a zoster outbreak, VZV can spread to blood vessels of the brain, leading to vasculopathy, particularly in immunocompromised patients.

Therapy for herpes zoster is aimed at accelerating healing, limiting the severity and duration of acute or chronic pain, and reducing complications associated with the infection. In patients who are immunocompromised, therapy should be aimed at reducing the risk of viral dissemination. Acyclovir, valacyclovir and famciclovir are all used in the U.S. for the treatment of herpes zoster. Acyclovir is approved in the U.S. for the treatment of both chickenpox and herpes zoster in the normal host. Oral acyclovir therapy in normal children, adolescents and adults shortens the duration of lesion formation by about a day, reduces the total number of new lesions by about 25 % and reduces many of the symptoms in about one-third of patients (Snoeck et al. 1999; Gnann and Whitley 2002).

29.2.2.3 Summary of Productive Infection and Latency

VZV contains many genes similar to HSV-1 suggesting they are functional homologues. Thus, the general steps during productive infection are similar for VZV and HSV-1, and

will not be discussed in detail. In contrast to HSV-1, VZV infectious virus produced during productive infection is tightly cell associated making it difficult to obtain high yields of virus.

VZV appears to be latent only in ganglia and viral genomes are primarily found in sensory neurons. Analysis of latent VZV is restricted to human ganglia obtained at autopsy. Based on *in situ* hybridization (ISH) studies combined with sequencing, four transcripts corresponding to VZV genes 21, 29, 62, and 63 have been identified in latently infected human ganglia (Gilden et al. 2007). A monospecific polyclonal antiserum directed against VZV ORF 63 protein detected this protein in the cytoplasm of neurons [reviewed in (Mitchell et al. 2003)]. These VZV proteins are primarily in the nucleus during productive infection (zoster), suggesting that the cytoplasmic location might maintain VZV in a latent state. In contrast to HSV-1, VZV does not appear to encode a LAT that is abundantly expressed during latency. Several independent studies have demonstrated that T cells, CD8⁺ T lymphocytes in particular, are crucial for controlling HSV infection in sensory ganglia (Nash et al. 1987; Simmons et al. 1992). A persistent cell-mediated immune response occurs in TG during HSV-1 latency, and that CD8⁺ as well as CD4⁺ T lymphocytes reside in TG and inhibit reactivation from latency long after acute infection is over (Nash et al. 1987; Simmons and Tschärke 1992; Simmons et al. 1992; Khanna et al. 2003; Liu et al. 1996, 2000a, b, 2001; Prbhakaran et al. 2005). In sharp contrast, persistent infiltration of lymphocytes in human or macaque TG latently infected with human VZV (Verjans et al. 2007) or simian VZV (Ouwendijk et al. 2013) does not occur. However, infiltration of lymphocytes does occur in TG when simian VZV reactivates from latency (Ouwendijk et al. 2013).

29.2.2.4 Neurological Disorders Associated with VZV

In general, neurological diseases occur as a result of reactivation from latency. The neurological complications after VZV reactivation are serious and can be life-threatening. VZV is the causal agent in 29 % of 3231 cases of encephalitis, meningitis, and myelitis, and the most common cause of encephalitis in patients over the age 65 (Rantalaiho et al. 2001). More than 500,000 Americans develop zoster (severe dermatomal distribution pain and rash) every year. Zoster is frequently followed by postherpetic neuralgia (pain that persists for months and often years after the rash disappears), myelitis, or unifocal or multifocal vasculopathy. Many cases of VZV vasculopathy, myelitis, and polyneuropathy occur in the absence of rash. Since VZV causes a wide spectrum of neurological disorders, testing for VZV DNA and antibody in cerebrospinal fluid (CSF) should be routinely performed. Proper diagnosis is critical because antiviral treatment can be curative, even after weeks to months of chronic VZV infection.

Reactivation of latent VZV in dorsal root ganglia results in a localized cutaneous eruption termed “herpes zoster” (or shingles). The annual incidence is about 1.5–3.0 cases per 1000. Zoster occurs during the lifetime of 10–20 % of all persons (Liesegang 1999). The epidemiology of herpes zoster differs from that of chickenpox. Decreasing virus-specific cell-mediated immune responses, which occur naturally as a result of aging or from immunosuppressive illness or medical treatments, increase the risk of shingles (Gnann and Whitley 2002). Most patients who develop herpes zoster have no history of exposure to other persons with VZV infection at the time of the appearance of lesions. Over 90 % of adults in the U.S. have serologic evidence of VZV infection and are at risk for herpes zoster. Patients with cancer, those receiving immunosuppressive agents and organ-transplant recipients are thus at an increased risk of shingles. Furthermore, individuals who are positive for human immunodeficiency virus also develop herpes zoster at a higher frequency than those who are negative (Liesegang 1999).

29.2.2.5 Potential Models to Study VZV Neuropathogenesis

Unlike HSV-1, VZV does not reactivate from ganglia after experimental infection of primates or rodents. However, after footpad inoculation of rats with VZV, the protein encoded by gene 63 can be detected in lumbar ganglia 1 month after infection (Debrus et al. 1995). Viral protein is also detected in neurons, both in the nuclei and cytoplasm of infected cells. An independent study using the same rat model detected VZV gene 63 DNA in 5–10 % of neurons and VZV RNA in neurons and non-neuronal cells (Kennedy et al. 1999). Simian varicella virus may also be a valuable model to study the pathogenesis of varicella virus-host interactions. Finally, VZV infection of humanized immuno-deficient mice (SCID-hu) has provided new information about viral genes that regulate viral growth and pathogenesis (Ku et al. 2005).

29.2.3 Human Cytomegalovirus (HCMV) Induced Encephalitis

29.2.3.1 Epidemiology of HCMV

HCMV is widely distributed among humans from developed and industrial nations as well as isolated aboriginal groups (Pass 1995). The prevalence of HCMV increases with age in every group that has been studied. In general, prevalence is greater and acquired earlier in life in developing countries, and in lower socioeconomic sections of developed countries (Pass 2001).

29.2.3.2 Clinical Features of HCMV

Although HCMV acute infection is usually asymptomatic in patients with intact immune systems, it is a common opportunistic pathogen in immunocompromised patients.

In HIV-infected individuals, HCMV disease appears to be due to reactivation of the latent virus. The clinical manifestations of HCMV disease are generally seen when the CD4⁺ T-lymphocyte count falls below 100 cells/ml. Retinitis is perhaps the most well known disease associated with this infection. Other features associated with infection include gastro-intestinal disease, pneumonitis and neurologic disease. HCMV is associated with various neurologic infections in persons infected with HIV, particularly inflammation of the ventricle accompanied by encephalitis (ventriculoencephalitis) and ascending polyradiculopathy (disease or injury involving multiple nerve roots). Ventriculoencephalitis usually occurs in advanced HIV infection of patients with a prior HCMV diagnosis. Polyradiculopathy is the most common CNS infection caused by HCMV and is characterized by urinary retention and progressive bilateral leg weakness. Clinical symptoms generally progress over several weeks to include loss of bowel and bladder control, and paralysis. A spastic myelopathy has been reported and sacral paresthesias may also occur. The CSF often shows a greater than normal number of cells, less than normal content of glucose, and elevated protein levels (Cheung and Teich 1999; Cheeseman et al. 2004).

29.2.3.3 Summary of Virus Lifecycle

HCMV is a β -herpesvirinae subfamily member that is an important causative agent of congenital disease, and a significant opportunistic infection (Knipe and Howley 2001). Like other herpesviruses, HCMV is a large DNA virus that is estimated to encode approximately 165 genes. In contrast to HSV-1, HCMV exhibits a restricted host range in cell culture. Primary differentiated fibroblasts show the greatest susceptibility to infection. As with other herpesvirus members, HCMV immediate early genes regulate viral transcription, and inhibit cellular responses to infection (apoptosis and interferon induction for example).

29.2.3.4 Pathogenesis and Persistence of HCMV

Unlike HSV-1 and VZV, HCMV does not establish latency in the nervous system. Myeloid-lineage hematopoietic cells (granulocytes, macrophages, and dendritic cells for example) are important targets for lifelong latency. It is now well established that one site of HCMV latency in vivo is cells of the myeloid lineage including CD14⁺ monocytes and their CD34⁺ progenitors, reviewed in (Poole et al. 2014). Low passage HCMV strains (Toledo and FIX for example) can efficiently establish latency in CD34⁺ cells whereas strains extensively passaged in cell culture (AD169 and Towne) do not because the low passage strains contain additional genes (UL133–UL138). The UL138 ORF is required for HCMV to establish and maintain a latent infection in CD34⁺ hematopoietic progenitor cells infected in vitro (Goodrum et al. 2007).

The UL138 protein localizes to the Golgi apparatus and a mutant that expresses the UL138 transcripts, but not the protein, has an impaired loss of latency phenotype (Petrucelli et al. 2012), which raises the possibility that additional viral genes located between UL133-UL138 are important for latency.

29.2.3.5 HCMV Induced Encephalitis

Approximately, two out three newborn children that have symptomatic congenital CMV infection have CNS involvement (Bopanna et al. 1992). CNS pathology includes microcephaly, elevation of cerebrospinal fluid protein, and neurologic abnormalities (poor feeding, lethargy, or generalized hypotonia for example). Cranial computed tomography scans are abnormal in 75% of symptomatic newborns, with the most common abnormality being periventricular calcification (Bopanna et al. 1997). Fortunately, only 5–10% of newborns with congenital HCMV infection are symptomatic.

29.2.4 Human Herpesvirus 6 (HHV-6) Induced Encephalitis

Like HCMV, HHV-6 is a β -herpesvirinae subfamily member. HHV-6 was originally isolated from peripheral blood leukocytes of patients with lymphoproliferative disease (Salahuddin et al. 1986) and is now recognized as the causative agent of exanthem subitum, also referred to as roseola, (Yamanishi et al. 1988). Following acute infection, HHV-6 can persist in several different cell types, including peripheral lymphocytes. Since the receptor for HHV-6 (complement-regulatory trans membrane protein CD46), is widely expressed in humans, HHV-6 has the potential to infect many cell types (Santoro et al. 1999).

HHV-6 DNA and certain viral transcripts, but not viral antigens, have been detected in brains of healthy immunocompetent humans suggesting the virus is latent, reviewed in (Reynaud and Horvat 2013). Several studies have concluded that HHV-6 can cause encephalitis (Reynaud and Horvat 2013; Yoshikawa and Asano 2000; Nagasawa et al. 2007). The clinical symptoms associated with HHV-6 induced encephalitis appear to be distinct relative to other herpesviruses because there is a cluster of convulsions during the eruptive stage. The features of HHV-6 induced encephalitis are summarized below (Nagasawa et al. 2007). First, primary infection occurs in a normally developing infant followed by the initial convulsion with the onset of encephalopathy occurring on the second day of febrile illness. Following the first convulsion, consciousness is disturbed. Frequent convulsions occur within one day after fever is resolved, which is associated with the onset of exanthem subitum (eruptive stage). Conversely, “typical” encephalitis that features convulsions and severe disturbance of consciousness has been described following initial stages of disease: then a slow recovery ensues.

29.2.5 γ -Herpesvirus Induced Encephalitis

Although Epstein Barr Virus (EBV) can cause encephalitis (Fujimoto et al. 2003; Hussain and Hussain 2013; Bhatti et al. 1990), it is a relatively rare event when compared to HSV-1 or CMV. Since EBV acute infection is primarily initiated in pharyngeal tonsils (infectious mononucleosis) and then establishes a latent infection in B cells, reviewed by (Young and Rickinson 2004; Cohen 2000), it is not surprising that encephalitis occurs only rarely.

29.2.6 Therapeutic Agents Available to Treat Herpesvirus Infections and Encephalitis

29.2.6.1 Acyclovir

Acyclovir is an acyclic guanine nucleoside analog that lacks a 3'-hydroxyl on the side chain. Acyclovir was the first drug clearly demonstrated to be effective against herpes simplex virus infections (Wagstaff et al. 1994). Acyclovir selectively inhibits viral DNA synthesis because it is preferentially activated in virally infected cells. Cellular uptake and initial phosphorylation are facilitated by the herpes virus thymidine kinase. The affinity of acyclovir for HSV thymidine kinase is about 200-fold greater than for the mammalian enzyme. Cellular enzymes subsequently convert the monophosphate to acyclovir triphosphate. Acyclovir triphosphate is present in 40- to 100-fold higher concentrations in HSV-infected than in uninfected cells, and competes for endogenous deoxyguanosine triphosphate. The triphosphate competitively inhibits viral DNA polymerases to a much greater extent than cellular DNA polymerases. The triphosphate is also incorporated into viral DNA where it acts as a chain terminator as a result of the lack of the 3'-hydroxyl group. The terminated DNA template containing acyclovir binds the enzyme and leads to irreversible inactivation of the DNA polymerase (Wagstaff et al. 1994; Scholar 2000). The oral bioavailability of acyclovir is poor and ranges from 10–30%. The drug is poorly protein bound but is widely distributed throughout body fluids and tissues including the cerebrovascular fluid. It is primarily excreted unchanged in the urine (Wagstaff et al. 1994).

Acyclovir is useful for treating infections caused by HSV, herpes zoster and for VZV infections (Whitley and Roizman 2001). Acyclovir is the major therapy used for HSV encephalitis. It should be given intravenously at a dose of 10 mg/kg every 8 h for 14–21 days. It is usually well tolerated with few side effects. Although HCMV is relatively resistant to acyclovir, some cytomegalovirus infections have responded marginally to large doses of acyclovir and it seems to be effective for the prophylaxis of cytomegalovirus infections in immunocompromised patients. Epstein-Barr virus is not sensitive to acyclovir and clinical infections do not respond to the drug.

Oral acyclovir is recommended for the treatment of VZV infection (chickenpox) in patients over 13 years-of-age who are otherwise healthy, children over 12 months of age with a chronic cutaneous or pulmonary condition, or receiving long-term salicylate therapy, and in children receiving short or intermittent courses of aerosolized corticosteroids. Intravenous acyclovir should be used for treatment of varicella infection in immunocompromised children, including those receiving high doses of corticosteroids. In general, acyclovir is the most effective agent for the treatment of infections caused by VZV (Snoeck et al. 1999).

Parenteral acyclovir is the drug of choice for the treatment of initial and recurrent mucosal or cutaneous herpes simplex infections in immunocompromised patients and for the treatment of disseminated, neonatal, encephalitic, and severe first episodes of genital herpes simplex infections in immunocompetent patients (Whitley 1997). Intravenous acyclovir should also be used for severe diseases such as encephalitis (Brady and Bernstein 2004). Acyclovir is generally well tolerated whether administered topically, orally or by the intravenous route. Gastrointestinal disturbances, headache, and rash may occur. Renal dysfunction due to crystalline nephropathy is more likely with IV administration, rapid infusion, and in patients in the dehydrated state and with underlying renal disease and large doses. CNS effects are rare but include encephalopathy, tremors, hallucinations, seizures, and coma. Due to an elevated pH, intravenous administration may also cause phlebitis and inflammation at sites of extravasation (Wagstaff et al. 1994).

29.2.6.2 Valacyclovir

Valacyclovir is an ester prodrug of acyclovir. It provides significantly better oral bioavailability compared to acyclovir. This advantage results in substantially higher serum concentrations than is possible with oral acyclovir. In addition, fewer daily doses are required with valacyclovir (Curran and Noble 2001). Valacyclovir and famciclovir are alternative drugs used for the treatment of HSV encephalitis (Griffiths 1995; Stanberry et al. 2004; Stanberry et al. 2002).

After oral administration and absorption, valacyclovir is converted to acyclovir which is the active antiviral component of valacyclovir. The antiviral activity and mechanism of action of valacyclovir is identical to that of acyclovir (Perry and Faulds 1996). The oral bioavailability of valacyclovir is significantly greater than that of acyclovir. Oral administration of valacyclovir results in plasma acyclovir concentrations comparable to those observed with intravenous acyclovir.

Valacyclovir is effective for the treatment of herpes zoster (shingles), for the treatment of initial and recurrent episodes of genital herpes, and for suppression of recurrent genital herpes in immunocompetent and HIV-infected patients. It is also indicated for the reduction of transmission of genital herpes in immunocompetent individuals, and for the treatment of cold

sores. Valacyclovir appears to be equally effective in treating herpes zoster and recurrent genital herpes in immunocompetent adults. Valacyclovir has shown efficacy in the prophylaxis of cytomegalovirus infections in transplant patients (Perry and Faulds 1996).

Like acyclovir, valacyclovir is a well-tolerated drug. The most common adverse effects of valacyclovir are headache and nausea. Other adverse effects associated with valacyclovir administration include vomiting, weakness, dizziness, and abdominal pain. Thrombotic thrombocytopenic purpura/hemolytic uremic syndrome has also been reported in a few patients after high doses of valacyclovir as has confusion, hallucinations and nephrotoxicity (Perry and Faulds 1996; Curran and Noble 2001).

29.2.6.3 Famciclovir

Famciclovir is an oral prodrug of the antiviral agent penciclovir. Famciclovir lacks intrinsic viral activity and this drug owes its activity to formation of penciclovir. Penciclovir is an acyclic guanine nucleoside analog. Penciclovir and its prodrug famciclovir are chemically similar to acyclovir. Penciclovir differs structurally from acyclovir only by the presence of an additional hydroxyl group. Famciclovir is the diacetyl 6-deoxy analogue of penciclovir. The mechanism of antiviral activity of penciclovir is similar to acyclovir. Both drugs inhibit viral DNA synthesis (Scholar 2000). Penciclovir is rapidly and selectively phosphorylated in virus-infected cells by viral thymidine kinase to the monophosphate and this is followed by further phosphorylation to the triphosphate, which is the active form of the drug. Over 90% of penciclovir triphosphate in virus cells is the (S)-enantiomer, a competitive inhibitor of DNA polymerases with respect to the natural substrate deoxyguanosine triphosphate (dGTP). Inhibition of the polymerase results in prevention of viral replication by inhibition of viral DNA synthesis. The R-enantiomer of penciclovir triphosphate has only minimal activity on viral DNA polymerases (Scholar 2000). In contrast to acyclovir triphosphate, which is an obligate DNA chain terminator, penciclovir triphosphate allows DNA chain extension owing to its free hydroxyl group; however, penciclovir appears at least as effective as acyclovir as an inhibitor of herpes virus DNA synthesis.

Famciclovir is rapidly converted to penciclovir in intestinal and liver tissue after oral administration. More than half of an oral dose of famciclovir is excreted in the urine as unchanged penciclovir. The plasma elimination half-life of penciclovir is about 2 h, similar to that of acyclovir; however, the intracellular half-life of penciclovir in herpes virus-infected cells is considerably longer than that of acyclovir.

Oral famciclovir is used for the treatment of immunocompetent patients with herpes zoster infections and for the treatment and suppression of recurrent genital HSV. It is also effective for the treatment of recurrent mucocutaneous her-

pes simplex in both immunocompetent and immunocompromised patients. Famciclovir is fairly well tolerated. Adverse effects include headache, dizziness, nausea, and diarrhea (Scholar 2000).

29.2.6.4 Ganciclovir

Ganciclovir is an acyclic guanine nucleotide analog with a structure similar to acyclovir but with an additional hydroxymethyl group on the acyclic side chain. It is inhibitory to all herpes viruses but is especially active against cytomegalovirus. Like acyclovir and penciclovir, ganciclovir inhibits viral DNA synthesis. Also like these other agents, the virus-induced enzyme to the monophosphate form first phosphorylates it intracellularly. Cellular enzymes catalyze further phosphorylation to the di and tri phosphates. Intracellular ganciclovir triphosphate concentrations are tenfold higher than those of acyclovir triphosphate and decrease much more slowly with an intracellular half-life of elimination greater than 24 h. These differences probably explain at least in part the greater activity of ganciclovir against HCMV and provide a rationale for single daily doses in suppressing HCMV infections. (Scholar 2000; Markham and Faulds 1994). Ganciclovir-triphosphate acts as an inhibitor and substrate for the cytomegalovirus DNA polymerase. Ganciclovir-triphosphate competitively inhibits the binding of deoxyguanosine triphosphate to DNA polymerase that results in the inhibition of DNA synthesis and termination of DNA elongation. Ganciclovir is incorporated into both viral and cellular DNA. It appears to limit viral DNA synthesis and packaging of viral DNA into infectious units.

The bioavailability of orally administered ganciclovir is quite low. In patients with normal renal function, the plasma half-life is about 2–4 h. Concentrations of ganciclovir in cerebrospinal fluid are lower than those in serum after intravenous administration (Markham and Faulds 1994). More than 90% of the drug is eliminated unchanged in the urine. The plasma half-life increases as creatinine clearance declines and may reach 28–40 h in patients with severe renal insufficiency. Ganciclovir is an effective antiviral agent for the treatment of serious life threatening or sight-threatening HCMV infections in immunocompromised patients. Intravenous ganciclovir, foscarnet or the combination of both are recommended for the treatment of HCMV neurological syndromes (Markham and Faulds 1994).

The dose limiting and most common adverse effects with intravenous and oral ganciclovir is bone marrow suppression (anemia, leukopenia, neutropenia and thrombocytopenia). These effects are usually reversible upon withdrawal of the drug. CNS side effects are less common and range in severity from headache to behavioral changes to convulsions and coma. Fever, edema, phlebitis, disorientation, nausea, anorexia, rash, and myalgias have also been reported with ganciclovir therapy (Markham and Faulds 1994).

29.2.6.5 Foscarnet

Foscarnet is an inorganic pyrophosphate analog that has antiviral activity against all herpes viruses and the human immunodeficiency virus. Foscarnet is a pyrophosphate analogue that acts as a noncompetitive inhibitor of many viral RNA and DNA polymerases as well as HIV reverse transcriptase (Chrisp and Clissold 1991). It is approximately 100 fold more effective against the herpes virus DNA polymerase than the cellular DNA polymerase- α ; however, some human cell growth suppression has been observed with high concentrations in vitro. Inhibition of DNA polymerase results in inhibition of pyrophosphate exchange which prevents elongation of the DNA chain (Scholar 2000). Similar to ganciclovir, foscarnet is a virostatic agent. Foscarnet is not a nucleoside and thus is not phosphorylated. It reversibly blocks the pyrophosphate-binding site of the viral DNA polymerase in a noncompetitive manner and inhibits cleavage of pyrophosphate from deoxynucleotide triphosphates.

The oral bioavailability of foscarnet is poor so intravenous therapy is needed to treat viral infections. It is fairly well distributed throughout the body with CSF levels averaging two-thirds of those in plasma. Foscarnet is taken up slowly by cells, and biotransformation of foscarnet does not occur. The drug is excreted mainly unchanged in the urine (Chrisp and Clissold 1991). Foscarnet is frequently used for treatment of HCMV retinitis and mucocutaneous acyclovir-resistant HSV infections. It may also be beneficial in other types of CMV or HSV infections (Wagstaff and Bryson 1994).

In contrast to ganciclovir, foscarnet is not associated with dose-limiting neutropenia, enabling it to be used in combination with zidovudine and other bone marrow suppressant drugs. Nephrotoxicity and hypocalcemia are the major dose-limiting toxicities. Renal toxicity can be minimized with adequate hydration. Changes in serum calcium and phosphate levels may be related to incorporation of the drug into bone. Other adverse effects of the drug include tremor, headache, fatigue, nausea, and vomiting. Some cases of penile and vaginal ulceration have also been reported.

29.2.6.6 Cidofovir

Cidofovir is an antiviral cytidine nucleotide analog with inhibitory activity against HCMV and other herpes viruses. Cidofovir is first converted to an active diphosphate form by cellular enzymes. Antiviral effects of cidofovir are due to inhibition of viral DNA polymerase by the diphosphate metabolite (Scholar 2000; Neyts and De Clercq 1994; Plosker and Noble 1999). The diphosphate probably interacts with DNA polymerase either as an alternate substrate (incorporation at the 3'-end or within interior of DNA chain) or as a competitive inhibitor (with respect to the normal substrate dCTP). Cidofovir inhibits HCMV DNA synthesis at intracellular concentrations 1000-fold lower than are

required to inhibit cellular DNA synthesis (Neyts and De Clercq 1994). For HSV-1 and HSV-2, corresponding concentrations are at least 50-fold lower.

Cidofovir has poor oral bioavailability (<5%) and is therefore administered intravenously. It has a long intracellular half-life that allows for a prolonged interval between maintenance doses. Cidofovir is excreted extensively by the kidneys and is eliminated almost entirely as unchanged drug in the urine (Plosker and Noble 1999). Intravenous cidofovir is well tolerated. The major treatment limiting toxicity of this drug is irreversible nephrotoxicity (Plosker and Noble 1999). Intravenous pre-hydration with normal saline and administration of oral probenecid must be used with each cidofovir infusion to lessen the effects on the kidney. Serum creatinine and urine protein must be monitored with each infusion and adjusted accordingly. Other adverse effects associated with its use are neutropenia and peripheral neuropathy (Plosker and Noble 1999).

Cidofovir, ganciclovir, and foscarnet are effective at treating HCMV disease, and are approved for use in the U.S. (Cheeseman et al. 2004; Cheung and Teich 1999). Antiviral therapy for esophagitis, enterocolitis, encephalitis, peripheral neuropathy, polyradiculoneuropathy and pneumonitis is usually treated with intravenous ganciclovir or foscarnet regimens similar to those used for retinitis. Patients with CNS or neurologic disease often respond best to combination therapy (Arribas et al. 1996).

29.3 Arboviruses

Arboviruses are a group of RNA viruses transmitted by arthropod vectors. Clinical symptoms occur between 3–15 days after exposure and typically consist of fever, headache, and malaise, reviewed in (Kuno and Chang 2005). Less frequently, life-threatening encephalitis and hemorrhagic fever can occur. Arboviruses are maintained in nature by cycling between a vector, (mosquitoes, ticks, sandflies, as well as other arthropods) that consumes the blood of vertebrates. Vertebrates that have their blood consumed act as the hosts, with each vector usually preferring the blood of a specific species, making those species the host. Transmission between the vector and the host occurs when the vector feeds on the blood of the vertebrate, consequently the virus establishes

infection in the salivary glands of the vector. High levels of infectious virus must be present in the blood of the host to allow transmission to the vector. If high levels of virus are not present in the host, viremia is not achieved and this infection is referred to as a “dead end host”, which cannot be transmitted back to the vector. Below is a discussion of the major arboviruses that pose a health threat to humans.

29.3.1 West Nile Virus (WNV) Induced Encephalitis

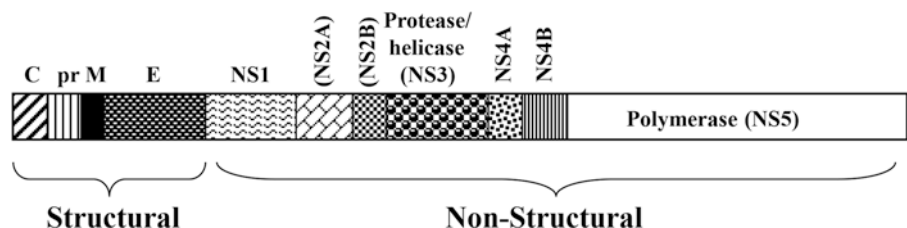
29.3.1.1 Summary of Virus Lifecycle and Virus Transmission

West Nile virus (WNV) is a member of the Flaviviridae family (Lindenback and Rice 2001), and is a small positive strand RNA virus that is approximately 11,000 bases long. Genomic RNA serves as a messenger RNA that is translated into a single polypeptide. This polypeptide is then cleaved into at least 10 discrete proteins by cellular proteases and a virally encoded serine protease. Three structural proteins (C, prM, and E) are produced from the polypeptide (Fig. 29.1). Seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) are generated from the polypeptide. NS5 is the most conserved protein in the Flaviviridae family because this protein is the viral encoded RNA dependent RNA polymerase. The other non-structural proteins are not as well conserved suggesting they have virus-specific functions.

WNV has a broad range of antigenic variation and restriction-length polymorphisms indicating WNV has a highly variable genome. Strains from Africa, Europe, and the Middle East form a distinct group relative to strains isolated in India and the Far East (Price and O’Leary 1967). WNV is widely disseminated throughout Africa, Europe, the Middle East, and the Far East. During the summer of 1999, WNV was introduced to the east coast in the United States, and has since spread westward.

WNV can be readily grown in a variety of mammalian cell lines, and *Drosophila* cells (Lindenback and Rice 2001). In the wild, WNV is vectored by female mosquitoes of the genus *Culex*, and can infect a variety of small rodents and birds. In addition, WNV can infect soft and hard ticks under natural and experimental conditions. Almost all species of birds tested (wild species, chickens, and pigeons) develop viremia. During the recent US outbreak, a number of crows have been killed by

Fig. 29.1 Schematic of West Nile Virus genome. The various genes of the WNV genome are presented, and those comprising the non-structural proteins are denoted. For details, see text



the virus, which has been used to track virus spread (Anderson et al. 1999). Sporadic cases of naturally acquired infections can occur in horses, and these infections can lead to encephalitis. Bovine species do not develop viremia, but antibodies in cattle are prevalent. Finally, dogs are susceptible to infection, but low viremia levels preclude a significant role in virus transmission. Humans are a dead end host and human to human transmission is rare, but as described below infections can be serious.

29.3.1.2 Pathogenesis of encephalitis

The incubation period is 1–6 days, and the typical case is mild. Typical clinical features are fever, headache, backache, generalized myalgia, and anorexia. Rash occurs in about 1/2 of the cases, the rash is usually roseolar or maculopapular, and usually involves the chest, back, and upper extremities. The disease usually runs its course in 3–6 days, and is usually milder in children. WNV can also cause severe, potentially fatal neurological disease, including encephalitis, meningitis, paralysis, and anterior myelitis. Although neurons are the primary target of WNV infection, a hallmark of WNV encephalitis is the accumulation of inflammatory infiltrates extending from the meninges in the brain parenchyma. These infiltrates are primarily comprised of lymphocytes and macrophages (Kelley et al. 2003). Most cases of WNV induced encephalitis generally occur in older individuals.

29.3.1.3 Animal Models for Studying WNV Induced Encephalitis

Mice have been used extensively to examine virus host interactions, and to examine virus induced encephalitis. The adaptive immune response plays a crucial role in controlling WNV infections in mice, including encephalitis (Diamond et al. 2003; Shresta and Diamond 2004; Wang et al. 2003). WNV infection leads to a toll-like receptor 3 dependent inflammatory response, which promotes brain penetration of the virus, neuronal injury, and enhanced encephalitis (Wang et al. 2004). Since toll-like receptor 3 recognizes double stranded RNA and promotes innate immune responses, this was somewhat surprising. The innate immune response also protects against WNV induced encephalitis because when mice lacking the alpha/beta interferon receptor are infected with WNV an increase in the frequency of encephalitis is seen in mice (Samuel and Diamond 2005). With the availability of a number of knockout mice, future studies should lead to identification of additional cellular factors that regulate WNV induced encephalitis.

29.3.2 Other Arboviruses Associated with Encephalitis

In addition to WNV, there are several additional encephalitic viruses that are transmitted via an insect vector, reviewed in (Stahl et al. 2011). With respect to North and South America,

the notable arboviruses that cause encephalitis include Saint Louis encephalitis, Eastern encephalitic virus, Western equine encephalitic virus, La Crosse virus, and Colorado Tick Fever virus. In addition, there are several arboviruses that can cause encephalitis in other parts of the world, and these include Japanese encephalitis virus, Tick-borne encephalitis virus, and Toscana virus. Like WNV, these are RNA viruses.

29.3.3 Therapy

Currently there is no specific treatment for arboviruses. Treatment consists only of supportive and symptomatic care, including support of respiration, intravenous fluids and prevention of secondary infections. In addition, insect repellents and other antivectorial measures are used to reduce the incidence of human transmission.

29.4 Other Encephalitic Viruses

The final category of encephalitic viruses are non-herpesviruses that are not arbovirus members, reviewed in (Stahl et al. 2011). All viruses included in this category are transmitted via human to human and have no intermediate vector. The RNA viruses included in this category are Mumps virus, Measles, Rubella, Hendrix, and Enteroviruses. The only other DNA virus known to cause encephalitis is the JC virus, which belongs to the Polymavirinae subfamily.

29.5 Review Questions

1. What are the common non-specific symptoms of a patient in the early stages of encephalitis?
2. If you are a physician and you diagnose a patient with encephalitis, which virus family would you first expect and how would you treat these patients?
3. A patient who has been on vacation in a tropical setting exhibits signs of encephalitis, what family of viruses would you suspect to cause this disease? What is the logic for your choice?
4. What are the major differences between herpesviruses and arboviruses?
5. Can West Nile Virus be spread from human to human?
6. If you develop shingles and then develop headaches and other symptoms consistent with encephalitis, what virus might be the culprit?
7. There are three subfamily of herpesviruses? Which one would you suspect is the most common causative agent of encephalitis and why?

29.6 Answers

1. Patients in the early stages of encephalitis suffer from fever, headache, seizures, and photophobia. Less frequently, patients can also experience stiffness of the neck, stiffness of the limbs, slowness in movement, and clumsiness.
2. A member of the herpesviridae family would be expected. In particular, HSV-1, would be a reasonable guess because it causes encephalitis more frequently than other herpesviruses. Acyclovir or derivatives of acyclovir would be effective because this family of drugs inhibits the viral encoded thymidine kinase.
3. I would suspect arboviruses because they are frequently spread by insect bites, in particular mosquitoes.
4. Herpesviruses are large double stranded DNA viruses (>130,000 base pairs) that encode more than 80 viral proteins. Conversely, arboviruses are all RNA viruses that have a genome of approximately 10,000 bases that encode approximately 10 viral proteins. Transmission of arboviruses to humans is usually via an insect vector. In contrast, herpesviruses are spread via human to human spread; most common mechanism of spread is by aerosol and to a lesser extent by sexual transmission.
5. No. Human to human spread does not normally occur. Humans are considered to be a dead-end host. Birds commonly develop viremia and virus replication is high in birds.
6. Varicella Zoster Virus (VZV), the "Chicken Pox Virus". The rationale for this suspicion is many elderly suffer from VZV reactivation from latency, which frequently leads to chicken pox. When VZV reactivates from latency in the elderly, the virus may also enter the central nervous system where it can cause encephalitis.
7. ANSWER: Since α -herpesvirinae subfamily members establish a latent infection in sensory neurons, there ability to infect neurons is why they cause encephalitis more frequently. β - or γ -herpesvirinae subfamily members typically have a more restricted cell tropism and establish a latent infection in non-neural cells. Thus, they do not cause encephalitis as frequently as members of the α -herpesvirinae subfamily.

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Abstract

AD is the most common form of elderly dementia and has no effective therapy. Clinical diagnosis is based on the evaluation of cognitive function and lab tests. Pathological diagnosis is based on post-mortem neuropathology, defined by three hallmarks: senile plaque, NFT, and neuronal cell loss. Senile plaque mainly consists of A β aggregates and serves as a focal point of astrocyte and microglial activation, while NFT contains hyperphosphorylated tau. A β is produced during the processing of APP by BACE and the γ -secretase complex that includes PS1. Both APP and PS1 are causative genes of AD, while apoE4 is a risk factor allele. The A β load in CNS is maintained by balancing its production and clearance, which is mediated by passive diffusion to blood stream, degradation by A β degrading enzymes (IDE and neprilysin), and microglial phagocytosis. A β production, deposition, and neurodegeneration are significantly regulated by astroglial and microglial activation via direct contact as well as secretion of a number of neurotoxins. Specific pro-inflammatory cytokines, chemokines, and excitotoxins have been characterized as mediators of neuroinflammation and neurotoxicity. Currently their specific roles in mediating the pathogenesis in AD are being explored. Those diverse studies are generating a large number of potential therapeutic targets for treating AD. Beneficial effects have been reported for anti-inflammatory drugs, such as NSAIDs, to treat glial inflammation and disease progression.

Keywords

Amyloid precursor protein (APP) • Apolipoprotein E (apoE) • β -amyloid peptide (A β) • β -amyloid precursor protein converting enzyme (BACE) • Insulin degrading enzyme (IDE) • Neurofibrillary tangle (NFT) • Oxidative damage • Presenilin-1 (PS1)

30.1 Introduction

Alzheimer's disease (AD) is the most frequent neurodegenerative disease and the most common cause of dementia. While AD affects multiple systems of the central nervous system (CNS), the classic and most frequent initial symptom is the loss of short-term memory that progresses to profound

cognitive failure. As initially observed by Alzheimer, brains of AD patients exhibit two hallmark neuropathological features: (1) senile plaques containing depositions of amyloid- β peptide (A β) and (2) neurofibrillary tangles (NFT) containing hyperphosphorylated microtubule-associated protein tau. Quantitative evaluation has revealed that in early stages of AD there is a significant loss of neurons in brain regions specifically involved in memory. Genetic studies have identified causative genes, amyloid precursor protein (APP), presenilin-1 (PS1), and presenilin-2 (PS2), which established that aberrant proteolytic processing of the APP leading to the formation of the A β 1-40 and 1-42 is primarily responsible for the pathogenesis of familial AD. However,

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the mechanisms by which APP processing leads to senile plaque deposition, NFT formation, and neuronal cell death remain elusive. The objective of this chapter is to review the current understanding of these mechanisms and the role played by neuroimmune interactions, primarily innate immunity in the CNS, in the disease process.

30.2 Clinical Features, Epidemiology, and Pathology

30.2.1 Symptoms (Memory Loss to Progressive Dementia)

Alzheimer's Disease is the leading cause of dementia, without effective therapy (Sisodia 1999). The symptoms of classic AD begin with a loss of short-term memory that slowly progresses to affect all cognitive functions, including long-term memory and executive functions (Corey-Bloom et al. 1994). Initially, impaired short-term memory often manifests itself as losing objects or forgetfulness in conversations. Subsequently, other early cognitive deficits occur; they include difficulty in word-finding, problem solving and spatio-temporal awareness. These symptoms are commonly accompanied by mental disorders, such as depression and apathy, and alternatively or subsequently agitation and irritability may occur. With further disease progression, memory failure becomes more profound and longer-term memory is affected. Patients with these deficits are frequently disoriented and can easily become lost, thus a close supervision of patients become necessary. About this stage, their language and complex motor skills also deteriorate. Psychosis and hallucinations can occur along with bradykinesia and rigidity. Patients ultimately become bed-ridden, unable to speak, and die from infection (such as pneumonia) or other medical conditions. Average survival time of Alzheimer's patients is about 8 years after onset of initial symptoms, although the rate of decline is quite variable.

30.2.2 Diagnosis

The elements for the diagnosis of AD include several components: (1) a history obtained from someone who knows the patient well, (2) a physical exam and an psychiatric exam to exclude other neurodegenerative diseases, (3) blood tests to exclude potential metabolic disorders involving the kidneys, liver, or thyroid, (4) a mental status test to determine dementia progression and (5) brain imaging (see Neuroimaging chapter for details). The first cognitive assessment widely used was the Mini-Mental State Examination (MMSE). MMSE assesses multiple cognitive tasks including spatio-temporal orientation, immediate/delayed word recall, naming, verbal repetition,

reading, writing, and spatial ability (Folstein et al. 1975). There are multiple reports of reduced cognitive performance in pre-clinical AD, notably impaired function in abstract reasoning, episodic memory, and new learning (see Fratiglioni for review) (Fratiglioni et al. 2001). Episodic memory deficits are dominant among early clinical and preclinical phases of AD. A diagnosis of dementia is usually determined using DSM-IV (Diagnostic and Statistical Manual of Mental Disorders) of the American Psychiatric Association (Association 1994). The final diagnosis is typically determined by autopsy using the criteria of NINDS-ADRDA (National Institute of Neurological Disorders and Stroke and Alzheimer's Disease and Related Disorders Association), which has been developed to document the neuropathology and underlying etiology (McKhann et al. 1984). Other diagnosis standard includes International Classification of Diseases (ICD)-10 guideline (Organization WH 1992). Since DSM-IV and ICD-10 criteria closely follow NINDS-ADRDA guidelines, diagnostic procedures for AD are notably consistent throughout the world. Given that AD is the primary cause of dementia, AD is often considered to be the likely cause of dementia while clinical tests are used to exclude other causes.

30.2.3 Epidemiology

Alzheimer's Disease affects estimated 5.2 million in the United States in 2014 and prevalence studies demonstrate an exponential rise of dementia with age (Katzman 2001). By 2050, the number of people age 65 and older with AD may nearly triple, from five million to as many as 16 million, barring the development of medical breakthroughs to prevent, slow or stop the disease. (Katzman and Fox 1999). AD is officially the sixth leading cause of death in the United States and the fifth leading cause of death for those aged 65 and older (Ewbank 1999).

In addition to age, other factors are associated with an increased risk of AD. In developed countries, AD appears to be more common in women than men (Mielke et al. 2014). Lack of education is a risk factor for senile dementia in China and Europe (Zhang et al. 1990; Schmand et al. 1997). Head trauma is also a risk factor for both sporadic (Mortimer et al. 1991) and familial AD (Guo et al. 2000). Silent myocardial infarcts and coronary stenosis triple the risk for AD (Aronson et al. 1990; Sparks et al. 1990), suggesting the importance of vascular risk factors. Other potential risk factors being studied include diabetes and hypertension (Ott et al. 1999; Peila et al. 2002; Qiu et al. 2005; Reitz and Mayeux 2014). As discussed below, a large number of genetic mutations are now associated with either early-onset AD or with increased risk of late-onset AD.

Identification of genetic mutations that cause early onset AD has helped to dramatically increase the understanding of

the origins of AD. Although cases of familial AD are relatively rare, they represent identified molecular mutations that cause AD. The genes identified to be responsible for early onset familial AD code for the amyloid precursor protein (APP), presenilin-1 and presenilin-2; these are found on chromosomes 21, 14, and 1, respectively. In addition to these dominantly-transmitted disease-causing mutations in early onset AD, still other studies have identified over 25 genetic risk factors that influence the appearance of the more common sporadic late onset AD. Current research is actively using genetically-modified mice to determine how specific protein modifications lead to the pathology of AD.

30.2.4 Genetic Studies of AD

Alzheimer's Disease is classified into early onset AD (EOAD, onset <65 years) and late-onset AD (LOAD, onset ≥65 years). EOAD and LOAD account for 1–5 % and >95 % of all cases, respectively. EOAD is generally associated with a more rapid rate of progression and are mostly familial AD. Three genes (*APP*, *PSEN1* and *PSEN2*) are identified in the EOAD pedigrees. All of them encode proteins involved in APP breakdown and Aβ generation. AD-linked mutations in these three genes exhibit high penetrance (>85 %), are mostly autosomal dominantly inherited. In contrast, the genes involved in LOAD increase disease risk in a non-Mendelian fashion. For two decades, only one genetic risk factor, the apolipoprotein E4 isoform (*APOEε4*) allele, located on chromosome 19q13, was an established risk gene in LOAD. APOE is a lipid-binding protein and is expressed in humans as three common isoforms coded for by three alleles, APOEε2, ε3, and ε4. A single *APOE-ε4* allele is associated with a two to threefold increased risk of LOAD; having two copies is associated with a five-fold or more increased risk (Kuusisto et al. 1994). A detailed discussion of ApoE biology can be found in Section 32.3.5.

Extensive genome-wide association studies (GWAS) have been conducted in a large cohort of LOAD subject in multiple institutions (Hardy and Singleton 2009; Sherva and Farrer 2011). The first set of studies identified *CLU*, *PICALM*, *CR1* and *BIN1* as susceptibility loci (Harold et al. 2009; Lambert et al. 2009; Seshadri et al. 2010). These loci mainly cluster in four pathways, namely immune response, APP processing, lipid metabolism and endocytosis. The second set of large GWAS studies identified additional susceptibility genes (*CD33*, *MS4A4A/MS4A4E/MS4A6E cluster*, *ABCA7*, *CD2AP* and *EPHA1*) (Hollingworth et al. 2011; Naj et al. 2011). All of these five loci are likely involved in the immune system while the *ABCA7* is in addition involved in lipid metabolism and APP processing. Finally, the largest GWAS to date, the International Genomics of Alzheimer's Project, performed a mega-meta analysis that included 74,046 sub-

jects (Lambert et al. 2013). In addition to *APOE*, *CR1*, *BIN1*, *CD2AP*, *EPHA1*, *CLU*, *MS4A6A*, *PICALM*, *ABCA7* and *CD33* this GWAS identified 12 additional susceptibility loci at genome-wide significance (*HLA-DRB5/HLA-DRB1*, *PTK2B*, *SORL1*, *SLC24A4/RIN3*, *DSG2*, *INPP5D*, *MEF2C*, *NME8*, *ZCWPW1*, *CELF1*, *FERMT2*, *CASS4*). Most of the 12 novel loci cluster in the specific pathways identified by the earlier GWAS, i.e. immune response (*HLA-DRB5/DRB1*, *INPP5D*, *MEF2C*), APP processing (*SORL1*, *CASS4*), Tau pathology (*CASS4*, *FERMT2*), cell migration (*PTK2B*) and lipid transport and endocytosis (*SORL1*). These results strongly reinforce the importance of these pathways in the etiology of LOAD.

TREM2: In the context of neuroimmune mechanism of LOAD, triggering receptor expressed by myeloid cells-2 (TREM2) is a transmembrane signaling molecule for phagocytosis and anti-inflammatory response and is genetically linked to LOAD, polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOS, also known as Nasu-Hakola disease), behavioral variant frontotemporal lobar degeneration (FTLD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) (Guerreiro et al. 2013a, b; Jonsson et al. 2013; Paloneva et al. 2002; Giraldo et al. 2013; Rayaprolu et al. 2013; Cady et al. 2014). The detailed biology of TREM2 in microglia is discussed in Sect. 30.4.1.

30.2.5 Pathology

At autopsy, AD brains appear to have widespread neuronal and neuropil loss that thins cortical layers and expands ventricular and sulci spaces. However, there is not an obvious global loss of neurons in the cerebral cortex. Overall neuronal number appears relatively unchanged despite significant loss of brain tissue. Some of this apparent discrepancy is due to preferential loss of large pyramidal neurons in the cortex and relative sparing of smaller, densely packed neurons. Particularly vulnerable regions such as the entorhinal cortex and the CA1 of the hippocampus, show profound neuronal loss early in the disease. Wider involvement of temporal, frontal, and parietal cortices and a preferential loss of large neurons in these areas is seen as the disease progresses.

AD is characterized by an accumulation of senile plaques containing aggregated protein deposits of Aβ fibrils, numerous NFTs, reactive astrocytes, and activated microglia in the neocortex and hippocampus (Alzheimer 1907; Selkoe 1997). Senile plaques can contain either a diffuse amyloid deposition (presumably early senile plaques) or a dense core of insoluble Aβ with neuritic structures (mature senile plaques). Although Aβ deposition and NFT are commonly observed in non-demented elderly 80 years and older, increased levels of senile plaques are seen in AD brains (Berg et al. 1993; Schmitt et al. 2000). The degree of both Aβ deposition and

NFT formation are correlated with cognitive decline (Naslund et al. 2000; Berg et al. 1993; Wilcock and Esiri 1982; Braak and Braak 1995).

Although inflammation is a well-recognized phenomenon and is intensively investigated, the precise definition of brain inflammation has not yet been established (McGeer and McGeer 2001). Clinically, AD patients exhibit few of the classic signs of inflammation. Histopathologically, the classical acute inflammatory response (neutrophil recruitment), immunoglobulin precipitation, and T-cell accumulation are not seen suggesting that humoral or classical cellular immune-mediated responses are not involved in AD progression (Eikelenboom et al. 1994). However, AD brains show an increase in activated microglial clusters on senile plaques surrounded by astrocytes, indicating focal glial inflammation (Eikelenboom et al. 2002; Wyss-Coray and Mucke 2002). In particular, volume density of CD68⁺ mononuclear phagocytes (brain perivascular macrophages and microglia) is correlated with volume density of congophilic compact plaque deposits (Arends et al. 2000). CD68⁺ cells are present from very early preclinical phases to terminal phases of AD and are involved in disease progression, which will be further described later (Fonseca et al. 2004a; Akiyama et al. 2000). Moreover, deactivation of microglia has implications for therapy since long-term treatment of affected patients with non-steroidal anti-inflammatory drugs (NSAIDs) has been shown to inactivate microglia and retard the development and progression of AD (In't Veld et al. 2002), although NSAIDs may also directly affect the APP processing.

30.3 Molecular Pathogenesis, Animal Models, and Neuroinflammation

The molecular pathogenesis of AD is centered around A β production and its clearance. A β is generated by the processing of APP by β and γ -processing enzymes, and cleared from brain by its diffusion, export to vascular system, phagocytosis, or degradation. The following molecules are essential for understanding the molecular mechanism of A β processing.

30.3.1 Amyloid Precursor Protein (APP)

APP is the first gene to be identified as a causative gene of familial AD (FAD) and has been a key molecule in the study of the molecular mechanism of AD. As shown in Fig. 30.1, the APP molecule can be cleaved at different specific cleavage sites by various proteases to generate fragments of different sizes. APP is primarily expressed in neurons and cleaved at the α , β , and γ sites by APP processing enzymes (called α , β , and γ -secretases), producing A β (β - and γ -processing product), p3 (α - and γ -processing product), and

other APP fragments (Selkoe 1994). A β is composed of 39 to 43 amino acids, mainly 1-40 (A β 40) and 1-42 (A β 42). A β 42 is more amyloidogenic (faster to be aggregated) than A β 40, and early A β deposits are usually detectable with anti-A β 42 antibodies, but not with anti-A β 40 antibodies (Selkoe 1994). FAD mutations on APP gene are all linked to abnormal APP processing (increase in A β 42/A β 40 ratio), A β aggregation, and A β deposition (Scheuner et al. 1996).

30.3.2 PS1 and γ -Processing Enzyme Complex

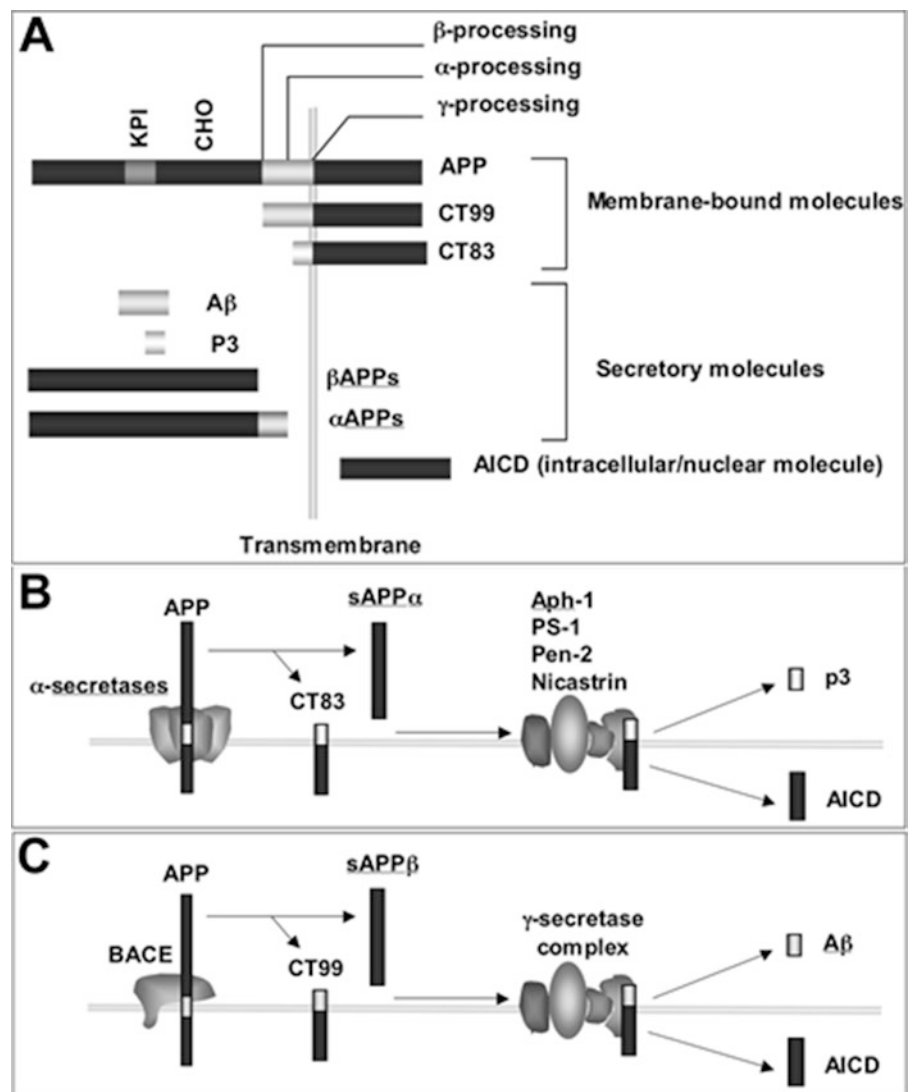
A combination of the presenilin genes (PS1 and 2), aph-1, pen-2, and nicastrin forms the γ -secretase complex (De Strooper 2003). The γ -secretase complex cleaves not only APP but also other transmembrane molecules, such as Notch, ErbB-4, and sterol regulatory element binding proteins (SREBPs). PS1 and PS2 have been established as causative genes of early onset FAD (Sherrington et al. 1995; Levy-Lahad et al. 1995). Transgene expression of PS1 FAD mutants enhances the A β 42/A β 40 ratio and accelerates A β aggregation and deposition in APP mice, which over-express Swedish FAD APP mutant in brains (Duff et al. 1996; Borchelt et al. 1997; Holcomb et al. 1998). PS2 FAD mutant over-expression converts A β 40 to A β 42, whereas PS2 wild type over-expression reduces both A β 40 and A β 42 (Mastrangelo et al. 2005). Taken together, these *in vivo* studies demonstrate the effect of PS1/2 FAD mutations on A β aggregation through transition of A β 40 to A β 42.

30.3.3 APP and PS1 Animal Models

30.3.3.1 Conventional APP and PS1 Mouse Models

Several established AD mouse models have made significant contributions to the understanding of AD pathogenesis (Price et al. 1998; Spire and Hyman 2005). Analyses of AD brain tissue obtained from autopsies provide only limited insight into the dynamic nature of the disease process. Currently, no AD mouse model has all of the characteristics of the human disease. However, several lines of transgenic mice expressing mutated APP and PS1 simulate prominent behavioral and pathological features of AD. These include, but not limited to, PDAPP expressing a platelet-derived growth factor gene promoter-driven human APP (isoforms 695, 751, and 770) with the V717F (Indiana) mutation (Games et al. 1995), Tg2576 expressing hamster prion gene promoter-driven human APP (isoform 695) gene with K670N/M671L (Swedish mutation) (Hsiao et al. 1996), APP23 expressing Thy1 gene promoter-driven human APP (isoform 751) with Swedish mutation (Sturchler-Pierrat et al. 1997), TgCRND8 expressing hamster prion gene promoter-driven human APP

Fig. 30.1 Scheme of APP processing. A, Cleavage sites and processing enzymes of APP. B, P3 and sAPP α production by α and γ -processing of APP. C, A β and sAPP β production by β and γ -processing of APP



695 gene with Swedish and Indiana mutations (Chishti et al. 2001), APP^{swe} x Hu PS1-A246E expressing mouse prion gene promoter-driven human APP 695 gene with Swedish mutation and human PS1 gene with A246E mutation (Borchelt et al. 1997), PS/APP expressing hamster prion gene promoter-driven APP 695 gene with Swedish mutation and human PS1 gene with M146L mutation (Holcomb et al. 1998), and 3xTg-AD mice expressing Thy1.2 gene promoter-driven human APP 695 gene with Swedish mutation, human tau gene with P301L mutation, and homozygotic P146L knock-in mutation of mouse PS1 gene (Oddo et al. 2003). Phenotypes displayed by these mice include age-related impairments in learning and memory, electrophysiological abnormalities, neuronal loss, microgliosis, astrogliosis, neuritic changes, amyloid deposition, oxidative stress, and abnormal tau phosphorylation (Price et al. 1998; Smith et al. 1998) (Fig. 30.2). Microglia in close proximity to fibrillar A β deposits are immunoreactive to interleukin (IL)-1 β and TNF- α , whereas activated astrocytes are immunoreactive to IL-1 β ,

transforming growth factor (TGF)-1 β , and IL-10 in Tg2576 mice (Frautschy et al. 1998; Benzing et al. 1999; Apelt and Schliebs 2001).

Tg2576 mice which express Swedish familial AD mutant (K670N/M671L) of APP695 under a hamster prion promoter show memory and learning impairments as determined by Morris water maze (Hsiao et al. 1996). The memory recall is impaired as early as 6 months of age, which persists without significant progression for up to 12 months of age, followed by impairment of both memory acquisition and recall after 12 months of age (Lesne et al. 2006). There is a strong correlation of such age-related memory decline to A β accumulation in the brain (Fig. 30.3) (Janus et al. 2000; Chen et al. 2000). A recent study in Tg2576 mice suggests that 9- or 12-mer A β oligomers are responsible for such memory loss, which can be isolated by Tg2576 and can induce transient memory loss in injected rodents (Lesne et al. 2006). Contextual fear conditioning tests, which represents hippocampal learning, show memory impairments in PDAPP mice

Fig. 30.2 3D reconstruction image of amyloid plaque deposition and glial accumulation in APP mouse brain. Cortical section of Tg2576 (14 months of age) was stained by thioflavin-S (compact amyloid plaque, purple), anti-IBA1 rabbit polyclonal (microglia, red), and anti-GFAP mouse monoclonal (astrocytes, green), and imaged using a laser scanning confocal microscopy (Zeiss 510 Meta). Z-stack images were deconvolved and 3D reconstructed using image processing software (AutoQuant)

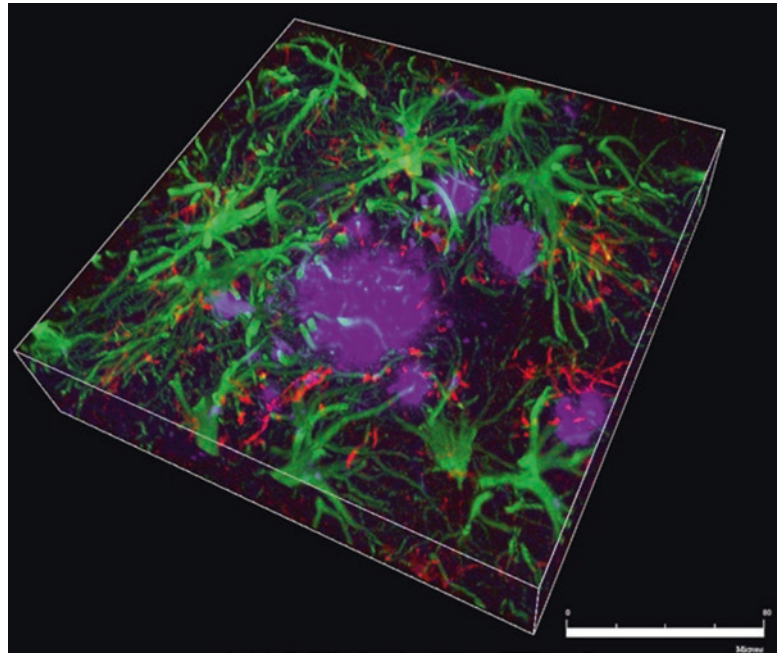
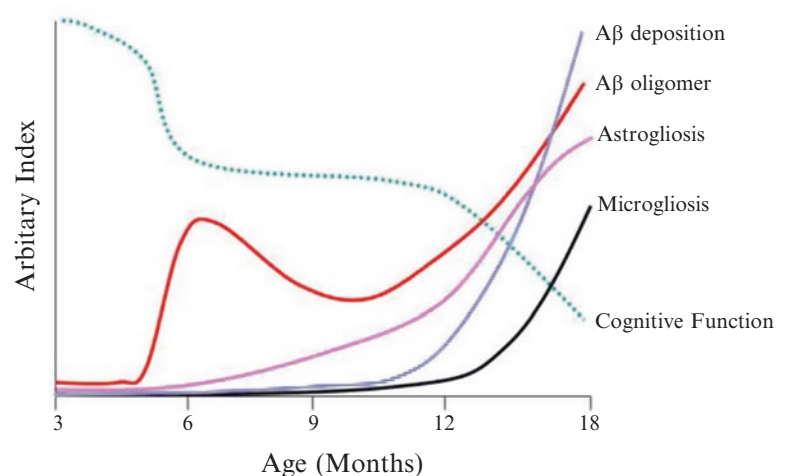


Fig. 30.3 Schematic representation of the time course of A β deposition, A β oligomer accumulation, cognitive dysfunction, astrogliosis, and microgliosis in Tg2586 mice



as early as 4 months of age preceding the A β deposition (Jacobsen et al. 2006). These studies strongly suggest that onset of memory loss precedes the A β deposition, is more correlated with A β oligomer formation in the brain, may also be relevant to the clinical symptoms of AD patients.

30.3.3.2 APP Knocked-In Mouse Models

Transgene expression of recombinant molecules under the control of exogenous promoter derived from genetic fragment of other species are often associated with artificial phenotypes not predicted or commonly seen in human disease. Understanding the effects of elevated A β from those due to APP overexpression is a common challenge when interpreting the phenotypes of APP transgenic mice. To overcome this

issue, Saito *et al.* created a new APP knocked-in mouse models (APP^{NL-F} and APP^{NL-G-F}) (Saito et al. 2014). These mouse models avoid potential artifacts introduced by APP overexpression by using a knock-in approach to express APP at wild-type levels and with appropriate cell-type and temporal specificity. APP is not overexpressed, but levels of pathogenic A β are elevated due to the combined effects of two (in APP^{NL-F}) or three mutations (in APP^{NL-G-F}) linked to familial AD. Both APP^{NL-F} and APP^{NL-G-F} mice accumulate A β and recapitulate several AD-associated pathologies, including amyloid plaques, synaptic loss, and microgliosis and astrogliosis, especially in the vicinity of plaques. The presence of the Arctic mutation in APP^{NL-G-F} accelerates the pathology relative to APP^{NL-F} mice, and leads to particularly severe pheno-

types. A β deposition is observed starting at 2 months and is nearly saturated by 7 months. In contrast to APP^{NL-F} mice, the APP^{NL-G-F} mice develop subcortical amyloidosis in addition to cortical amyloidosis, consistent with the neuropathology observed in patients with the Arctic mutation. The mice also display age-associated cognitive impairment, specifically memory impairment as measured by the Y maze starting at 6 months. The cognitive impairment in APP^{NL-G-F} mice is more severe than the impairment in APP^{NL-F} mice. These mice will be suitable for future studies using APP mouse models.

30.3.4 β and α -Processing Enzymes

Beta-site APP-cleaving enzyme (BACE1, also called Asp2 or Memapsin2), a β -secretase, is an aspartic transmembrane proteinase (Vassar et al. 1999). BACE2 (also called Asp1 and DRAP) is a homolog of BACE1 and contains the same overall structural organization. BACE1 is essential for A β production as demonstrated by BACE1 gene-targeted mouse models (Cai et al. 2001). BACE over-expression enhances total A β production, deposition of both diffuse and compact A β plaques in brain parenchyma, and reduces cerebrovascular amyloid angiopathy in aged APP/BACE double transgenic mice (Willem et al. 2004; Mohajeri et al. 2004). However, this effect seems to be dose-dependent, since high BACE expression rather inhibits A β deposition (Lee et al. 2005). Thus, the effect of BACE upregulation in A β pathology in vivo is not conclusive. A Disintegrin And Metalloprotease (ADAM) 9, 10, and 17 have been identified as putative α -secretases (Asai et al. 2003). Overexpression of ADAM10 prevents A β deposition and hippocampal defect in APP mice (Postina et al. 2004), suggesting its therapeutic application for enhancing α -processing of APP.

30.3.5 ApoE

ApoE is the most well characterized genetic risk factor of AD. Evidence indicates that endogenous apoE in mouse brain enhances A β deposition in APP animal models (Bales et al. 1999; Sadowski et al. 2004). Tg2576 mice lacking mouse apoE exhibit diminished compact plaque deposition, while diffuse plaque load remained unchanged (Bales et al. 1999). Mouse apoE is rather amyloidogenic since apoE disruption significantly reduces the compact plaque formation in APP mice (Bales et al. 1999). In contrast, human apoE3 enhances A β clearance in mouse brain, while apoE4 weakly promotes A β clearance (Holtzman et al. 1999, 2000; Fagan et al. 2002). Although many studies concluded that astrocytes are the main apoE-producing cell in brain (Danik et al. 1999), recent reports suggest that microglial cells are also a source of apoE (Saura et al. 2003; Mori et al. 2004). A recent study of EGFP-ApoE knock-in mice demonstrated that apoE

is expressed in 75 % of astrocytes, and <10 % of microglia after stimulation. Thus, apoE is primarily an astrocyte protein (Xu et al. 2006).

30.3.6 A β Degrading Enzymes

The current knowledge of A β degradation biology is limited since only a few of the molecules involved have been characterized, namely insulin degrading enzyme (IDE) and neprilysin, which is also known as enkephalinase, and neutral endopeptidase metalloendopeptidase (NEP) (Selkoe et al. 2001; Iwata et al. 2000; Yamin et al. 1999). IDE was originally identified as a microglia-secreted A β degrading enzyme (Qiu et al. 1998), and is also found in neurons (Vekrellis et al. 2000). Neprilysin protein level is downregulated during aging, but is locally upregulated in activated astrocytes surrounding A β plaques in APP mice (Apelt et al. 2003). Thus the amount of IDE and neprilysin can be altered by chronic inflammatory reaction. Neprilysin, a neutral zinc metallopeptidase, plays a role in degrading insoluble and/or aggregated A β 42 (Selkoe et al. 2001; Iwata et al. 2000). Transgenic expression of both IDE and neprilysin show inhibition of A β accumulation in APP mice (Leissring et al. 2003). In addition, endothelin converting enzyme and matrix metalloprotease-2 and 3 are also new candidates of A β degrading enzymes (Choi et al. 2006; White et al. 2006).

30.3.7 Neurofibrillary Tangle Formation

Presence of NFT is one of the original neuropathological hallmarks of AD. NFT are fibrous tangles composed of insoluble, conformationally abnormal, hyperphosphorylated tau protein deposited in neuronal cell bodies. Hyperphosphorylated tau can form a specific insoluble structure known as a paired helical filament (PHF) (Lee et al. 2001; Buee et al. 2000). Six tau protein isoforms are generated by alternative mRNA splicing of the *tau* gene. Based on the repeat of the intermediate microtubule-binding domain, the majority of tau is classified as three repeat (3R) or four repeat (4R) tau. The primary function of tau is to bind to and stabilize microtubules, thereby promoting microtubule polymerization. Many tau phosphorylation sites flank microtubule binding domains and are believed to play a significant role in its microtubule binding (Lee et al. 2001; Planel et al. 2002). Hyperphosphorylation of tau appears to cause tau to dissociate from microtubules and form tau protein aggregates, which becomes PHF (Biernat et al. 1993; Bramblett et al. 1993; Alonso et al. 1996). Glycogen synthase kinase 3- β (GSK3 β), cyclin dependent protein kinase 5 / p25 complex, microtubule affinity regulating kinase, and recently tau-tubulin kinase 1 have been extensively characterized as

tau kinases that seem to be involved in the hyperphosphorylation of tau (Takashima et al. 1993; Ishiguro et al. 1992; Patrick et al. 1999; Drewes et al. 1997; Sato et al. 2006).

Tau-related neurodegenerative disorders, or “tauopathies”, include AD, argyrophilic grain dementia, corticobasal degeneration, diffuse neurofibrillary tangles with calcification, Down’s syndrome, frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17), Gerstmann-Straussler-Scheinker disease, Niemann-Pick disease, non-Guamanian motor neuron disease with neurofibrillary tangles, Pick’s disease, progressive supranuclear palsy, subacute sclerosing panencephalitis, and tangle only dementia (Lee et al. 2001). Genetic studies show that mis-sense mutations in the *tau* gene (*Mtapt*) induce FTDP-17 supporting its importance in tauopathies (Hutton et al. 1998; Lee et al. 2001), although such mutations have not been found in AD.

These tau mutations develop similar pathology in transgenic animal models, such as P301L tau mouse (Gotz et al. 2001a; Lewis et al. 2000), R406W tau mouse (Sato et al. 2002), G272V tau mouse (Gotz et al. 2001b), and V337M tau mouse (Tanemura et al. 2002). However, P301L tau mice develop NFT-like tau aggregation not only in CNS but also in peripheral motor neurons, astrocytes and oligodendrocytes, that is correlated with gait disturbances (Lewis et al. 2000).

rTg4510 mice express FTDP-17-linked tau P301L mutant under the control of tetracycline response element (TRE), to which Ca^{2+} /calmodulin-dependent kinase II (CaMKII) promoter-driven tTA binds and suppresses the gene transcription in the presence of doxycycline (Dox) (Santacruz et al. 2005; Mayford et al. 1996). The CaMKII promoter is specifically activated in and largely restricted to the hippocampus, cortex, olfactory bulb, and striatum, and Tg4510 show age- and Dox-dependent tau aggregation and neurodegeneration in the brain subregions (Santacruz et al. 2005). This is, so far, one of most advanced tauopathy animal models with a clear neurodegeneration phenotype, which can be controlled by Dox treatment.

In the context of neuroinflammation, P301S tau mice, another FTDP-17 mouse model, show age-dependent neurodegeneration in entorhinal cortical and hippocampal regions, which is preceded by extensive microglial activation and can be suppressed by an immunosuppressive agent, FK506 (Yoshiyama et al. 2007), indicative of non-autonomous neuronal cell loss. Similarly, another P301S tau mouse model shows extensive neurodegeneration in brain stem and spinal cord, which is accompanied with neuroinflammation and M1-type mononuclear phagocyte accumulation (Allen et al. 2002; Bellucci et al. 2004). These studies reveal that specific type of neuroinflammation may be one of the mechanisms in the tauopathy-related neurodegeneration and a potential therapeutic target.

30.3.8 Neurotoxicity and Synaptic Dysfunction

30.3.8.1 A β -Mediated Neurotoxicity

It has been widely accepted that toxicity of A β requires aggregation of native A β monomers (Pike et al. 1993; Hardy and Higgins 1992). A β (A β 40 or A β 42) self-assembles to form low-n oligomers (dimers-hexamers), protofibrils, and fibrils (Roher et al. 1996; Lambert et al. 1998; Harper et al. 1997; Walsh et al. 1997). Since intermediates can further associate into higher-ordered aggregates, it is difficult to determine the oligomerization state of A β that causes pathogenicity. Nonetheless, several groups succeeded in isolating spherical non-fibrillar assemblies of synthetic A β (A β -derived diffusible ligands; ADDLs and amylospheroid; ASPD) (Lambert et al. 1998; Hoshi et al. 2003). These A β oligomers show more potent toxicity than fibrillar A β at lower concentrations (Lambert et al. 1998) and induce reversible synaptic loss through N-methyl-D-aspartate (NMDA) receptor interaction in organotypic hippocampal culture systems (Shankar et al. 2007). Thus, A β oligomers are a likely therapeutic target (De Felice et al. 2004).

A β can also potentially mediate neurotoxicity by activating microglia and glia and hereby promoting their secretion of a variety of neurotoxic factors. A β and secreted β -amyloid precursor protein (sAPP)-induce microglial activation and neurotoxicity (Giulian and Baker 1986; Meda et al. 1995; Barger and Harmon 1997; Ikezu et al. 2003). Neurotoxicity can be mediated through secretion of neurotoxic agents such as FasL, tumor necrosis factor (TNF)- α , reactive oxygen species (ROS), proteases, excitatory amino acids, and nitric oxides. Excitatory amino acids (glutamate, quinolinic acid, D-serine, among others) activate N-methyl-D-aspartate (NMDA) receptors, which are significantly involved in microglial-mediated neurotoxicity (Giulian et al. 1994; Piani et al. 1992). Indeed, both A β and sAPP activated microglia increase glutamate secretion (Barger and Basile 2001; Ikezu et al. 2003). Glutamate and ROS can exacerbate each other’s neurotoxic effects due to their relationships with the cystine/glutamate exchange system (Xc⁻) antiporter. A β /sAPP-induced microglial activation leads to ROS production and glutathione consumption, that in turn promotes cystine import to regenerate glutathione and, as a consequence, increased glutamate is exported in exchange through the Xc⁻ antiporter (Ikezu et al. 2003).

30.3.8.2 A β -Mediated Synaptic Dysfunction

There are a number of studies documenting the synaptic dysfunction of APP mice, which starts as early as 4 months of age, including enhanced paired-pulse facilitation, disturbed high frequency stimulation (HFS) burst, and rapid decay of LTP in the CA1 field of hippocampal slices (Larson et al. 1999; Hsia et al. 1999). In addition, impaired synaptic

transmission and LTP in both the CA1 and DG regions are also evident in Tg2576 mice (Chapman et al. 1999). A β oligomers block hippocampal long-term potentiation (LTP) *ex vivo* at nanomolar concentrations. *In vivo* applications of A β oligomers potently inhibit rat hippocampal LTP and cognitive function (Walsh et al. 2002; Cleary et al. 2005). These studies strongly suggest that A β oligomers induce memory impairment through inhibition of synaptic transmission and long-term potentiation in brain. One of the potential mechanisms of A β -induced LTP inhibition is through the activation of a number of inflammation-related molecules, such as c-jun N-terminal kinase, CDK5, p38 mitogen-activated kinase, inducible nitric oxide synthase, and superoxide, (Wang et al. 2004a, b). These data suggest significant involvement of microglial activation in the inhibitory mechanism.

30.3.8.3 Neurotoxicity By Tau Aggregation

Although NFT is a hallmark of AD, its direct involvement in neuronal cell death is still unclear. Presence of NFT does not appear to be highly toxic since clinical studies suggest long-term survival of NFT-bearing neurons (Morsch et al. 1999; Hof et al. 2003). These data are also supported by studies using FTDP-17 tau mutant mice showing NFT formation in the absence of neuronal cell loss (Santacruz et al. 2005) and a lack of cognitive impairment as assessed by a water maze test (Arendash et al. 2004). A new doxycycline-inducible tau transgenic mouse model suggests accumulation of intracellular tau protein rather than NFT formation is responsible for neurodegeneration and cognitive impairment (Santacruz et al. 2005; Ramsden et al. 2005). However, tau is known to be involved in A β aggregate-induced neurotoxicity in conjunction with protein kinase activation *in vitro* (Rapoport et al. 2002; Hoshi et al. 2003; Medina et al. 2005). Thus, it is possible that acute neuronal cytoskeletal destabilization by tau phosphorylation and its end-product (globular tau aggregates) are neurotoxic, but its subsequent subcellular compartmentalization as NFT/PHF is a preventive neuroprotective process used to contain neurotoxic aggregates. Further study will be necessary to characterize its effect on neurodegeneration or dementia.

30.4 Neuroimmunological Mechanism of AD

Neuronal loss, edema, and recruited mononuclear phagocytes (moving from blood to brain), are all classical hallmarks of brain inflammation in AD (Arvin et al. 1996). Gliosis, characterized by activation, proliferation, and hypertrophy of astrocytes and microglia, is one important feature of neuroinflammation. Amyloid plaques contain a number of proteins including complement proteins and pro-inflammatory cytokines and chemokines. Many of these proteins

are secreted by activated microglia and reactive astrocytes. Activated microglia integrate deeply into neuritic plaques and express major histocompatibility complex type II (MHC-II), a marker for microglial activation. MHC-II expression is significantly upregulated in AD brains (Rogers et al. 1988; Styren et al. 1990).

Neurotoxicity can be mediated through secretion of various factors, ROS, proteases, excitatory amino acids, and nitric oxides. Pro-inflammatory cytokines and chemokines can inhibit fibrillar A β phagocytosis by a murine microglial cell line (Koenigsknecht-Talboo and Landreth 2005). Such T-cell-mediated cytokines also inhibit post-phagocytosis intracellular A β degradation in human monocyte-derived macrophages. A β -microglia interaction results in two outputs: phagocytosis and signaling for priming and activation. A β binding to a complex of cell surface proteins (including CD36, integrin-associated protein (CD47), and $\alpha_6\beta_1$ -integrin) leads to src-like tyrosine kinase activation and ROS production (Bamberger et al. 2003). CD36 is necessary for full A β -induced mononuclear phagocyte activation and chemotaxis (El Khoury et al. 2003). These findings strongly suggest that A β aggregates activate microglia by its binding to specific cell surface molecules, which activates src-like tyrosine kinases and other signaling for A β clearance and neuroinflammation in AD brains.

30.4.1 Microglia-Mediated Phagocytosis

30.4.1.1 A β Scavenger Receptors

Our current knowledge of A β clearance is limited compared to the more detailed understanding of A β synthesis and aggregation mechanisms. It is well known that A β stimulation enhances microglial phagocytosis, *in vitro* (Kopec and Carroll 1998). The internalized A β in phagosomes initially migrate to acid hydrolase-containing late endosomes and lysosomal compartments, and then moves into perinuclear vesicles, that are morphologically similar to lysosomes, where it stays up to 20 days (Paresce et al. 1997). The long-term accumulation of A β has also been observed by using unlabeled A β aggregates in bone marrow-derived macrophages (Yamamoto et al. 2007b). In addition to microglia, astrocytes also contribute to the uptake and removal of A β in a manner enhanced by a CC-type chemokine, CCL2 (Wyss-Coray et al. 2003).

Microglia constantly take-up A β via potential A β receptors, including scavenger receptor type A (SR-A), CD36, and receptor for advanced glycation end product (RAGE) (El Khoury et al. 1996, 2003; Yan et al. 1996). Although SR-A was characterized as a major receptor for microglial phagocytosis of A β (Paresce et al. 1996), APP mice deficient in SR-A show no difference in amyloid plaque formation or synaptic degeneration (Huang et al. 1999). This suggests that

other scavenger receptors or A β -binding molecules, such as CD36, CD47, and $\alpha_6\beta_1$ -integrin, can compensate for the deficiency of SR-A. On the other hand, RAGE seems to enhance A β deposition. APP mice deficient in RAGE exhibit reduced A β deposition (unpublished observations), which is consistent with enhanced A β deposition in mice over-expressing RAGE (Arancio et al. 2004). P-glycoprotein (Pgp), an ATP binding cassette transporter/multidrug resistance-1 α and β , is another molecule involved in A β clearance from brain parenchyma. Disruption of Pgp exhibits enhanced A β deposition, suggesting its role in endothelial efflux of A β from the brain (Cirrito et al. 2005).

30.4.1.2 TREM2/TYROBP Pathway for Phagocytic Clearance and Disease

TREM2 is mostly expressed in myeloid cells (immature dendritic cells, microglia and osteoclasts). In the CNS, TREM2 is expressed at levels greater than 300-fold in microglia compared to astrocytes (Hickman and El Khoury 2014). Inflammation decreases TREM2 expression in microglia and their ability to phagocytize apoptotic neurons (Takahashi et al. 2007). TREM2 requires the interaction with the adaptor protein, TYRO protein tyrosine kinase binding protein (TYROBP; formerly DAP12) that facilitates the downstream signaling of phagocytosis (Paloneva et al. 2000). Thus, healthy coupling of TREM2 and TYROBP is essential for the phagocytic function of myeloid cells. No endogenous ligand has been confirmed for TREM2, but TREM2 is shown to bind to Gram⁺ and Gram⁻ bacteria and yeast in a charge-based method (Daws et al. 2003). Stimulation of TREM2 by a cross-linked TREM2 antibody is shown to activate TREM2. This activation causes actin polymerization and restructuring of the cytoskeleton (Takahashi et al. 2005).

TYROBP is a transmembrane adaptor protein that binds to TREM2 and signals through immunoreceptor tyrosine-based activation motifs (ITAMs). The ITAM signal is important for phagocytosis activation in microglia (Linnartz and Neumann 2013). Tyrosine phosphorylation of ITAM by Src family proteins activates TYROBP. The phosphorylated ITAM binds to protein spleen tyrosine kinase (Syk) (Fig. 30.1). Syk activates many downstream cascades linked to phagocytosis (Linnartz and Neumann 2013). The ITAM activation is negatively regulated by the immunoreceptor tyrosine-based inhibition motifs (ITIMs). When a regulatory ligand binds to receptor containing an ITIM, recruitment of SHP1/SHP2 occurs. SHP1/SHP2 can dephosphorylate and inhibit ITAM activation and other downstream cascades (Linnartz and Neumann 2013). TYROBP deficient mice show enhanced inflammatory phenotypes (Hamerman et al. 2005).

Mutations in TREM2 or TYROBP are thought to be the main cause of Nasu-Hakola (Paloneva et al. 2003). Nasu-Hakola diseases is polycystic lipomembranous osteodyspla-

sia with sclerosing leukoencephalopathy (PLOS), a rare autosomal recessive disease (Paloneva et al. 2003). Patients who are diagnosed with Nasu-Hakola disease suffer from bone cyst-like lesions and bone fractures often on their lower legs and also experience rapid pre-senile dementia (Paloneva et al. 2001, 2003). Around the age of 40, patients have severe neurodegeneration and suffer from severe dementia, gait disturbances, sensory agnosia, agraphia and in some cases develop tumors in the CNS (Hakola and Puranen 1993). The link between neurodegeneration and TREM2/TYROBP function provided by Nasu-Hakola disease indicates that the phagocytic and anti-inflammatory function of TREM2/TYROBP signaling is important for healthy brain function.

The missense R47H mutation in TREM2 is associated with LOAD, has an effect on both folding and ligand binding, and decreases the clearance of damaging debris (Abduljaleel et al. 2014). Similarly, FTDL-linked missense mutations in TREM2 (T66M and Y38C) also reduce phagocytic activity (Kleinberger et al. 2014). TYROBP is recently highlighted as one of the central genetic nodes and networks in LOAD (Paloneva et al. 2000, 2001; Zhang et al. 2013). Since both TREM2 and TYROBP are specifically expressed in myeloid cells and physically interact to transduce phagocytosis and anti-inflammatory signaling (Linnartz and Neumann 2013; N'Diaye et al. 2009; Paradowska-Gorycka and Jurkowska 2013), the TREM2/TYROBP pathway may play an important role for regulating peripheral or central inflammation.

30.4.2 Glial Inflammation and Innate Immunity

Innate immune responses in AD are mediated by a large number of inflammatory proteins, such as complement factors, acute-phase proteins, pro-inflammatory cytokines, and chemokines (McGeer and McGeer 2001; Akiyama et al. 2000). Upregulation of the complement system includes activation of both classical and alternative pathways and enhanced secretion of cytokines and chemokines including, but not limited to, IFN- γ , IL-1 α/β , IL-6, IL-8, IL-12, TGF- β 1/2, TNF- α , CD40L, CCL2 (MCP-1), CCL3 (MIP-1 α), CCL4 (MIP-1 β), S100- β (for review, see (Akiyama et al. 2000)). The interplay between A β aggregates, microglia and astrocytes results in secretion of pro-inflammatory cytokines that, in turn, may affect local APP expression in nearby neurons. This process results in local upregulation of A β and seeding of “new” A β deposits, which may therefore be a potential mechanism for the plaque accumulation typically seen in AD brains. A detailed review on this topic can be found in our recent publication (Varnum and Ikezu 2012).

30.4.2.1 Cytokines

IL-1

IL-1 upregulation seems to occur early in A β plaque formation since it is associated with diffuse plaques and also found in young children with Down's syndrome (Griffin et al. 1989, 1995). IL-1 induces S100 β , an astrocyte-derived neurite promoting cytokine (Kligman and Marshak 1985), and α 1-antichymotripsin (Das and Potter 1995). IL-1 upregulates APP synthesis in primary astrocytes (Rogers et al. 1999), and A β production when co-stimulated with IFN- γ in vitro (Blasko et al. 2000).

IFN- γ

IFN- γ is produced by Th1 type T cells and natural killer cells (Young and Hardy 1995), as well as macrophages (Fultz et al. 1993), astrocytes, and microglia (DeSimone et al. 1998). IFN- γ affects A β production, A β degradation (Yamamoto et al. 2007a), and neurotoxicity (Meda et al. 1999). IFN- γ , in conjunction with TNF- α , has been shown to enhance APP mRNA transcription and A β production (Blasko et al. 1999), and indirectly induce neuronal loss by stimulating release of NO from microglia (Chao et al. 1995). In the Tg2576 mouse model, astrocytes significantly upregulate IFN- γ mRNA in vivo (Abbas et al. 2002). We have recently found that transgenic APP mice (Tg2576) lacking IFN- γ receptor type I show marked reduction of A β deposition, microgliosis, astrogliosis, and astrocytic BACE expression (Yamamoto et al. 2007a). Thus, IFN- γ plays an important role for AD pathogenesis.

IL-4

IL-4 is a prototypical anti-inflammatory cytokine derived from Th2 cells. IL-4 is a particularly important cytokine for M2a skewing (Lyons et al. 2007b; Maher et al. 2005; Nolan et al. 2005). Microglia stimulated by IL-4 have a decrease in TNF- α production and an increase in secretion of insulin-like growth factor-1 (IGF-1), and they also induce neurogenesis in vitro (Butovsky et al. 2005, 2006). Stimulation of microglia by IL-4 enhanced neural differentiation (Kiyota et al. 2010). Gene delivery of IL-4 into APP+PS1 mice partially suppressed glial accumulation in the hippocampus, directly enhanced neurogenesis, restored impaired spatial learning, and also reduced A β deposition (Kiyota et al. 2010). Gene delivery of either IL-4 also reversed the LPS-induced deficit in LTP in the rat hippocampus (Lynch et al. 2004; Nolan et al. 2005). Thus, IL-4 is an important therapeutic target of AD.

IL-6

IL-6 is a pleiotropic pro-inflammatory cytokine, secreted from T cells, macrophages, astro/microglia, and is involved in a number of inflammatory signals, including the acute phase response. IL-6 and its receptor complex, IL-6R and

gp130/CD130, are all upregulated in AD brains in a region-specific manner (Hampel et al. 2005). IL-6 immunoreactivity is co-localized with diffuse plaques but not with compact plaques (Hull et al. 1996), suggesting its role in diffuse plaque reaction rather than compact plaque formation. Astrocyte-derived IL-6 induces expression of complement component C3, and may be involved in complement cascade activation in AD (Barnum et al. 1996).

IL-10

IL-10 is an anti-inflammatory cytokine induced by either pro or anti-inflammatory activation of immune cells, including T cells and mononuclear phagocytes. Instead of inducing alternative activation of immune cells (such as seen by IL-4 stimulation), IL-10 suppresses intracellular signaling to restore the resting status of activated cells. Our group recently identified that resting microglia express high levels of TGF- β 1, extracellular matrix-related genes, and IL-10 (Freilich et al. 2013). With gene delivery of IL-10 into APP+PS1 mice, there was suppression of astro/microgliosis and a restoration of impaired spatial learning and neurogenesis, and while there was no reduction in A β deposition, there was enhanced vascular transport of A β (Kiyota et al. 2011). Gene delivery of either IL-10 also reversed the LPS-induced deficit in LTP in the rat hippocampus (Lynch et al. 2004; Nolan et al. 2005). In accord, we recently identified that IL-10-stimulated microglia enhanced proliferation but not differentiation of neural stem cells in vitro (Kiyota et al. 2011). Thus, IL-10 has a significant potential as a therapeutic target of AD.

TGF- β 1/2/3

TGF- β 1/2/3 are expressed in the CNS and are involved in development, homeostasis, and repair (Finch et al. 1993). TGF- β 1 has been detected in plaques (van der Wal et al. 1993) and is elevated in cerebrospinal fluid and serum in AD (Chao et al. 1994a, b). TGF- β 2 has also been detected in reactive astrocytes, microglia, and in tangle-bearing neurons in AD brains (Wyss-Coray et al. 2000). TGF- β 1 transgenic mice show cerebrovascular amyloid deposition (Wyss-Coray et al. 1997). Over-expression of TGF- β 1 gene in APP mice accelerates vascular amyloid deposition although plaque burden was reduced in brain tissue (Wyss-Coray et al. 1997, 2001), suggesting its role in clearing A β from brain parenchyma. Since TGF- β 1 increases APP expression in astrocytes and microglia (Gray and Patel 1993; Monning et al. 1994; Harris-White et al. 1998), it may also be involved in TGF- β 1 glial A β production. Disruption of TGF- β 1 gene induces neurodegeneration and microgliosis (Brionne et al. 2003). APP mice over-expressing kinase-deficient TGF- β receptor type II, which inhibits endogenous TGF- β signaling, show enhanced A β deposition, neurodegeneration, and dendritic loss (Tesseur et al. 2006).

TNF- α

TNF- α , a pleiotropic pro-inflammatory cytokine, has been extensively investigated in AD. TNF- α is predominantly expressed by activated microglia, and to a lesser extent, astrocytes and neurons (Meda et al. 1995; Botchkina et al. 1997; Bezzi et al. 2001; Williams et al. 2005). In AD, TNF- α is upregulated in cerebrospinal fluid (Tarkowski et al. 1999), serum (Fillit et al. 1991), and cortex (Tarkowski et al. 1999). The effect of TNF- α on neurons is different dependent on the neuronal preparation and perhaps species; it is neurotrophic to rat cortical, hippocampal, and septal neurons (Cheng et al. 1994; Barger and Harmon 1997), while directly neurotoxic (or enhances glutamate neurotoxicity) to human cortical neurons (D'Souza et al. 1995; Williams et al. 2005; Chao and Hu 1994). This discrepancy could be due to the diverse signaling of two distinctly different TNF- α receptors, type I (p55 TNFR-1) and type II (p75 TNFR-2) (Botchkina et al. 1997; Dziewulska and Mossakowski 2003). TNFR-1, but not TNFR-2, contains an intracellular death domain, common to proteins in the TNF receptor family. TNFR-1 mediates both apoptosis and NF- κ B-mediated cell survival (Wallach et al. 1999; Gupta and Gollapudi 2005). Importantly, primary cultured hippocampal neurons derived from TNFR-1 null mice were resistant to A β -mediated neurotoxicity (Li et al. 2004). Thus, TNF- α -mediated neurotoxicity is bidirectional and the outcomes of TNF signaling depend distinctly on cell type and species.

CD40L

CD40L belongs to the TNF family and one of the most important T-cell mediated cytokines. CD40, the receptor of CD40L, is upregulated by A β -stimulation of primary cultured microglia in vitro (Tan et al. 1999) and is found to be expressed in reactive microglia in AD patient brain tissue (Togo et al. 2000). Transgenic APP (Tg2576) mice deficient in CD40L show reduced A β deposition, astro/microgliosis, and hyperphosphorylation of tau (Tan et al. 2002). In addition, chronic treatment of APP/PS1 bigenic mice with neutralizing anti-CD40L antibody reduces A β deposition and causes a modest improvement in cognitive performance (Tan et al. 2002; Todd Roach et al. 2004), suggesting its therapeutic application for AD.

CD200

CD200 is a type I transmembrane glycoprotein with an immunoglobulin superfamily (IgSF) domain (Clark et al. 1985). It is expressed on a number of cells involved in the immune response, including T cells, B cells, and particularly in neurons in the brain (Barclay 1981; McMaster and Williams 1979; Webb and Barclay 1984). The CD200R is expressed largely on cells of myeloid lineage, namely, microglia (Barclay 1981; Koning et al. 2010). It is a cell surface glycoprotein with IgSF domains and a single transmem-

brane region (Wright et al. 2000). Unlike CD200, however, it has a large cytoplasmic region allowing for intracellular signaling initiated by tyrosine phosphorylation (Wright et al. 2000). CD200 expression decreases with age where it is simultaneously associated with increased microglial activation (Lyons et al. 2007a). It is likewise decreased in the brains of AD patients and in the brains of A β -treated mice (Lyons et al. 2009; Walker et al. 2009). Interestingly, hippocampal slices from mice lacking CD200 also showed significant impairments in synaptic plasticity, specifically in LTP, tying the age-dependent decrease in CD200 with the decreased ability to form new memories in AD patients (Costello et al. 2011).

In animal models of AD, where the age-related increase in microglial activation was accompanied by an age-related or A β -induced decrease in CD200 expression, IL-4 treatment was able to restore that deficit (Kiyota et al. 2010; Lyons et al. 2007a, b). These tissues and glia from AD models lack IL-4 and have enhanced inflammatory responses to LPS (Lyons et al. 2009). In accord, our laboratory recently identified that over-expression of CD200 significantly reduced A β deposition and inflammatory changes in Tg2576 APP mice (unpublished observation). These studies indicate that CD200 is a novel therapeutic target of AD.

30.4.2.2 Chemokines

CCL2 (MCP-1)

CCL2 (MCP-1) is a member of the β chemokine subfamily and a candidate molecule for stimulating monocyte chemotaxis into the CNS (Charo et al. 1994). Activated astrocytes and cells of monocytic origin (such as microglia and macrophages) have been shown to express CCL2 in the brain (Calvo et al. 1996; Glabinski et al. 1996). CCL2 has been detected in senile plaques, reactive microglia and microvessels in AD brains (Ishizuka et al. 1997; Xia and Hyman 1999; Grammas and Ovase 2001), and is upregulated in cerebrospinal fluid and serum of AD cases and AD animal models (Sun et al. 2003; Galimberti et al. 2005; Janelins et al. 2005). Over-expression of CCL2 resulted in increased diffuse plaque deposition, microglial accumulation, and apoE expression in APP mice (Yamamoto et al. 2005), accompanied by cognitive impairment (Kiyota et al. 2009b). Intracerebral over-expression of dominant-negative CCL2 ameliorate the spatial learning of APP/PS1 mice (Kiyota et al. 2009a). On the otherhand, genetic disruption of CCR2 increases A β accumulation and neurodegeneration when crossed with APP mouse model, suggesting that physiological CCL2/CCR2 signaling is necessary for the A β clearance in brain (El Khoury et al. 2007). These results show that CCL2/CCR2 signaling is critical in mononuclear phagocyte accumulation and A β deposition in CNS.

CXCR Family

A number of other chemokines and their receptors have been identified in AD. CXCR2 is a member of the CXC chemokines receptor family and is a receptor for multiple ligands, including CXCL1/2/3 (GRO α / β / δ) (Zlotnik and Yoshie 2000). Both CXCR2 and CXCL1 have been detected in AD brains. In particular, CXCR2 expression has been identified in dystrophic neurites of senile plaques (Xia et al. 1997). CXCR3, another CXC receptor family, and its ligand CXCL10 (IP-10) have been found in both cortical and subcortical neurons in normal and AD brains. CXCL10 was significantly upregulated in AD brain (Xia et al. 2000). In addition, expression of CC chemokine receptors, such as CCR3 and CCR5, and their ligands, CCL4 (MIP-1 β) and CCL5 (RANTES), is also enhanced on reactive microglia in AD brain (Xia et al. 1998). Although the exact physiological functions of these molecules have yet to be characterized in the context of AD pathogenesis, modulation of such chemokine signaling might be beneficial to correct the detrimental imbalance of metabolic homeostasis and neuroinflammation in brain.

30.4.2.3 Toll-Like Receptors

One of the most important recent findings in the effort to understand innate immunity has been the identification of Toll-like receptors (TLRs). TLRs define a major class of pattern-recognition receptors critical to the initiation and tailoring of both innate and subsequent adaptive immune responses (Beutler 2004; Iwasaki and Medzhitov 2004). For example, bacterial cell wall components are recognized by TLR2, and lipopolysaccharide and viral envelope proteins are recognized by TLR4. Double strand RNA, single strand RNA, and unmethylated CpGs are recognized by TLR3, TLR7/8, and TLR9, respectively (for review, see (Akira et al. 2006)). Primary cultured human and mouse microglia express mRNA for TLR1-9, whereas human and mouse astrocytes express high levels of TLR3, and low-level TLR 1, 2, and 4-6 (Jack et al. 2005; Olson and Miller 2004) (McKimmie and Fazakerley 2005; Carpentier et al. 2005). A recent study on aged mouse brains demonstrated that TLR1, TLR2, TLR4, TLR5, TLR7 and CD14 expression were upregulated in correlation with age, whereas TLR9 was downregulated (Letiembre et al. 2007). Interestingly, a TLR4 polymorphism was also associated with successful aging (Candore et al. 2006), which further indicates a role of innate immune receptors in aging and potentially AD.

The role of TLRs in neuroinflammation during AD is poorly characterized. However, recent reports indicate that TLR9 ligand (CpG-containing oligonucleotide) induced enhancement of A β uptake by N9 microglia cell line (Iribarren et al. 2005), and A β stimulation modulates TLR-specific inflammation (NO and TNF- α release) in primary cultured mouse microglia (Lotz et al. 2005). These results

suggest that there is crosstalk between A β and TLR signaling that may regulate glial inflammation and A β clearance in CNS.

30.4.2.4 Matrix Metalloprotease

A β stimulation induces robust expression of matrix metalloprotease (MMP) 1, 3, 10, 12, 19, and disintegrin and metalloprotease (ADAM) 8 in primary cultured human microglia (Walker et al. 2005). Increased expression of MMP 1, 3 and 9 has been documented in AD brains (Leake et al. 2000; Yoshiyama et al. 2000; Backstrom et al. 1996) and may contribute to tissue damage (Cuzner and Opdenakker 1999). These molecules may be involved in A β degradation, cytokine/chemokine processing, and shedding of cell adhesion molecules for enhancing glial motility.

30.4.2.5 Other Inflammatory Molecules: Complement Complex and CD45

Complement C1q, the first complement of the classical complement pathway, is upregulated in AD brains and is found in senile plaques (Afagh et al. 1996). C1q binds to soluble A β and modulates its uptake by mononuclear phagocytes (Webster et al. 2000). C1q also directly induces neurotoxicity through its interaction with cell surface calreticulin, a C1q receptor (Luo et al. 2003). However, APP mice lacking C1q exhibited reduced glial and astroglial activation without changes in A β deposition (Fonseca et al. 2004b). Thus, C1q may be involved in AD pathogenesis by influencing neurotoxicity and glial inflammation, but its effect on A β metabolism is modest.

Complement C3 is upregulated in APP/TGF- β 1 bigenic mice and AD brains (Wyss-Coray et al. 2001). Overexpression of soluble complement receptor-related protein y (sCrry), a complement C3 inhibitor, resulted in enhanced A β accumulation and hippocampal degeneration (Wyss-Coray et al. 2002). Since C3 is known to promote phagocytosis by binding to specific C3 receptor on specialized cells, C3 is likely to be positively involved in A β clearance.

CD45, a transmembrane protein tyrosine phosphatase critically involved in negative regulation of T- and B-cell activation, and an antagonizing partner of the TNF superfamily (Tan et al. 2000b), is also upregulated in AD brains (Masliah et al. 1991). APP mice deficient in CD45 have increased brain levels of TNF- α and NO, suggesting CD45 negative regulation of neuroinflammation in AD (Tan et al. 2000a).

30.4.3 Oxygen Free Radicals

Production of ROS, factors involved in the aging process (Finkel and Holbrook 2000), is elevated in AD brain and may be an important cause of AD (Martins et al. 1986). Elevated

levels of oxidized lipids (lipid peroxidation, malondialdehyde, 4-hydroxynonenal) (Markesbery and Carney 1999), proteins (advanced glycation end product modifications, tyrosine nitration) (Takeda et al. 1998; Good et al. 1996), and nucleic acids (8-hydroxy-deoxyguanosine) have been documented in AD brains (Lyras et al. 1997). Mitochondria and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex dysfunction can generate large amounts of ROS (Beal 1998), and down regulation of mitochondrial NADPH 15-kD gene have been found in AD cases (Manczak et al. 2004; Beal 1998). Markers of oxidative stress are elevated not only in the pathologic lesions in AD brain (Good et al. 1996), but also in cerebrospinal fluid of AD patients (Lovell et al. 1997). A β peptide can directly activate the NADPH oxidase complex of mononuclear phagocytes (Bianca et al. 1999) and microglia, which are mediated through the interaction of fibrillar A β to cell surface molecules (CD36, CD47, and α 6 β 1-integrin) and tyrosine kinase Vav signaling (Wilkinson et al. 2006). ROS is also known to mediate A β -induced neurotoxicity (Behl et al. 1994; Ikezu et al. 2003). In addition to NADPH oxidase, myeloperoxidase (MPO) is expressed by microglia in close proximity to A β plaques, and surprisingly, in neurons of AD brains (Reynolds et al. 1999; Green et al. 2004). MPO can catalyze both apoE and A β , generating oxidated and fragmented apoE (Jolivald et al. 1996) and stable A β 42 dimers through dityrosine bridge formation (Galeazzi et al. 1999; Jolivald et al. 1996).

30.4.3.1 Oxidative DNA Damage

DNA damage may also have a role in AD pathology. Recent studies indicate that aging-mediated DNA damage, including alterations in neuronal network and cognitive function-related gene expression profiles, is significantly increased after 40 years of age, (Lu et al. 2004). DNA damage induces cell cycle activation (Kruman et al. 2004), and cell cycle activation has been documented in AD brains (Nagy et al. 1998; Smith et al. 1999). DNA damage also potently induces p53, a tumor suppressor and transcriptional factor involved in neuronal apoptosis (Morrison et al. 2003; Kruman et al. 2004; Culmsee and Mattson 2005). Neuronal expression of p53 is increased in AD brains (de la Monte et al. 1997) and in APP mouse brains (LaFerla et al. 1996). These data suggest that DNA damage is involved in neurodegeneration in AD brain, or the result of neurodegeneration.

30.5 Immunotherapeutics Inventions

A detailed review on the immunotherapeutics of AD is referred to Sect. 3.2.1 (David Cribbs). Epidemiological studies using non-steroidal anti-inflammatory drugs (NSAIDs) has led to the proposal that NSAIDs reduce the risk of AD

(McGeer et al. 1996). These studies have been supported by data showing that treatment of APP mice with NSAIDs (such as ibuprofen and NO-flurbiprofen) in vivo results in reduced A β deposition and pro-inflammatory cytokine production (Lim et al. 2000; Jantzen et al. 2002; Yan et al. 2003). Microglial activation is suppressed by ibuprofen but rather enhanced by NO-flurbiprofen. Other studies have found that NSAID also affects γ -processing of APP through inhibition of the small GTP-binding protein Rho, which converts A β processing from A β 42 to A β 40 both in vitro and in vivo (Eriksen et al. 2003; Weggen et al. 2001; Zhou et al. 2003). Based on these observations, a new series of drugs derived from NSAIDs having a more specific effect on γ -processing has been developed (Kukar et al. 2005; Weggen et al. 2003; Czirr and Weggen 2006).

Proliferator-activated receptor γ (PPAR γ) agonists, such as the thiazolidinedione family, are also NSAIDs and can efficiently clear A β deposition in vitro and in vivo (Camacho et al. 2004; Heneka et al. 2005). PPAR is a nuclear transcription factor and its effect is different from ibuprofen and its derivatives. PPAR γ transcription factors play important physiological roles in the regulation of lipid metabolism (Mangelsdorf et al. 1995; Lemberger et al. 1996), suppression of inflammation (Heneka et al. 2000), and clinical treatment of diabetes type II (Dormandy et al. 2005). PPAR γ agonists enhance A β degradation (Camacho et al. 2004), as well as suppress BACE expression and microglial activation (Heneka et al. 2005).

Other anti-inflammatory/anti-oxidant dietary applications include curcumin, the yellow pigment in turmeric that targets multiple AD pathogenic cascades (Yang et al. 2005; Lim et al. 2001). The dietary omega-3 fatty acid, docosahexaenoic acid (DHA), also reduced amyloid, oxidative damage and synaptic and cognitive deficits in a transgenic mouse model (Lim et al. 2005; Calon et al. 2004). Both DHA and curcumin have favorable safety profiles, epidemiology and efficacy, and may exert general anti-aging benefits (Cole et al. 2005).

30.6 Review Questions

- Which is NOT the causative gene of familial Alzheimer's disease?
 - APP
 - Presenilin-1
 - Presenilin-2
 - Tau*
 - None of the above
- Presenilin-1 is a member of:
 - α -secretase
 - β -secretase
 - γ -secretase complex

- (d) A β degrading enzyme
- (e) None of the above
3. Amyloid-beta peptide is a processing product of amyloid precursor protein (APP) through a combination of
 - (a) α -secretase and Beta-site APP cleaving enzyme (BACE1)
 - (b) α -secretase and γ -secretase complex
 - (c) BACE1 and γ -secretase complex
 - (d) BACE1 and insulin-degrading enzyme
 - (e) none of the above
4. Microglia-mediated inflammatory reaction affects AD progression via
 - (a) cytokine production
 - (b) reactive nitrogen/oxygen production
 - (c) excitotoxin production
 - (d) *all of the above*
 - (e) none of the above
5. A β deposition in APP mouse brain can be reduced by vaccination with the following antigens except
 - (a) A β
 - (b) *apoE4*
 - (c) Copaxon-1
 - (d) transgenic potato expressing amyloid-beta peptide
 - (e) none of the above
6. Non-steroidal anti-inflammatory drugs (NSAIDs) can reduce A β deposition in APP mouse brain through
 - (a) suppression of brain inflammation
 - (b) suppression of A β 42 generation
 - (c) suppression of BACE expression
 - (d) *all of the above*
 - (e) none of the above
7. Describe the available animal models for studying AD. What is the limitation of the animal models?
8. Describe the potential immunotherapy of AD.

30.7 Answers

7. Common animal models of AD includes transgenic mice expressing familial-AD-linked mutation of APP (such as Tg2576, PDAPP, and APP23) and PS1 (such as PS1/APP and 3xTg-AD). These mouse models consistently develop amyloid plaques, neuroinflammation, cognitive dysfunction and neurophysiological impairment as determined by electrophysiology. The limitation of these animal models are the lack of two other neuropathology in AD: prominent neurofibrillary tangle formation and neuronal cell loss in cortical and subcortical regions. Many of the mouse model also takes many months to develop disease-like pathology, often over 1 year.
8. ANSWER: A conventional approach is either active or passive immunotherapy against A β . Active immunization of AD mouse models and human patients with synthetic

A β peptide was successful in reducing the clearance of amyloid deposition in the brain. However, this approach is largely obsolete due to the significant side effect associated with active immunization, such as meningoencephalitis. Passive immunization is also effective in lowering the amyloid load in the brain, although in a transient manner, without significant side effects. Passive immunization against A β is active in a large scale clinical trials, such as Anti-amyloid Treatment in Asymptomatic Alzheimer's disease (A4).

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Abstract

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by motor abnormalities and a loss of primarily, but not exclusively, dopaminergic neurons. Other neuropathological hallmarks of PD include the proteinaceous inclusions, Lewy bodies and various inflammatory changes. The inflammation is made up of resident innate and, to lesser extent, adaptive immune cells. These inflammatory changes are found in both sporadic and familial non-PD parkinsonian syndromes and in experimental models of PD. There are studies that suggest that inflammation initiates PD, but most investigations rather suggest that, in PD, inflammation is secondary to the demise of the neurons. Mounting evidence indicates that inflammation in PD and in related conditions can modulate the neurodegenerative process. However, there is still a lack of consensus about whether inflammation in PD plays a beneficial or a detrimental role.

Keywords

Adaptive immune system • Astrocyte • Chromogranin • Cyclooxygenase-2 • Dopamine FAS • Ferritin • Glial-derived neurotrophic factor • Glial fibrillary acidic protein • HLA-DR • 6-Hydroxydopamine • Inflammation • Innate immune system • Interferon-gamma • Interleukine-1beta • Lewy body • Leucine-rich repeat kinase 2 • Lipopolysaccharide • Metallothionein-I/II • Microglia • MPP+ • MPTP • Neuromelanin • Nigrostriatal pathway • Nitric oxide • Paraquat • Parkin • Reactive nitrogen species • Reactive oxygen species • Rotenone • Substantia nigra • Striatum • Alpha-synuclein • T-cell • Toll-like receptors • Transcription nuclear factor-k-B • Tumor necrosis factor-alpha

31.1 Introduction

In this chapter, selected topics on inflammation in Parkinson's disease (PD) and in models of this human disease will be reviewed. To set the stage, the biological, clinical and pathological hallmarks of PD and related degenerative conditions

are reviewed. A more comprehensive view of PD can be found in the following references (Fahn and Przedborski 2005; Dauer and Przedborski 2003).

Parkinson's disease is the second most common neurodegenerative disorder of the aging brain after the dementing disorder, Alzheimer's disease (AD). The average age of onset is in the fifth to sixth decades of life with a reduced life expectancy for all onset ages but, as reported by Ishihara et al. (2007), this reduction is greatest in individuals with a young-onset PD (i.e. age at onset ≤ 40). It is estimated that more than one million individuals in the United States alone are affected with PD and, around 50,000 new cases are identified each year. The main clinical features of PD include resting tremor, slowness and paucity of voluntary move-

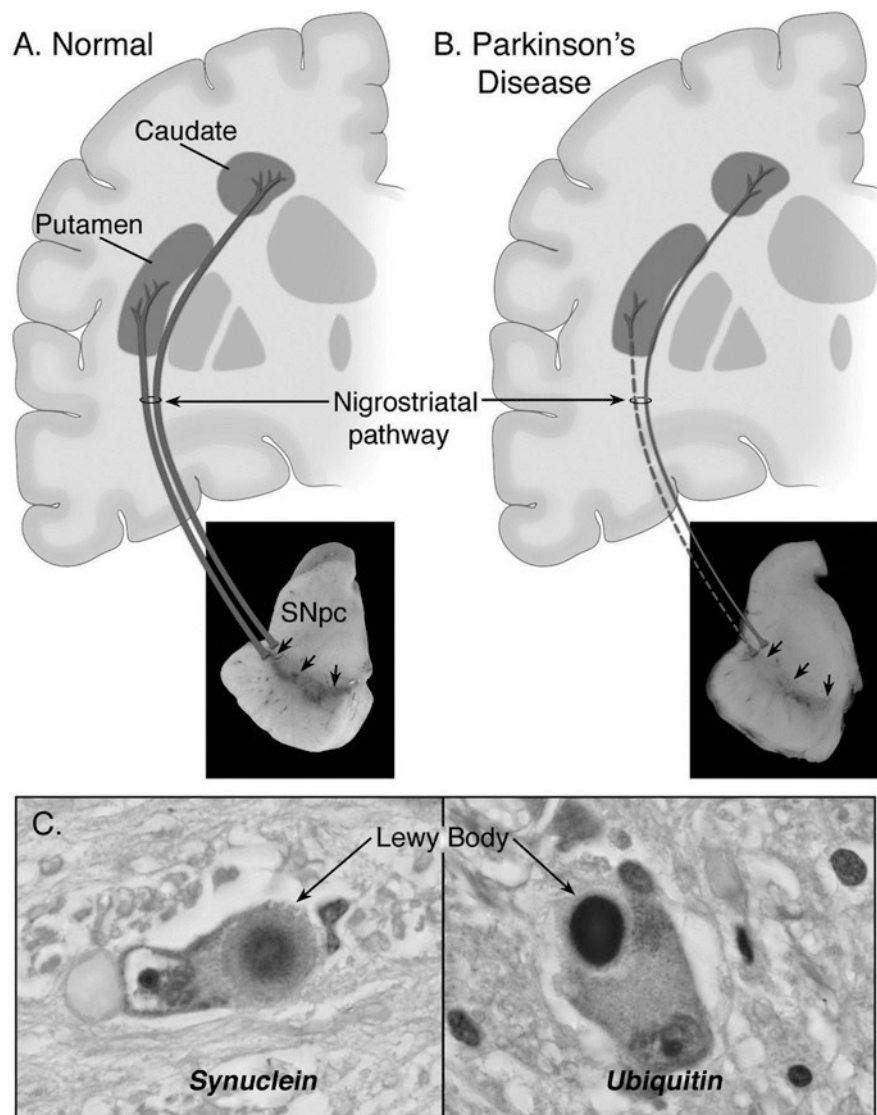
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ments (i.e. bradykinesia), increased muscle tone (i.e. rigidity), and postural instability. Similar to many other prevalent neurodegenerative disorders discussed in this book, including AD and amyotrophic lateral sclerosis (ALS), PD presents itself essentially as a sporadic condition. However, as with AD and ALS, only a small fraction of PD patients (~10%) inherit the disease. In some of these familial cases, the genetic defect has been identified and linked to mutations in a variety of genes. Currently, over 15 disease-causing genes for PD have been identified (Trinh and Farrer 2013). The most salient motor features of PD cited above are similarly seen in both the sporadic and the familial forms of PD and have, especially for bradykinesia and rigidity, been attributed to a profound deficit in brain dopamine (Hornykiewicz and Kish 1987). Given this fact, it is not surprising that most of the attention has been geared toward the study of the dopaminergic pathways in PD.

Autopsy investigations have revealed that most of the ascending dopaminergic pathways in PD are affected, although to different degrees (Hornykiewicz and Kish 1987), the nigrostriatal pathway being consistently more affected than any other (Hornykiewicz and Kish 1987). The nigrostriatal pathway is made up of dopaminergic neurons whose cell bodies are located in the substantia nigra pars compacta and their projecting axons and terminals in the striatum (Fig. 31.1a). Among these dopaminergic neurons, it appears that those with the highest content of brown pigment called neuromelanin are the ones most prone to degeneration (Hirsch et al. 1988). In contrast to the damage to the ascending pathways, the descending dopaminergic pathways are generally spared (Hornykiewicz and Kish 1987). If the most salient clinical features of PD are indeed related to the damage in the dopaminergic system in the brain, it cannot be stressed enough that degenerative changes in PD are restricted neither to the nigrostriatal pathway nor to other

Fig. 31.1 Neuropathology of Parkinson's disease. **(a)** Schematic representation of the normal nigrostriatal pathway (in red). It is composed of dopaminergic neurons whose cell bodies are located in the substantia nigra pars compacta (SNpc; see arrows). These neurons project (thick solid red lines) to the basal ganglia and synapse in the striatum (i.e., putamen and caudate nucleus). The photograph demonstrates the normal pigmentation of the SNpc, produced by neuromelanin within the dopaminergic neurons. **(b)** Schematic representation of the diseased nigrostriatal pathway (in red). In Parkinson's disease, the nigrostriatal pathway degenerates. There is a marked loss of dopaminergic neurons that projects to the putamen (dashed line) and a much more modest loss of those neurons that project to the caudate (thin red solid line). The photograph demonstrates depigmentation (i.e., loss of the dark-brown pigment neuromelanin; arrows) of the SNpc due to the marked loss of dopaminergic neurons. **(c)** immunohistochemical labeling of intra-neuronal inclusions, termed Lewy bodies, in a SNpc dopaminergic neuron. Immunostaining with an antibody against α -synuclein reveals a Lewy body (black arrow) with an intensely immunoreactive central zone surrounded by a faintly immunoreactive peripheral zone (left photograph). Conversely, immunostaining with an antibody against ubiquitin yields more diffuse immunoreactivity within the Lewy body (right photograph). From Dauer and Przedborski, *Neuron* 39:889–909, 2003, with permission



dopaminergic systems (Hornykiewicz and Kish 1987; Agid et al. 1987). For instance, abnormal histological features can also be found in many non-dopaminergic cell groups including the locus coeruleus, raphe nuclei, and nucleus basalis of Meynert (Braak et al. 1995).

In PD, all affected areas of the brain also contain intra-neuronal proteinaceous inclusions called Lewy bodies (Shults 2006). These bodies contain alpha synuclein and neuromelanin and can readily be visualized by either classical histological methods, e.g. hematoxylin & eosin, or by immunohistochemistry using antibodies raised against alpha-synuclein or ubiquitin (Fig. 31.1b, c). Often, Lewy bodies are large and round, and occupy most of the cytoplasmic area of the few spared dopaminergic neurons. While identification of these proteinaceous inclusions is usually used for neuropathological diagnostic purposes, the actual pathogenic role of Lewy bodies remains controversial. Another, but often overlooked, salient neuropathological feature of PD is the presence of inflammatory changes within affected regions (Forno et al. 1992; McGeer et al. 1988). This inflammatory response is the topic of this chapter and will be described in further detail below.

31.2 Inflammatory Response in Parkinson's Disease

Before discussing the issue of inflammation in PD *per se*, it is worth reviewing briefly the question of innate resident immune cell topography in the normal brain. In a healthy adult brain, microglia constitute roughly 10% of all glial cells and are not evenly distributed in the brain (Lawson et al. 1990). In an unstimulated situation, microglia exhibit elongated, bipolar shaped cell bodies with spine-like processes that often branch out perpendicularly. Among the areas of the brain susceptible to the PD process, the density of microglia identified by the labeling of the specific plasma membrane glycoprotein F4/80 (a well-characterized macrophage marker that is part of the EFG-TM7 family and that shares a 68% amino acid homology with human EGF-like module-containing mucin-like hormone receptor-like 1) is higher in the substantia nigra compared to other regions of the brain, at least in adult mice (Lawson et al. 1990). In contrast to microglia, astrocytes in the normal adult brain are homogeneously distributed, except in the midbrain where the estimated density of glial fibrillary acidic protein (GFAP)-positive cells varies among the different catecholaminergic groups (Damier et al. 1993). The density of GFAP-positive cells is moderate in those midbrain areas most severely affected in PD, such as the SNpc, and high in those areas least affected, such as the central gray matter (Damier et al. 1993). Furthermore, it has been shown that the density of GFAP-positive cells within the SNpc is lowest in the

calbindin-D_{28K} SNpc where the loss of dopaminergic neurons is the highest (Damier et al. 1999).

Because of the notoriety of nigrostriatal dopaminergic damage in PD, information regarding inflammation in this common neurodegenerative disorder pertains essentially to the substantia nigra and the striatum. Consistently, it has been found that the loss of dopaminergic neurons in post-mortem PD brains is associated with microgliosis and astrogliosis (McGeer et al. 1988; Forno et al. 1992; Banati et al. 1998; Mirza et al. 2000). However, these changes have always been found to be more intense in the SNpc than in the striatum (McGeer et al. 1988). This situation contrasts with that of the loss of dopaminergic structures, which is more intense in the striatum than in the substantia nigra.

At the cellular level, the majority of the immunostained astrocytes in PD exhibit a resting-like morphology, with thinly shaped cell bodies and elongated processes, while only few exhibit a reactive morphology with hypertrophic cell bodies and short processes (Mirza et al. 2000; Forno et al. 1992; McGann et al. 2012). Furthermore, there seems to be only a minimal increase in astrocyte numbers when GFAP- or metallothionein I/II-positive cells are quantified (Mirza et al. 2000). Unlike the astrocyte alterations discussed above, the microglial changes in PD are striking (McGeer et al. 1988; Banati et al. 1998; Mirza et al. 2000; Imamura et al. 2003). Morphologically, microglial cells in both the substantia nigra and the striata of PD patients, unlike controls, typically exhibit thick, elongated processes (McGeer et al. 1988; Banati et al. 1998; Mirza et al. 2000). The number of these activated microglia, identified by HLA-DR or ferritin immunostaining, is much higher in PD than in controls (Mirza et al. 2000; Imamura et al. 2003). Activated microglia are found mainly near free neuromelanin in the neuropil and remaining neurons in the substantia nigra (McGeer et al. 1988). Similar microglial activation is also found in the striatum (Imamura et al. 2003). However, do these activated microglia contribute to the PD disease process via active processes .g. release of cytokines) or rather by passive ones, merely by failing to function properly (Kaushik and Basu 2013)?

31.3 Inflammatory Response in Parkinsonian Syndromes

While PD is the most common of the parkinsonian syndromes, more than 30 different neurological syndromes share the clinical features of PD (Dauer and Przedborski 2003). Many of these non-PD parkinsonian syndromes are sporadic (Table 31.1) and often exhibit both clinical (e.g., ocular movement) and neuropathological features typically not seen in PD (e.g., striatal or corticospinal track pathology). Of these syndromes, most, if not all, show evidence of nigrostriatal neurodegeneration (not always associated with

Table 31.1 Parkinsonian syndromes

<i>Primary parkinsonism</i>
Parkinson disease (sporadic, familial)
<i>Secondary parkinsonism</i>
Drug-induced: dopamine antagonists and depletors
Hemiatrophy-hemiparkinsonism
Hydrocephalus: normal pressure hydrocephalus
Hypoxia
Infectious: post-encephalitic
Metabolic: parathyroid dysfunction
Toxin: Mn, CO, MPTP, cyanide
Trauma
Tumor
Vascular: multiinfarct state
<i>Parkinson-plus syndromes</i>
Cortical-basal ganglionic degeneration
Dementia syndromes: Alzheimer disease, diffuse Lewy body disease, frontotemporal dementia
Lytico-Bodig (Guamanian Parkinsonism-dementia-ALS)
Multiple system atrophy syndromes: Striatonigral degeneration, Shy-Drager syndrome, sporadic olivopontocerebellar degeneration (OPCA), motor neuron disease-parkinsonism
Progressive pallidal atrophy
Progressive supranuclear palsy
<i>Familial neurodegenerative diseases</i>
Hallervorden-Spatz disease
Huntington disease
Lubag (X-linked dystonia-parkinsonism)
Mitochondrial cytopathies with striatal necrosis
Neuroacanthocytosis
Wilson disease

MPTP 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, *ALS* amyotrophic lateral sclerosis (From Dauer and Przedborski, *Neuron* 39:889–909, 2003, with permission)

Lewy bodies) and inflammation (Oppenheimer and Esiri 1997) at autopsy. Unfortunately, inflammation is casually mentioned in most published reports on non-PD parkinsonian syndromes, and when the authors of these reports do mention inflammation, it is often limited to vague, qualitative statements. For instance, in the initial reports on progressive supranuclear palsy (Steel et al. 1964) and on striatonigral degeneration (Adams and Salam-Adams 1986), *gliosis* was recognized as a prominent feature of the pathological changes seen in these syndromes, but this is the only reference to inflammation. However, whether or not gliosis, as mentioned in these studies, refers to alterations in astrocytes, microglia, or in both, remains unknown. Despite this limitation, it is remarkable that gliosis is not only described in these non-PD parkinsonian syndromes at the level of the nigrostriatal pathway, but also at the level of other regions in the brain, which are normally not involved in PD. This indicates a rather wide-spread neuropathology which is consistent

with the usual multisystemic nature of many of these parkinsonian syndromes (Table 31.1).

Aside from sporadic parkinsonian syndromes, several familial forms of PD also exist (Vila and Przedborski 2004). Like in sporadic PD, histological examinations revealed similar glial alterations in most of the familial forms of parkinsonian syndromes, whether the underlying genetic defect has (Vila and Przedborski 2004) or has not been identified (Dwork et al. 1993). Among these familial parkinsonian syndromes, the situation of the autosomal dominant form of PD linked to mutations in the gene coding for leucine-rich repeat kinase 2 (LRRK2) is interesting (Zimprich et al. 2004). In the six autopsies from patients carrying a LRRK2 mutation reported by Zimprich and collaborators (2004), all had nigrostriatal dopaminergic neuronal loss and gliosis in the substantia nigra. However, some of these patients had dopaminergic neuronal loss and gliosis without Lewy bodies, whereas others had dopaminergic neuronal loss and gliosis with Lewy bodies (Zimprich et al. 2004). In the latter cases, Lewy bodies were restricted to the brainstem, or widespread in the brainstem and cortex. In one case, there were also *tau*-immunoreactive lesions not only in neurons, but also in glial cells (Zimprich et al. 2004). In the autosomal recessive parkinsonian syndrome linked to *parkin* mutations there is also a loss of dopaminergic neurons associated with astrogliosis and microgliosis but, as in several LRRK2 mutation cases, not typically with Lewy bodies (Hayashi et al. 2000). These findings support the view that neuroinflammation is a generic feature that arises from neuronal death irrespective of the type of parkinsonian syndrome or neuropathological picture. This also suggests that the presence of Lewy bodies is not a prerequisite for the occurrence of inflammation in PD and related conditions.

31.4 Inflammatory Response in Experimental Models

As discussed above, autopsy studies from patients afflicted with parkinsonian syndromes have led to the conclusion that inflammation is part of the neuropathological picture of these neurodegenerative disorders. While the descriptive data provided by these studies are invaluable, the generated information remains correlative by nature and fails to provide mechanistic insights. This is why researchers rely heavily on experimental models of PD. These models help to define the temporal and topographical relationships between inflammation and dopaminergic neuronal death and, more importantly, to determine the potential pathogenic role of inflammation in the death of dopaminergic neurons.

There are a variety of animal models of PD (Lee et al. 2012) to study the basis of neurodegeneration in this disorder, ranging from invertebrates to non-human primates. These are either genetic or toxic in nature (Dauer and

Przedborski 2003). There are generally two types of animal models. The first type involves genetically mutated genes (α -synuclein or LRRK2) which are associated with an increased risk of PD. The second type involves the administration of toxic agents, such as MPTP, rotenone and paraquat, which have been associated with PD-like symptoms in humans. The neuropathological picture created in these animal models, particularly in the toxic models, is very close to that described for PD including, neurodegeneration and motor and behavioral effects. In almost all of the PD models, whether or not there is an overt loss of nigrostriatal dopaminergic neurons, a glial response is found in the substantia nigra. Some data on inflammation are available in rodent models produced by the herbicide paraquat (McCormack et al. 2002) and the mitochondrial poison rotenone (Sherer et al. 2003). Data can also be found from transgenic mice expressing mutant alpha-synuclein (Gomez-Isla et al. 2003). The limited amount of data on inflammation in these models however is in striking contrast to the wealth of information available for the 6-hydroxydopamine (6-OHDA) and the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxic models of PD. Perhaps this can be explained by the fact that both the 6-OHDA and MPTP models are not only more popular among PD researchers, but also they have been used for much longer than the other models cited above. Supporting the generic nature of the inflammatory response in neurodegeneration are the observations that both the type and magnitude of the glial alterations in rodents following the administration of 6-OHDA (Nomura et al. 2000; Przedborski et al. 1995; Sheng et al. 1993; Stromberg et al. 1986; Rodrigues et al. 2001; He et al. 1999; Akiyama and McGeer 1989) are comparable to those seen following the administration of MPTP (see below).

In the early eighties, several drug-addicts became intoxicated with MPTP after self-injecting contaminated street-batches of a meperidine analog (Langston et al. 1983). Among these individuals, some developed an acute, irreversible parkinsonian syndrome indistinguishable from PD (Ballard et al. 1985). Of the few MPTP-intoxicated individuals who died and underwent autopsy, post-mortem examination showed a profound loss of nigrostriatal dopaminergic neurons (Davis et al. 1979; Langston et al. 1999). This dramatic loss of large neuronal cells was in striking contrast with the obvious abundance of small cells immunoreactive for either the astrocyte marker GFAP or the microglia marker HLA-DR (Langston et al. 1999). Almost all of the GFAP- and the HLA-DR-positive cells exhibited morphological characteristics of, respectively, reactive astrocytes and activated microglia (Langston et al. 1999). Images of neuronophagia were also observed (Langston et al. 1999). Similar glial alterations were found in six monkeys, which survived 5–14 years after an acute MPTP intoxication (McGeer et al. 2003). In all of these monkeys, there was also evidence of extracellular neuromelanin and activated microglia in the

substantia nigra (McGeer et al. 2003). These monkeys also had an abundance of reactive astrocytes positive for the intracellular adhesion molecule-1 (Miklossy et al. 2005). The presence of intracellular adhesion molecule-1-positive reactive astrocytes, of activated microglia, and of neuronophagia in humans and monkeys subjected to an acute MPTP intoxication many years prior is remarkable as these neuropathological features suggest an active, ongoing inflammatory process. Microglial activation and neuronophagia would be expected to be seen in PD tissues in light of its progressive neurodegenerative nature. However, as stressed by McGeer and collaborators (2003), these neuropathological findings challenge the belief that MPTP, “produces an acute loss of cells, followed by healing and long-term stabilization of surviving neurons.” Alternatively, these neuropathological data raise the possibility that acute MPTP intoxication can set into motion a self-sustained cascade of detrimental effects on dopaminergic neurons. Corroborating this view is a positron emission tomography study in which 10 individuals exposed acutely to MPTP were scanned twice, seven years apart (Vingerhoets et al. 1994). This work revealed a decrease of [18 F]fluorodopa uptake in the striata of these patients between the first and second imaging studies (Vingerhoets et al. 1994). Also remarkable is the fact that three of the 10 MPTP-intoxicated participants were asymptomatic at the time of the first scan but became parkinsonian by the time of the second scan (Vingerhoets et al. 1994) supporting a progressive nature of the neurodegenerative process in this toxic parkinsonian syndrome, at least in primates.

In small animals such as mice, time course experiments have been performed to define not only the kinetics of dopaminergic neuronal death but also of the glial response. These studies demonstrated that the appearance of reactive astrocytes paralleled the destruction of dopaminergic structures in both the striatum and the substantia nigra in mice (Czlonkowska et al. 1996; Kohutnicka et al. 1998; Liberatore et al. 1999). Worth noting, GFAP expression remained high even after the main phase of neuronal degeneration (Czlonkowska et al. 1996; Kohutnicka et al. 1998; Liberatore et al. 1999). Also important to note is the fact that, on inhibition of the entry of the active metabolite of MPTP, 1-methyl-4-phenylpyridinium (MPP^+), into dopaminergic neurons, there is not only a protection of substantia nigra dopaminergic neurons, but also a lack of glial response (O’Callaghan et al. 1990). This critical observation indicates that inflammation in the MPTP model is a consequence of the demise of neurons and not the reverse. This interpretation may not be valid for all types of injury used to model PD as we will see for the endotoxin of gram-negative bacteria lipopolysaccharide (LPS). The activation of microglial cells is also quite robust after MPTP administration to mice (Fig. 31.2); this has been extensively documented in the MPTP mouse model (Czlonkowska et al. 1996; Kohutnicka et al. 1998; Liberatore et al. 1999; Dehmer et al. 2000). When the astrocyte and

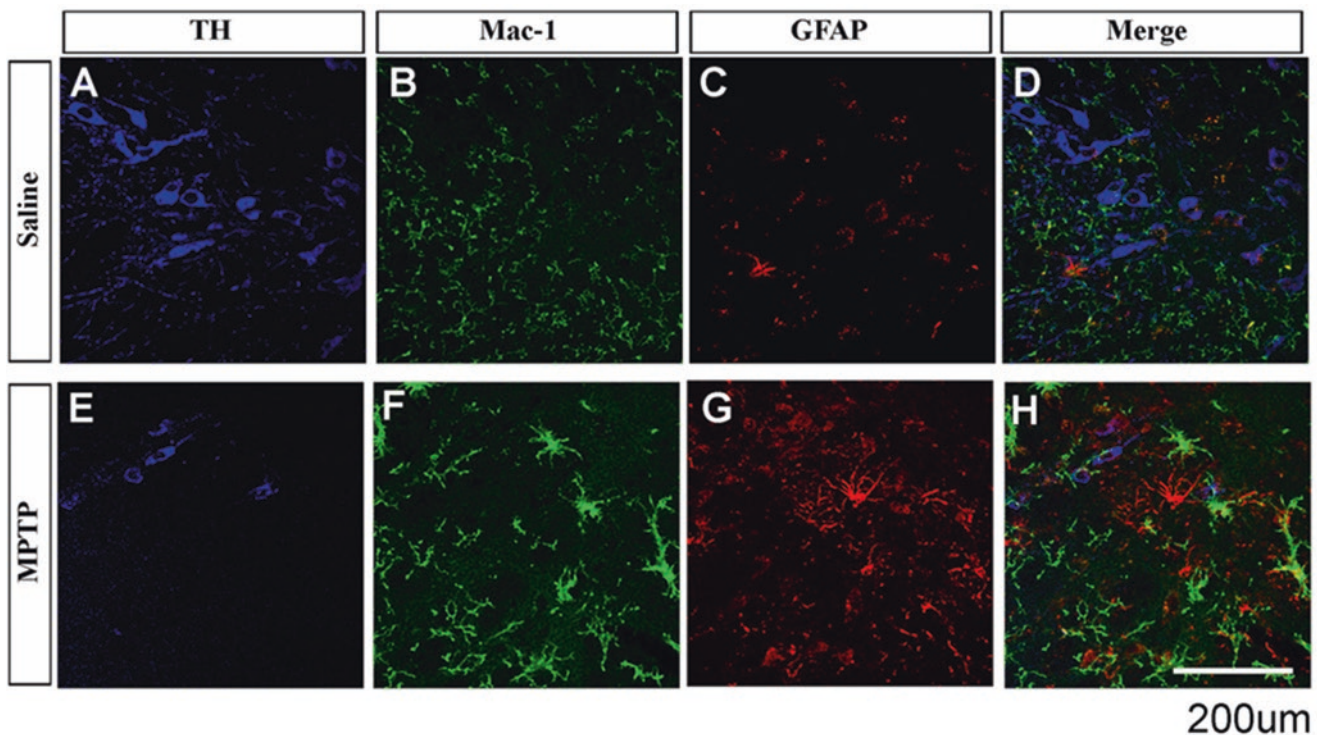


Fig. 31.2 Effects of MPTP on dopaminergic neurons and glial cells in ventral midbrain of mice. For this illustration, mice received either 0.3 ml of MPTP (2 mg/ml) or vehicle (saline) in one day. (a, e) Two days after the last injection of MPTP, TH immunofluorescence (blue) reveals a marked reduction in dopaminergic neurons in the substantia nigra pars compacta compared to controls (saline). (b, c, f, g) The loss of TH labeling coincides with an increase in immunofluorescent labeling of microglial cells (Mac-1; green) and of astrocytes (GFAP; red).

(d, h) Overlay of the three cellular markers. When MPTP and saline tissue sections are compared, these images show that, upon reduction in the dopaminergic neuronal marker, there is an increase in the two glial markers. Ventral midbrain tissue sections (30 μ m) were immunostained with a rabbit polyclonal anti-TH (1:1000; Calbiochem, San Diego, CA), a rat anti-MAC-1 (1:1000; Serotec, Raleigh, NC), and a chicken anti-GFAP (1:500, Chemicon, Temecula, CA). Scale bar = 200 μ m

microglial responses are compared, however, it appears that microglial activation occurs earlier than that of astrocytes, and peaks just before or coincidentally with, the climax of dopaminergic neurodegeneration (Liberatore et al. 1999).

In addition to the response of the innate immune system in the MPTP mouse model, there has also been some descriptive data from Czelonkowska and collaborators (Kurkowska-Jastrzebska et al. 1999a, b) about the response of the adaptive immune system. Although this question will be developed in-depth in the chapter authored by Dr. Lee Mosley, herein it is included as a brief informative aspect. In the series of investigations performed by Czelonkowska and collaborators cited above, a marked increase of MHC-II antigen expression by microglia accompanied by a recruitment of T-cells in both the ventral midbrain and striatum of MPTP-injected mice were found. In contrast to the infiltration of the diseased areas of the brain with T-cells, there were no B-cells in the tissue samples. The investigators went further into their characterization of the adaptive immune system in this model by showing that the infiltrating T-cells were mainly of the CD8+ type, but some CD4+ were present too, and more than 50 % of the observed lymphocytes expressed the CD44 antigen.

One problem with any animal model of PD and, in particular, rodent models, is that the PD symptoms presented in these models appear acutely. In contrast, human PD is a late onset neurodegeneration that occurs over many decades.

31.5 Initiation of the Inflammatory Response in Parkinsonian Brains

If indices of inflammation are well recognized in tissues from virtually all neurodegenerative disorders including PD, how inflammation begins, remains. In reviewing the literature, two main hypotheses are emerging. One hypothesis posits that inflammation precedes and triggers the actual loss of dopaminergic neurons. Based on this model, the starting point of the neurodegenerative process would be an insult to the brain by pro-inflammatory factors such as LPS or other infectious-related molecules. Although there is no evidence that septic individuals with high levels of LPS develop PD (Lee et al. 2012), administration of this molecule to mice has been used to initiate dopaminergic neuronal death and the ensuing neuroinflammatory response (Lee et al. 2009). Relevant to this possibility is the demonstration that a

stereotaxic injection of LPS into a normal SNpc produces a local inflammatory response that is associated with the degeneration of dopaminergic neurons in adult rats (Liu et al. 2000). Similarly, LPS-induced inflammation led to the neurodegeneration of dopaminergic MES 23.5 cells or primary ventral midbrain neurons co-cultured with purified microglia (Le et al. 2001). Based on a series of investigations in rodents, it seems that even a prenatal exposure to LPS could produce a long-lasting inflammatory response in the brain (Ling et al. 2004). This striking observation led the authors to propose that “individuals exposed to LPS prenatally, as might occur if their mothers had bacterial vaginosis, would be at increased risk for PD.”

As much as the first hypothesis appears tantalizing, most of the available data are rather consistent with this second hypothesis which posits that inflammation in PD results from the detection of pathological alterations in neighboring neurons by the immune system. According to this model, inflammation in PD and related disorders is thus not the initiator of a primary event, but rather the consequence of neurodegeneration. However, how dysfunctional or dying neurons elicit the inflammatory response remains unknown. Many glial cells are located in close proximity to neurons. Therefore, it may very well be that inflammation could be initiated by some type of change in the nature or the quality of the neuronal contact with glial cells. In healthy cells including neurons, phosphatidylserine is located at the cytoplasmic side of the plasma membrane, while the outer monolayer is composed of phosphatidylcholine and sphingomyelin. A loss of this phospholipid asymmetry of the plasma membrane, with appearance of phosphatidylserine in the outer membrane leaflet, is a feature of cells undergoing apoptosis (van den Eijnde et al. 1998). It is thus possible that such an alteration of the neuronal membrane could be detected by the neighboring glial cells, leading to their activation.

Cell culture studies also show that inflammation can be triggered by soluble factors, either secreted or leaked by neurons. For instance, chromogranin-A is a glycoprotein widely distributed in the nervous system, and was shown to accumulate in areas of neurodegeneration (Nishimura et al. 1994). Noteworthy is the fact that once released or leaked by neurons, chromogranin-A could activate microglia (Ciesielski-Treska et al. 1998). Furthermore, dopaminergic neurons in both PD and experimental models of PD are the site of a robust induction of cyclooxygenase-2 (Teismann et al. 2003), which is a key prostaglandin-synthesizing enzyme. It is also plausible that once synthesized, prostaglandins exit neurons and activate their cognate receptors expressed by glial cells, thereby triggering inflammatory events. Similarly, neuromelanin, upon its release into the extracellular space from dying neurons, can strongly activate glial cells (Wilms et al. 2003). Furthermore, it has been demonstrated that human neuromelanin, extracted from PD brains, can cause a robust

inflammatory response when injected into the substantia nigra of normal rats (Zhang et al. 2011). Finally, it has been suggested that misfolded neuronal proteins and protein aggregates may contribute to glial activation in PD. This view has been prompted by the observations that mutations in the genes encoding for parkin and ubiquitin C-terminal hydrolase L1—two enzymes of the ubiquitin/proteasome pathway—and for alpha-synuclein—a main component of the intraneuronal proteinaceous inclusions Lewy bodies—are linked to familial PD (Vila and Przedborski 2004).

Although more work needs to be done to identify the factors that initiate inflammation in PD and related disorders, mounting evidence indicates that this immune response is probably not a standard event of dysfunctional and dying neurons. Currently, most researchers favor the idea that *specific* neuronal-derived molecules bind to *specific* transmembrane receptors, such as toll-like receptors (TLRs) present on glial cells (Bowman et al. 2003). Among the ten different TLRs, each is activated by a distinct ligand (Iwasaki and Medzhitov 2004), for instance, TLR9, according to Ros-Bernal et al. 2011, is up-regulated in PD brains and in the brains of animal models of PD. TLRs activation, studied in the past in the context of infection, is now being examined in relation to DA neuron death as it is now believed that inflammation in neurodegenerative disorders arises from the ligation of the particular TLR subtypes by molecules originating from dysfunctional and dying neurons. Relevant to this idea is the demonstration of wild-type alpha-synuclein, which upon overexpression can cause a familial form of PD (Eriksen et al. 2005), is secreted by neurons through unconventional exocytosis (Lee et al. 2005). Furthermore, it appears that the expression of markers of microglial activation parallels the extent of alpha-synuclein deposits in the substantia nigra of PD patients (Croisier et al. 2005). At this point, it would be quite interesting to know whether alpha-synuclein or any other mutant proteins linked to familial PD can bind to TLRs.

31.6 Role of Inflammation in Parkinson's Disease

Over the past decade, the idea that brain inflammation (also referred to as neuroinflammation) may modulate the neurodegenerative process in disorders like PD has attracted major interest among neuroscientists. How can this be explained given the fact that inflammation is likely a consequence of neurodegeneration? Neurons in all degenerative diseases die in an asynchronous manner (Pittman et al. 1999). Accordingly, in PD, dopaminergic neurons in the SNpc do not die simultaneously. Instead, only a small number of dopaminergic neurons are dying at any given time and, among these, many are in various stages along the cell death

process. Based on these premises, it may thus be envisioned that the very first neurons to succumb trigger inflammation. Thereafter, the cellular environment in which compromised but still living neurons are embedded becomes inflamed. Should this scenario be correct, it would be significant in opening the door to fostering the understanding of how inflammation could modulate the neurodegenerative process. While most experts do agree on the idea that glial cells play a role in the neurodegenerative process, the significance and, more importantly, the direction of the effect remains a matter of fierce debate (Streit 2002). This dispute is fueled, in part, by the fact that both *in vitro* and *in vivo* system models of human diseases have revealed both detrimental and beneficial effects of inflammation. For instance, the blockade of microglial activation by minocycline has been associated with either reduction or augmentation of dopaminergic neurodegeneration after MPTP administration (Du et al. 2001; Wu et al. 2002; Yang et al. 2003; Diguët et al. 2004). These divergent results may stem from the fact that inflammation is capable of exerting both neuroprotective and neurodestructive functions. Thus, depending on the local factors, the extent of the degenerative process, and even the etiology of the disease, inflammation can give rise to quite a distinct molecular and cellular phenotype.

31.7 Beneficial Role of Inflammation in Parkinsons' Disease

Several *in vivo* studies support the capability of inflammation to mediate neuroprotective and neurodegenerative actions in the nervous system (Drouin-Ouellet and Cicchetti 2012). One such example is the facial nerve axotomy paradigm in newborn rats and rabbits. In this system model, axotomized motor neurons recover coincidentally with the development of the glial response (Moran and Graeber 2004). Furthermore, one month after implantation of innate immune resident cells, such as microglia, into a small mechanically-produced cavity in the rat spinal cord, prominent neuritic growth was observed in the microglial grafts (Rabchevsky and Streit 1997). These results are agreed upon by some experts who believe that, in both normal and pathological situations, the function and survival of neurons rely on the presence of glial cells (Streit 2002).

The basis of the glial-derived beneficial role is likely multifactorial involving both cell contact and soluble mediators. For instance, glial cells play a critical role in maintaining ion and pH homeostasis, as well as extracellular volume. Glial cells can also protect neurons by scavenging and taking-up toxic molecules released by dysfunctional and dying neurons. A striking example of glial assistance in the process of neuronal well-being is glutathione, which is a tripeptide of great importance in the protection of neurons against reac-

tive oxygen species (ROS). Indeed, unlike glial cells, neurons do not possess an uptake system for cysteine (Pow 2001) which is necessary for the synthesis of glutathione. Instead, it has been proposed (Dringen 2000) that astrocytes assist neurons in their production of glutathione through a complex multistep process. First, the astrocytes would use extracellular substrates as precursors for glutathione. Second, glutathione would be released by astrocytes and then converted by the glial ectoenzyme gamma-glutamyl transpeptidase into the dipeptide CysGly. Third, CysGly would be taken-up by neurons and used as a precursor to neuronal glutathione. It has also been proposed (Dringen 2000) that glutamine produced by astrocytes would be used by neurons to produce glutamate, which is necessary for the synthesis of glutathione. Furthermore, astrocytes can avidly take up extracellular glutamate via the glutamate transporters GLT1 and GLAST. This fact may be quite relevant to PD pathogenesis as not only an excess amount of glutamate in the substantia nigra may emanate from dying neurons, but also from the subthalamic glutamatergic input (Benazzouz et al. 2000), which is increased in PD (DeLong 1990). Finally, through the production and maintenance of glutathione, astrocytes can protect against free radicals which can inhibit neurite outgrowth in the brain (Pizzurro et al. 2014). Thus, due to the ability of glial cells to maintain low concentrations of extracellular glutamate in the substantia nigra, the risk of excitotoxic injury to neurons here is prevented.

Aside from contributing to the protection of neurons against ROS and glutamate, glial and T-cells can produce trophic factors that are essential for the survival of dopaminergic neurons. Among these is the glial-derived neurotrophic factor (GDNF), which seems to be the most potent factor supporting nigrostriatal dopaminergic neurons during their period of natural, developmental death in post-natal ventral midbrain cultures (Burke et al. 1998). GDNF can be released by reactive astrocytes (Schaar et al. 1993) by the activation of microglia following a mechanical lesion of the striatum (Batchelor et al. 2000) and can be delivered locally through the implantation of astrocytes, a strategy that has been shown to be protective in 6-OHDA-injected rats (Drinkut et al. 2012). After MPTP administration to mice, however, it seems that reactive astrocytes in the substantia nigra do not engage in a significant production of GDNF (Benner et al. 2004) which may be due to the robustness of the MPTP dosing schedule. Brain-derived neurotrophic factor (BDNF) is another trophic factor that can also be released by reactive astrocytes (Rubio 1997; Stadelmann et al. 2002) and by activated microglia (Batchelor et al. 1999; Stadelmann et al. 2002), and can support the survival and development of dopaminergic structures in the striatum (Batchelor et al. 1999). Furthermore, stem cell-producing BDNF (Somoza et al. 2010) has been shown to be effective in PD animal models. Oligodendrocytes, whose main function is to provide

support to axons and to produce the myelin sheath which insulates axons, are also a type of glial cell capable of generating trophic factors (Du and Dreyfus 2002). For example, striatal oligodendrocytes have been shown to stimulate the survival and expression of phenotypic markers of mesencephalic dopaminergic neurons in culture (Sortwell et al. 2000). Taken together, these data support the contention that glial cells, especially astrocytes, could exert neuroprotective effects in PD. However, whether any of these slow the natural course of the disease in parkinsonian patients remains to be demonstrated.

31.8 Detrimental Role of Inflammation in Parkinsons' Disease

In addition to the body of literature on the beneficial role of inflammation, there is also a growing number of observations supporting the concept that activation of the innate immune system could worsen neurodegeneration (Drouin-Ouellet and Cicchetti 2012; Cappellano et al. 2013). Three theories have been proposed to explain the detrimental role of glial cells in diseases like PD. The first theory is called *glial cell senescence* (Streit 2004), which postulates that glial cells become progressively disabled during normal aging or disease progression. In this theory, it is assumed that glial cells lose their functional capacity to exert the type of beneficial effects described above. In this model, glial cells do not actively injure neurons; they simply stop supporting them, maybe due to aging (Streit and Xue 2009). Although still highly speculative, some investigations would suggest that glial cells may become dysfunctional in PD. For instance, Hishikawa et al. 2001 have found argyrophilic, alpha-synuclein-positive inclusions in glial cells from postmortem PD, but not from age-matched control brains. In addition, Gardai et al. 2013 found impaired cytokine release and phagocytosis in mice over-expressing alpha-synuclein, a finding that was extended to the human condition. The second theory about the detrimental role of inflammation in PD is called *facilitative neurotoxicity* (Streit 2002). During this theoretical process, glial cells would eliminate neurons that are compromised beyond viability and functionality by the primary pathological event. Based on this second model, glial cells would assume an active role in the demise of neurons that are destined to die and whose continued presence could hinder neuronal recovery. Finally, the third theory and probably the most popular in regards to the detrimental role of inflammation in PD is called *indiscriminate neurotoxicity*. Based on this third model, glial cells, upon activation, would engage in an array of cytotoxic events, which would stimulate neurodegeneration and promote both the progression and propagation of a disease such as PD. Over the past two decades, studies using experimental models of PD have

provided strong support for this last concept, which is based on the fact that activated glial cells, especially microglia, are known to produce a variety of noxious compounds including ROS, reactive nitrogen species (RNS), pro-inflammatory prostaglandins, and cytokines. Here, membrane proteins are repeatedly challenged which can detrimentally affect these proteins, thus leading to neuronal death (Kanfer et al. 1999).

Among the range of reactive species produced by glial cells, significant attention has been given to RNS due to the idea that the nitric oxide (NO)-mediated nitrating stress could play an important role in the pathogenesis of PD (Przedborski et al. 1996; Ara et al. 1998; Pennathur et al. 1999; Giasson et al. 2000; Przedborski et al. 2001). Along this line, it must be mentioned that inducible NO synthase (iNOS) is expressed in the SNpc of both PD patients (Hunot et al. 1996) and MPTP-intoxicated mice (Liberatore et al. 1999; Dehmer et al. 2000) in both astrocytes and microglial cells. Furthermore, the effects of NO are even noted at the molecular level as it has been shown that the s-nitrosylation of Parkin decreases its activity as a repressor of p53, which plays a role in apoptotic cell death (Sunico et al. 2013). Myeloperoxidase and NADPH-oxidase, two pro-inflammatory enzymes that produce strong oxidants, have also been identified in PD and MPTP brain tissues (Choi et al. 2005; Wu et al. 2003). Ablation of these three enzymes have been shown to attenuate MPTP-induced neurodegeneration in mice (Liberatore et al. 1999; Dehmer et al. 2000; Choi et al. 2005; Wu et al. 2003), supporting the involvement of inflammation-mediated oxidative/nitrative stress in the dopaminergic neurodegenerative process.

Prostaglandins and their synthesizing enzymes, such as Cox-2, constitute another group of potential offenders. Over the past few years, Cox-2 has emerged as a key cytotoxic factor associated with inflammation (O'Banion 1999). As mentioned above, compared to the normal basal ganglia where Cox-2 is minimally expressed, in the diseased basal ganglia in PD and in the MPTP mouse model, Cox-2 is robustly expressed (Teismann et al. 2003). Not surprisingly, the levels of Cox-2 products such as prostaglandin E₂ were also found to be increased in the substantia nigra of PD patients (Teismann et al. 2003; Mattammal et al. 1995). In the MPTP mouse model of PD, Cox-2 induction was shown to be mediated by a c-Jun N-terminal kinase (JNK)-dependent mechanism (Teismann et al. 2003; Hunot et al. 2004). Noteworthy, the inhibition of JNK activation, like inhibition of Cox-2, does attenuate MPTP-induced neurodegeneration (Hunot et al. 2004; Teismann et al. 2003). Conversely, while both inhibition and ablation of Cox-2 did attenuate MPTP-induced dopaminergic neurotoxicity, deletion of the constitutively expressed isoenzyme Cox-1 had no effect (Teismann et al. 2003).

Additional glial-derived molecules with cytotoxic potentials include the large group of pro-inflammatory cytokines. The expression level of many of these toxic

cytokines, assessed by gene profiling, appears increased in PD tissues (Mandel et al. 2005). In keeping with this result, tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) levels are increased in both SNpc tissues and cerebrospinal fluid of PD patients (Mogi et al. 2000; Mogi et al. 1996; Mogi et al. 1994). Of note, at this point, one cannot exclude that some of these alterations may be related to the chronic use of the anti-PD therapy levodopa (Bessler et al. 1999). Nevertheless, in autopsy tissue from PD patients, robust immunostaining for TNF- α , IL-1 β , and interferon- γ (IFN- γ) is observed in astrocytes at the level of the SNpc and in the MPTP-treated mouse at the level of the substantia nigra (Lofrumento et al. 2011). In terms of mechanism, these cytokines may operate in PD on at least two levels. First, once produced by reactive astrocytes, they can stimulate neighboring, quiescent astrocytes and microglia to elicit neurodegeneration (Mallajosyula et al. 2008). Consistent with this view is the upregulation of the microglial receptor Fc ϵ R11/CD23 in response to astrocyte-derived TNF- α , IL-1 β , and IFN- γ (Hunot et al. 1999). In this study, the authors show that, once upregulated, ligation of Fc ϵ R11/CD23 stimulates the expression of iNOS and the ensuing production of NO by microglial cells (Hunot et al. 1999). Second, astrocytic and microglial-derived cytokines may also operate directly on dopaminergic neurons by binding to specific cell surface cytokine receptors such as TNF- α receptor and FAS. However, the targeting of either TNF- α receptor (Rousselet et al. 2002; Ferger et al. 2004) or FAS (Hayley et al. 2004; Landau et al. 2005) has thus far generated conflicting results in the MPTP mouse model. Findings from several studies show that ablation of these receptors decreased MPTP-induced dopaminergic neurotoxicity (Ferger et al. 2004; Hayley et al. 2004), whereas other studies found that it increased MPTP-induced dopaminergic neurotoxicity (Rousselet et al. 2002; Landau et al. 2005). Upon activation, cytokine receptors trigger intracellular death-related signaling pathways, whose molecular correlates include translocation of the transcription nuclear factor- κ -B (NF- κ -B) from the cytoplasm to the nucleus. Relevant to this is the fact that PD patients show 70 times more dopaminergic neurons with nuclear NF- κ -B immunoreactivity compared to control subjects (Hunot et al. 1997). Despite the robust recruitment of NF- κ -B, it is not clear whether this transcriptional factor is instrumental in PD pathogenesis, as mice deficient in one of the main NF- κ -B polypeptides, P50, showed the same severity of MPTP-induced nigrostriatal pathway damage as that of their wild-type counterparts (Hunot et al. 2004). As exemplified above, at this point, experimental models of PD have not provided much insight into the role of cytokines in neurodegeneration for such conditions as PD and other related disorders.

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31.9 Review Questions

1. What are the clinical and neuropathological hallmarks of PD?
2. What is the difference between parkinsonian syndrome and Parkinson's disease?
3. Provide examples of experimental models of PD.
4. What is the topography and composition of the inflammatory response in PD?
5. What are the similarities and differences in the inflammatory response among PD, the various parkinsonian syndromes, and the common experimental models of PD?
6. What are the arguments in favor of inflammation being a primary or a secondary event in PD?
7. How can dying dopaminergic neurons trigger inflammation in PD?
8. By which mechanisms could inflammation be beneficial in PD?
9. Name the three theories about the detrimental role of inflammation in PD and explain their respective basis.
10. Summarize the different cellular and molecular factors of inflammation that can actively mediate the demise of dopaminergic neurons in PD?
11. Which statement is incorrect about PD?
 - a. Parkinson's disease is the second most frequent neurodegenerative disorder.
 - b. *The neurodegenerative process in Parkinson's disease is restricted to the dopaminergic system.*
 - c. Parkinson's disease is mainly a sporadic condition.
 - d. More than 30 other neurodegenerative diseases can clinically look like Parkinson's disease.
 - e. Aside from the loss of neurons the neuropathology of Parkinson's disease includes Lewy bodies and gliosis.
12. What are the main inflammatory cells encountered in the substantia nigra of Parkinson's?
 - a. Astrocytes
 - b. Microglia
 - c. Oligodendrocytes
 - d. T-cells
 - e. *a and b*
13. Which statement is correct concerning inflammation in parkinsonian syndromes?
 - a. *It is often noted, but more wide-spread and less detailed than in Parkinson's disease.*

- b. The type of inflammatory response differs between the sporadic and familial parkinsonian syndromes.
 - c. The syndrome multisystem atrophy is unique in that inflammation is primarily made up of infiltrating T-cells.
 - d. The neuropathological pleomorphism in patients carrying a LRRK2 mutation refers to the fact that neither Lewy bodies nor gliosis is a consistent finding.
 - e. None of the above.
14. Which statement about experimental models of Parkinson's disease is true?
 - a. Both genetic and toxic models exist, but only the former are commonly used.
 - b. *Inflammation has been described in all popular models of Parkinson's disease.*
 - c. The MPTP monkey model suggests that an acute intoxication produces an acute neurodegenerative event that is completed in a few days.
 - d. The MPTP mouse model suggests that the toxin provokes inflammation, which, in turn, kills dopaminergic neurons.
 - e. Neuronophagia which suggests ongoing inflammation has been described in genetics, but not in toxic models of Parkinson's disease.
 15. Which of the following statements is true about inflammation in Parkinson's disease?
 - a. Free neuromelanin fails to activate microglia.
 - b. Astrocytosis is as robust as microgliosis.
 - c. The propensity of the different dopaminergic structures to degenerate in Parkinson's disease correlates with the basal density of glial cells.
 - d. *Both prostaglandin and alpha-synuclein count among the factors potentially responsible for triggering inflammation in Parkinson's disease.*
 - e. It is proven that prenatal infection and subsequent inflammation predispose one to Parkinson's disease.
 16. Which of the following statements is most correct?
 - a. *Inflammation can exert both beneficial and detrimental effects.*
 - b. Most experimental models favor the beneficial role of inflammation
 - c. The detrimental role of inflammation in Parkinson's disease is due to the disease-related impairment of glial functions vital to neurons.
 - d. Contrary to astrocytes, oligodendrocytes play no role in Parkinson's disease.
 - e. Three different theories have been proposed to explain how inflammation may support the survival of dopaminergic neurons.
 17. Which of the following glial functions may improve neuronal survival or regeneration?
 - a. Inhibit phagocytosis.
 - b. Secrete chemotactic molecules to recruit polynuclear cells.
 - c. *Produce trophic factors.*
 - d. Assist in the synthesis of neuronal superoxide dismutase.
 - e. Stimulate the formation of myelin to guide new axons.
 18. Glial cells can exacerbate neurodegeneration in Parkinson's disease by?
 - a. Losing their ability to assist neighboring neurons.
 - b. Accelerating the demise of compromised neurons.
 - c. A process of indiscriminate toxicity.
 - d. Decreasing extracellular glutamate levels.
 - e. *a, b and c*
 19. Regarding the cytotoxicity of inflammation, which statement is not correct?
 - a. Both oxygen and nitrogen reactive species can participate in the deleterious effects of inflammation.
 - b. *Inactivation of NADPH-oxidase, but not of nitric oxide synthase, mitigates MPTP-induced neurodegeneration in mice.*
 - c. The detrimental effects of inflammation on dopaminergic neurons can be mediated by soluble factors.
 - d. Astrocytes and microglial cells can mutually modulate their degree of activation.
 - e. Dopaminergic neurons express receptors for various deleterious cytokines.
 20. Among the following inflammatory factors which ones have been identified in the substantia nigra of patients with Parkinson's disease?
 - a. Tumor necrosis factor
 - b. Inducible nitric oxide synthase
 - c. Interferon-gamma
 - d. Myeloperoxidase
 - e. *All of the above*

31.10 Answers

1. Motor abnormalities and a loss of primarily, but not exclusively, dopaminergic neurons. Other neuropathological hallmarks of PD include the proteinaceous inclusions, Lewy bodies and various inflammatory changes.
2. Many of these non-PD parkinsonian syndromes are sporadic and often exhibit both clinical (e.g., ocular movement) and neuropathological features typically not seen in PD (e.g., striatal or corticospinal track pathology). Of these syndromes, most, if not all, show evidence of nigrostriatal neurodegeneration (not always associated with Lewy bodies) and inflammation.
3. Animal models and cell culture studies
4. the density of microglia identified by the labeling of the specific plasma membrane glycoprotein F4/80 (a well-characterized macrophage marker that is part of the EFG-TM7 family and that shares a 68% amino acid

homology with human EGF-like module-containing mucin-like hormone receptor-like 1) is higher in the substantia nigra compared to other regions of the brain, at least in adult mice. The density of GFAP-positive cells is moderate in those midbrain areas most severely affected in PD, such as the SNpc, and high in those areas least affected, such as the central gray matter.

5. Gliosis is not only described in these non-PD parkinsonian syndromes at the level of the nigrostriatal pathway, but also at the level of other regions in the brain, which are normally not involved in PD. The neuropathological picture created in these animal models, particularly in the toxic models, is very close to that described for PD including, neurodegeneration and motor and behavioral effects. In almost all of the PD models, whether or not there is an overt loss of nigrostriatal dopaminergic neurons, a glial response is found in the substantia nigra.
6. LPS-induced inflammation led to the neurodegeneration of dopaminergic MES 23.5 cells or primary ventral midbrain neurons co-cultured with purified microglia. Inflammation in PD and related disorders is thus not the initiator of a primary event, but rather the consequence of neurodegeneration. However, how dysfunctional or dying neurons elicit the inflammatory response remains unknown.
7. It may very well be that inflammation could be initiated by some type of change in the nature or the quality of the neuronal contact with glial cells.
8. The blockade of microglial activation by minocycline has been associated with either reduction or augmentation of dopaminergic neurodegeneration after MPTP administration
9. The first theory is called *glial cell senescence*, which postulates that glial cells become progressively disabled during normal aging or disease progression. In this theory, it is assumed that glial cells lose their functional capacity to exert the type of beneficial effects described above. In this model, glial cells do not actively injure neurons; they simply stop supporting them, maybe due to aging (Streit and Xue 2009). Although still highly speculative, some investigations would suggest that glial cells may become dysfunctional in PD. For instance, Hishikawa and collaborators have found argyrophilic, alpha-synuclein-positive inclusions in glial cells from postmortem PD, but not from age-matched control brains. In addition, Gardai et al. found impaired cytokine release and phagocytosis in mice over-expressing alpha-synuclein, a finding that was extended to the human condition. The second theory about the detrimental role of inflammation in PD is called *facilitative neurotoxicity*. During this theoretical process, glial cells would eliminate neurons that are compromised beyond viability and functionality by the primary pathological

event. Based on this second model, glial cells would assume an active role in the demise of neurons that are destined to die and whose continued presence could hinder neuronal recovery. Finally, the third theory and probably the most popular in regards to the detrimental role of inflammation in PD is called *indiscriminate neurotoxicity*. Based on this third model, glial cells, upon activation, would engage in an array of cytotoxic events, which would stimulate neurodegeneration and promote both the progression and propagation of a disease such as PD. Over the past two decades, studies using experimental models of PD have provided strong support for this last concept, which is based on the fact that activated glial cells, especially microglia, are known to produce a variety of noxious compounds including ROS, reactive nitrogen species (RNS), pro-inflammatory prostaglandins, and cytokines. Here, membrane proteins are repeatedly challenged which can detrimentally affect these proteins, thus leading to neuronal death.

10. Also important to note is the fact that, on inhibition of the entry of the active metabolite of MPTP, 1-methyl-4-phenylpyridinium (MPP⁺), into dopaminergic neurons, there is not only a protection of substantia nigra dopaminergic neurons, but also a lack of glial response. This critical observation indicates that inflammation in the MPTP model is a consequence of the demise of neurons and not the reverse.

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Abstract

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease characterized by the loss of upper and lower motoneurons, eventually culminating in respiratory failure and death. Great insight as to what causes ALS has resulted from discovery of more than 20 gene disease-causing mutations. Clinical heterogeneity cannot be explained by altered gene expression alone, and accumulating evidence implicates neuroinflammation in modifying clinical phenotypes. In ALS neurons do not die alone; neuronal injury is non-cell-autonomous and depends on a well-orchestrated dialogue involving glia, T cells and motor neurons, mediating neuronal viability and neuronal injury. This neuroinflammatory response differs in different ALS patients: patients progressing rapidly have decreased regulatory T lymphocytic responses, while patients progressing slowly had normal to increased levels of Treg and FoxP3 expression. Therapeutic efforts targeting neuroinflammation have the potential of changing an acute rapidly progressive disease into a more slowly progressing disease and a more sustainable quality of life.

Keywords

Amyotrophic lateral sclerosis • Chemokines • Cytokines • Diverse clinical phenotypes • Microglia/macrophages • Neuroinflammation • Regulatory T lymphocytes

32.1 Introduction: Clinical Features

Amyotrophic lateral sclerosis (ALS) is a devastating, relentlessly progressive, neurodegenerative disease that severely compromises the quality of life as well as the length of life. (Haverkamp et al. 1995). The incidence of ALS is 1–2/100,000 and the prevalence is 4–6/100,000. Ninety percent of ALS cases are sporadic (sALS), while 10% are familial (fALS). Despite intensive efforts the factors initiating disease in sALS patients are not understood, and no known pharmacotherapy can significantly influence either disease progression or survival. In sALS, the mean age of onset is 57 years and the median survival is approximately 4

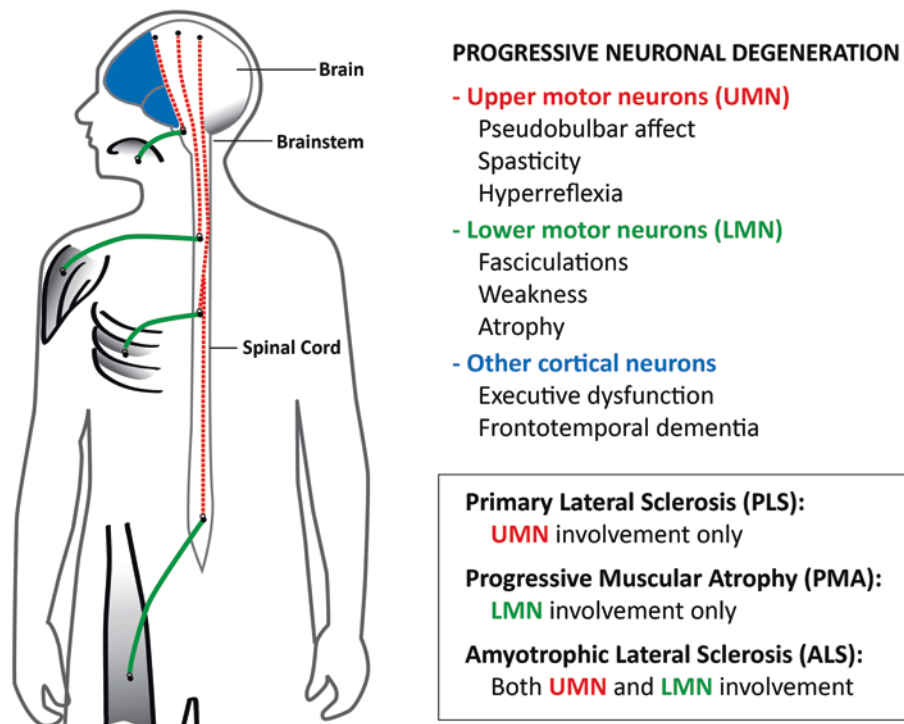
years with younger patients surviving longer than older individuals. Males develop sporadic ALS more frequently than females with an overall ratio 1.7:1. However, in younger individuals, the ratio of males to females may exceed 3:1, while in older individuals the frequency of female involvement is increased and the ratio approaches 1:1 over the age of 65, similar to the ratio in fALS.

In the last two decades more than 20 mutant genes that cause fALS have been discovered. A defect in the gene encoding copper-zinc superoxide dismutase (SOD1) was first described in 1993 and is present in 20% of fALS cases (Rosen et al. 1993). More recently, the presence of a hexanucleotide repeat (GGGGCC) in C9orf72 was reported in 30–50% of fALS cases (Renton et al. 2011; DeJesus-Hernandez et al. 2011) and is now recognized as the most common cause of fALS.

The clinical presentations of both sALS and fALS, can be remarkably diverse, and phenotypic heterogeneity is the rule

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Fig. 32.1 Neuroanatomy of amyotrophic lateral sclerosis



rather than the exception. As a result, it may be extremely difficult to predict the clinical course in any single patient which is a major challenge when developing clinical trials for this population.

The clinical hallmark of ALS is the presence of upper motor neuron and lower motor neuron disease of brainstem and spinal regions (Fig. 32.1) ALS can present with first symptoms and signs in the teenage years or in the 80's; disease duration can be as short as 6 months or as long as 40 years; and the initial presentation can be with limb onset, bulbar onset, or less commonly with breathing difficulty or neck weakness.

Symptoms and signs may start with complaints of arm or hand weakness, wasting, or functional impairment, such as difficulty writing, buttoning, or holding onto objects, or leg weakness with stumbling, tripping, and falling. Onset is usually asymmetrical, progressing from one leg to the ipsilateral arm or the contralateral leg, and then proceeding rostrally. Clinically, ALS may be misdiagnosed as a painless cervical radiculopathy and myelopathy when initial signs and symptoms are restricted to a single limb or adjacent root accompanied by hyperreflexia in the legs and before electromyography (EMG) studies are performed. Ruling out significant cervical spinal cord compression with neuroimaging is mandatory. However, many patients may have the presence of both ALS and suggestive cervical cord compromise. By recognizing the common asymmetrical presentation of ALS as well as the slow but relentless progression of atrophy and weakness, in addition to EMG evidence of lower motor neuron involvement, these patients could

avoid surgery that could aggravate the clinical syndrome. An additional dilemma in the diagnosis of ALS is the patient that presents with progressive atrophy and weakness and EMG findings characteristic of lower motor neuron involvement, yet with reflexes that are slightly decreased. The absence of hyperreflexia suggests a lower motor neuron syndrome such as progressive muscular atrophy. However, in this clinical context corticospinal tract involvement cannot be simply ruled out with a reflex hammer, yet we are still awaiting the development of meaningful surrogate markers of upper motor neuron, including neuroimaging modalities, to resolve this diagnostic dilemma.

In patients with bulbar involvement, symptoms of hoarseness, slurred speech, and drooling often precede choking or swallowing problems. Bulbar symptoms and signs, in association with tongue atrophy and fibrillations, may be the presenting feature in 20–25 % of patients, with a large percentage in women over the age of 60 (Haverkamp et al. 1995). In general, onset of bulbar signs during the disease course carries a poorer prognosis.

Cognitive dysfunction due to fronto-temporal lobe involvement is a frequently associated feature in 50 % of ALS patients and ranges from mild, frontal executive dysfunction to outright frontotemporal dementia (FTD) in 15 % (Ringholz et al. 2005). Whereas, up to 40 % of FTD cases have measureable motor neuron dysfunction with 15 % of cases with outright ALS, supporting that ALS and FTD are on a spectrum of disease (Bennion Callister and Pickering-Brown 2014). ALS-FTD patients typically have onset of

symptoms in their 50's, like ALS alone, it is slightly more common in men than women. The ALS symptoms may precede, co-occur, or follow the symptoms of FTD, although the most common finding is to have cognitive change first followed by weakness.

The interval between the cognitive symptoms and motor weakness may be a few months to up to 7 years, with a mean of 2 years (Achi and Rudnicki 2012). In patients with FTD apathy is often the first symptom reported by caregivers. FTD patients exhibit a lack of empathy and are unaware of the emotions of others. They may be more withdrawn and self-centered, and lack concern for their personal appearance. They may become disinhibited, and develop impulsive or socially inappropriate behavior. Their reasoning, judgment and organizational skills become markedly diminished, and their personal hygiene and overall care represent significant problems for their caregivers.

The presence of frontal dysfunction also contributes significantly to disease heterogeneity with patients presenting with varying clinical signs on a continuum from pure ALS to pure FTD; patients with ALS can have normal executive function, extensive frontal executive dysfunction plus ALS, or severe FTD with only minimal signs and symptoms of ALS.

Pseudobulbar affect is also a common symptom in ALS patients and characterized as a sudden outbursts of uncontrolled laughter or tearfulness/crying that can occur spontaneously or in response to provocative stimuli, such as television commercial or posed question. The displays are dissociated from the patient's underlying emotional state or mood or are out of proportion to it. Expectedly, this syndrome can have profound social consequences for the patient and family, however, can be effectively treated with a relatively new FDA approved drug, Nuedexta®, which has been shown to significantly decrease the number and severity of these outbursts in patients (Pioro et al. 2010)

The history and physical examination provide the foundation for the diagnosis and should demonstrate the combination of lower motor neuron (LMN) compromise, manifested by weakness and muscle atrophy, and upper motor neuron (UMN) compromise, evidenced by increased tone and hyperreflexia, with involvement in at least three areas, including the limbs, tongue, and back muscles. Needle EMG and nerve conduction studies of an upper and lower limb, thoracic paraspinal muscles and tongue should be performed to confirm the diagnosis in all patients with suspected ALS.

32.1.1 Pathogenesis

Most early studies of the pathogenesis of ALS focused on changes within motor neurons that could reflect the mechanisms of cell injury and cell destruction. In the early 1990's the presence of glutamate excitotoxicity was implicated (Rothstein

et al. 1990). Other studies emphasized the importance of increased intracellular calcium (Engelhardt et al. 1997; Siklos et al. 1996) and free-radical mechanisms in mediating cell death in ALS (Bowling et al. 1993; Simpson et al. 2004). However, none of these explanations are mutually exclusive because altered calcium homeostasis, free radical stress, and glutamate excitotoxicity may all participate in a cell injury cascade leading to motor neuron death. In fact, alterations in one parameter can lead to alterations in other parameters, and each can enhance and propagate the injury cascade. Increased intracellular calcium can enhance free radical production (Dyken 1994) and glutamate release (Coyle and Putterfarcken 1993; Carriedo et al. 1998) which in turn can further increase intracellular calcium. Increased free radicals can impair the glial uptake of glutamate, increasing the extracellular glutamate available to interact with neuronal AMPA/kainate receptors, which in turn can increase intracellular calcium and/or free radicals. Free radicals in turn can enhance lipid peroxidation leading to increased intracellular calcium (Kakkar et al. 1992). These changes of increased intracellular calcium, increased production of free radicals, and enhanced glutamate excitotoxicity could critically impair motor neuron structures such as mitochondria or neurofilaments, and compromise energy production and axoplasmic flow. Once initiated, the changes could become self-propagating.

32.1.2 Role of Mutant Genes in ALS

In the last several decades discovery of more than 20 genetic mutations has dramatically changed approaches to the pathogenesis of disease in ALS. Collectively these mutations cause alterations in diverse cellular pathways that contribute to disease pathogenesis and include mitochondrial dysfunction, increased reactive oxygen species, altered axonal transport, altered RNA metabolism, splicing, and transport, altered proteasomal function, impaired autophagy, and endoplasmic reticulum stress. A common denominator in many of these pathologies is the presence of misfolded aggregated proteins.

The first mutation to be described was in the gene for superoxide dismutase, (SOD1) a ubiquitously expressed protein that catalyses the detoxification of superoxide (Rosen et al. 1993). More than 160 SOD1 mutations have been described, all of which result in SOD1 protein misfolding and a toxic gain-of-function and lead to impaired mitochondrial function, axonal degeneration in a distal to proximal pattern ("dying-back"), impaired proteasomal function, and endoplasmic reticulum stress. The cytotoxic effects of aggregated SOD1 are not confined to mutant SOD1, but also are noted with misfolded wild-type SOD1, which has been identified in motor neurons of sALS patients, and could contribute to disease pathogenesis in sporadic cases.

The next major milestone was the identification of TDP-43 inclusions in ALS brain and spinal cords in 2006, and the subsequent discovery of TDP-43 gene mutations in fALS patients (Sreedharan et al. 2008; Kabashi et al. 2008). TDP-43 is ubiquitously expressed as a DNA/RNA binding protein involved in transcription, and RNA splicing and transport. In fALS patients mutant TDP-43 is cleaved and aggregated, with localization shifted from the nucleus to the cytoplasm impairing nuclear function. In 2009 mutations in FUS/TLS were described, with cytoplasmic aggregates and impaired nuclear function and nuclear transport, similar to the functional changes noted with mutant TDP-43, (Kwiatkowski et al. 2009; Vance et al. 2009).

In 2011 the most common cause of fALS, accounting for 30–50 % of cases, was discovered as a hexanucleotide repeat (GGGGCC) in the gene C9orf72 (Renton et al. 2011; DeJesus-Hernandez et al. 2011). Expansions in C9orf72 have also been reported in 5–10 % of sALS patients. The specific pathogenic mechanisms whereby C9orf72 leads to ALS are still incompletely defined, but three different potential pathogenic mechanisms have been proposed: 1. Haploinsufficiency, due to impaired C9orf72 transcription, 2. RNA toxicity due to binding of critical transcription or splicing factors by the C9orf72 hexanucleotide repeat; and 3. Non-ATG translation yielding polypeptides that readily aggregate and promote cytotoxicity.

Other genetic mutations initiate changes in vital motor neuron pathways that lead to an ALS clinical phenotype. Mutations in SOD1 lead to impaired axonal transport and axonal degeneration; mutations in the profilin-1 gene that normally mediates actin polymerization lead to axonal dysfunction (Wu et al. 2012); and mutations in dynactin lead to defects in axoplasmic cargo transport (Münch et al. 2004). Mutations in genes for ubiquitin 2 and p62 have been reported to compromise proteasomal function and autophagy and result in the clinical picture of ALS. (Deng et al. 2011; Fecto et al. 2011).

Such genetic heterogeneity was anticipated to provide a major explanation for phenotypic heterogeneity, since each distinctive mutant gene might initially compromise a relatively specific motor neuron metabolic pathway and result in a specific clinical phenotype. However, clinical phenotypes are so heterogeneous in both sALS and fALS patients that it is even difficult to classify patients as belonging to the 10 % with fALS or the 90 % with sALS based solely on the presenting clinical syndrome without taking a careful family history and doing genetic testing. Thus the clinical picture alone cannot classify the patients as having fALS or sALS. Phenotypic heterogeneity is noted even within affected members of a family with the same mutation, and in different families the same genetic mutation can give rise to multiple clinical phenotypes. Conversely, different mutant genes can give rise to the same clinical phenotype.

Phenotypic heterogeneity at the level of the gene could also be explained by epistasis, the interactions of multiple genes, as well as by epigenetic modifications including DNA methylation, histone acetylation, chromatin remodeling, and non-coding RNA regulation. In accord, the inheritability of sALS is estimated between 12–21 % of cases suggesting genetic influence on such cases. A recent study, reported that up to 28 % of sALS patient and 80 % of fALS have novel variants in genes associated with an earlier disease onset of 10 years in those with >1 variants detected (Cady et al. 2014). Such studies support an oligogenic inheritance in ALS that is relevant to disease pathogenesis. With continuing advances in next-generation sequencing and application in genomic wide association studies, the interaction of these genes upon penetrance, progression and survival for the individual patient will become clearer.

Another reason that distinctive mutations might not solely explain phenotypic heterogeneity, and might, in fact, give rise to similar clinical syndromes is the interaction and interdependence of metabolic pathways within the motor neuron. For example, mutations in mSOD1 may initially compromise mitochondria and subsequently increase free radical species, alter calcium homeostasis, compromise axonal function, alter proteasomal function, autophagy, and RNA metabolic pathways, and lead to ER stress and the unfolded protein response. Similarly, mutations in mTDP-43 or FUS may initially compromise transcription, RNA splicing or transport, and subsequently impair multiple diverse pathways within the motor neuron.

The involvement of multiple metabolic pathways in the progression from motor neuron injury to cell death suggests cell autonomous toxicity with neurons dying alone without the contribution of non-neuronal cells. Yet such is not the case, at least in the transgenic mouse of mutant SOD1 where cellular contributions can be identified. Expressing the mSOD1 primarily in motor neurons in transgenic mice does not lead to motor neuron disease and cell death. In these models, neurons do not die alone; the process is non-cell autonomous. Injury to motor neurons may be necessary as an early event, but may not be sufficient to cause severe injury and cell death. Neuronal injury and cell death do not occur in isolation, but require the participation of non-neuronal cells such as innate immune microglia and adaptive immune T lymphocytes in the pathogenesis of motor neuron cell death in ALS.

32.1.3 Neuroinflammation in ALS Transgenic Mice

The presence of activated innate (microglia) and adaptive immune cells (T lymphocytes) within the brain and spinal cord is characteristic of neuroinflammation. Neuroinflammation was originally thought to be a secondary effect

or consequence of neuronal injury, but is now increasingly recognized to contribute significantly to motor neuron pathophysiology and disease propagation (Henkel et al. 2009; Zhao et al. 2013). The most cogent evidence derives from transgenic rodent models of ALS.

Transgenic mice (Gurney et al. 1994) overexpressing mutant $\text{Cu}^{2+}/\text{Zn}^{2+}$ superoxide dismutase (mSOD1) develop a progressive motor neuron disease that resembles the clinical and pathological features of human familial amyotrophic lateral sclerosis (ALS). The motor neuron degeneration in these transgenic mice appears to be non-cell autonomous, i.e., non-neuronal cells contribute to disease pathogenesis. Expression of mSOD1 either in astrocytes (Gong et al. 2000) or in neurons (Pramatarova et al. 2001; Lino et al. 2002) does not induce an ALS-like disease in mice; nor does expression of mSOD1 in microglia cause disease (Beers et al. 2006). Data from chimeric mice, using the original mSOD1 promoter, in the same genomic location, and with the same number of copies, suggest that mSOD1-overexpressing neurons surrounded by normal glia remain relatively intact, whereas normal neurons surrounded by mSOD1-overexpressing glia showed signs of injury (Clement et al. 2003).

Non-cell autonomy suggests that neuroinflammation characterized by activated innate immune microglia and adaptive immune lymphocytes might contribute to the pathogenesis of motor neuron cell death in ALS models. At presymptomatic stages, spinal cords from mSOD1 transgenic mice have increased ICAM-1 expression, deposition of immunoglobulin G (IgG) within motor neuron cell bodies, and increased Fc receptor (FcR) expression on microglia (Alexianu et al. 2001). Activated microglia are present as early as 80 days of age, prior to evidence of motor weakness.

After disease onset in the mSOD1 transgenic mouse, there are two clinical phases: an initial “slow” phase where the mouse does not appear to worsen clinically, followed by a “rapid” phase of accelerated weakness and death (Beers et al. 2011b). The spinal cords of mSOD1 transgenic mice in the early slow phase are characterized by increased protective regulatory T lymphocytes and anti-inflammatory M2 microglia/macrophages, with increased expression of IL-10, IL-4, BDNF, GDNF, IGF-1, and TGF β . During the later accelerated phase of disease activated microglia have a pro-inflammatory M1 phenotype with increased expression of NOX2, IL-1 β , TNF α , and IL-6. In vitro, activated M2 microglia are neuroprotective for motor neurons while activated M1 microglia are neurotoxic.

However, the most cogent evidence for the neuroprotective effects of M2 microglia and the cytotoxic effects of M1 microglia derives from in vivo studies in the mSOD1 transgenic mice: the adoptive transfer of wild-type M2 microglia slowed disease progression while transfer of mSOD1 M1 microglia accelerated disease progression (Beers et al. 2006).

T-cell involvement in disease pathogenesis was documented in experiments that depleted the entire T cell population by crossbreeding the mSOD1 transgenic mice with RAG2 $^{-/-}$ or TCR $^{-/-}$ knockout mice. Surprisingly, the disease course significantly worsened in these mSOD1 T cell deficient mice, indicating that T cells had been neuroprotective (Beers et al. 2008; Chiu et al. 2008). The most prominent neuroprotective T cells are regulatory T and Th2 lymphocytes with the former suppressing the proliferation of potentially neurotoxic T effector cells and both Treg and Th2 suppressing the pro-inflammatory functions of activated M1 microglia (Zhao et al. 2012). Th2 CD4 $^{+}$ T cells and Tregs can express high levels of the anti-inflammatory factor IL-4 polarizing microglia to the neuroprotective (M2) phenotype. The most compelling evidence for the neuroprotective effects of T cells derives from in vivo experiments in which adoptive transfer of CD4 $^{+}$ and specifically Tregs early in the course of disease rescued these mice and extended survival (Banerjee et al. 2008; Beers et al. 2011a). These T cells not only promote microglia-mediated neuroprotection but also astroglia-mediated neuroprotection by increasing production of neurotrophic factors such as glial-cell-derived neurotrophic factor (GDNF).

In the “rapid” phase of accelerated weakness and death, spinal cords of the mSOD1 transgenic possess decreased regulatory T lymphocytes and increased proinflammatory Th1 lymphocytes and M1 microglia/macrophages. This shift from neuroprotective cells to cytotoxic cells results in a marked decrease in expression of neuroprotective cytokines and trophic factors and a dramatic increase in pro-inflammatory cytokines, accelerating a self-propagating cascade of motor neuron injury and cell death. Tregs transferred at this late stage are not able to reverse the ongoing progressive clinical deterioration.

32.1.4 In Vitro studies

To help define the interactions of motor neurons with non-neuronal cells such as microglia, we used primary MN cultures to investigate the effects of microglia activated by lipopolysaccharide or IgG immune complexes from patients with amyotrophic lateral sclerosis (Zhao et al. 2004). Following activation, microglia induced MN injury, which was preventable by a microglial inducible nitric oxide synthetase (iNOS) inhibitor as well as by catalase or glutathione. Glutamate was also required to elicit MN injury; inhibition of the motoneuron AMPA/kainate receptor by CNQX prevented the cytotoxic effects of activated microglia. In fact, peroxynitrite, a toxic product of nitric oxide metabolism, and glutamate were synergistic in producing MN injury. We subsequently demonstrated that misfolded mSOD1 protein but not wild-type SOD1 protein could activate microglia to

release free radicals and proinflammatory cytokines and provoke motor neuron cell death. The misfolded mSOD1 protein did not injure motor neurons in the absence of microglia (Zhao et al. 2010). It has also been demonstrated that mSOD1 released from motor neurons derived from transgenic mice could activate microglia, and, in turn, lead to motor neuron injury and cell death (Urushitani et al. 2006). Such evidence suggests that misfolded proteins or their peptide fragments may well be the “danger signals” released from injured motor neurons activating a neuroinflammatory response of proinflammatory microglia and T lymphocytes, and triggering a self-propagating motor neuron injury and cell death.

32.2 Human ALS Studies

Although early studies of ALS tissue did not describe the presence of inflammatory cells in ALS, reports beginning in 1988 suggested that inflammatory cell infiltration might be relatively common (Troost et al. 1988; Troost et al. 1989). Further studies (Lampson et al. 1990) demonstrated activated microglia and small numbers of T cells in degenerating white matter of ALS cords. Activated microglia are also prominent in the ventral horn of spinal cords from ALS patients. In contrast to resting microglia, which have long, highly branched processes, activated microglia have hypertrophied soma and thickened, retracted processes. Reactive microglia express surface markers indicative of their activated state. These include the complement receptor, CD11b; leukocyte common antigen (LCA); and major histocompatibility complex (MHC) class II glycoproteins, such as human leukocyte antigen DR (HLA-DR). In addition to serving a phagocytic function, activated microglia may also communicate with and affect the function of cells in the local environment, by expressing a broad array of cytokines, chemokines, proteases, and neurotrophic factors (Gonzalez-Scarano and Baltuch 1999).

Our early studies documented the presence of lymphocytes in the spinal cord in 18 of 27 consecutive ALS autopsies (Engelhardt et al. 1993). Lymphocytes were predominantly CD4-positive in the vicinity of degenerating cortical spinal tracts, and CD4 and CD8 cells were found in ventral horns. Kawamata et al. (1992) demonstrated the presence of significant numbers of CD8 T cells and, to a lesser extent, CD4 T cells marginating capillaries in the parenchyma of spinal cord and brains of 13 ALS patients. Lymphocytes and activated microglia expressing MHC Class I and Class II, leukocyte common antigen, FcγRI and β-2 integrins were present in ALS tissue.

Our studies of T cells in ALS spinal cord documented a greater expression of Vβ2 transcripts in ALS specimens, independent of the HLA genotype of the individual (Panzara et al. 1999). As additional confirmation, cells were assayed from the cerebrospinal fluid (CSF) of 22 consecutive ALS patients for the presence of Vβ2 transcripts. This specific T-lymphocyte receptor was demonstrated in the CSF of 17 of

22 ALS patients, while only 4 of 19 control patients had similar expression. Vβ2 is a T-cell receptor known to respond to superantigens, and the presence of T lymphocyte restriction supports the involvement of adaptive immune mechanisms in the pathogenesis of ALS. Further evidence supporting the importance of immune mechanisms is the presence of dendritic cells in ALS tissues (Henkel et al. 2004). Dendritic cells are potent antigen-presenting cells that initiate and amplify immune responses (Reichmann et al. 2002). Immature (DEC205, CD1a) and activated/mature (CD83, CD40) dendritic cell transcripts were significantly elevated in ALS tissues. Monocyte/macrophage/microglia transcripts (CD14, CD18, SR-A, CD68) were increased in ALS spinal cord, and activated CD68 (+) cells were demonstrated in close proximity to motor neurons. Increased mRNA expressions of the chemokine monocyte chemoattractant protein-1, CCL2, which attracts monocytes and myeloid dendritic cells and the cytokine macrophage-colony stimulating factor (M-CSF), which stimulates the differentiation of dendritic cells and macrophages, were found in ALS tissues. CCL2 protein was expressed in glia in ALS but not in control tissues and was increased in the CSF of ALS patients. Patients who clinically progressed most rapidly expressed significantly more dendritic transcripts than patients who progressed more slowly.

These results provide circumstantial evidence for the involvement of an adaptive immune/inflammatory response in amplifying motor neuron degeneration in ALS. Also prevalent in ALS are reactive astrocytes, which stain intensively for the intermediate filament, glial fibrillary acidic protein (GFAP). These reactive astrocytes, with a hypertrophied appearance, are present throughout areas of degeneration. Reactive astrocytes can have multiple roles including a neuroprotective function (Liberto et al. 2004).

Numerous biochemical markers of neuroinflammation have been shown to be elevated in ALS tissue, cerebrospinal fluid and serum, including cytokines, chemokines, complement proteins, prostaglandins, interleukins, interferons, integrins, acute phase reactants, apolipoproteins, and proteases (Table 32.1).

Some of the inflammatory mediators enhance the entry of leukocytes from the periphery into the areas of degeneration. CCL2 is well known to enhance the entry of monocytes and dendritic cells into tissues based on interaction with the cognate receptor CCR2 on invading cells. Leukocytes that are observed in the postcapillary venules also express leukocyte function antigen 1 (LFA-1), a surface molecule, which gives activated leukocytes the ability to adhere to endothelial cells and migrate into central nervous system CNS tissue. This binding involves a specific interaction between LFA-1 and intercellular adhesion molecule-1 (ICAM-1), an adhesion molecule that is up regulated on endothelial cells in areas of inflammation (McGeer and McGeer 2003). Another molecule that is up regulated in ALS tissue is matrix metalloproteinase-9 (MMP-9) (Lim et al. 1996). MMP-9 is a proteolytic

Table 32.1 Proteins associated with the inflammatory state in ALS tissues

LCA (leukocyte common antigen, CD45)	Membrane-tyrosine phosphatase; expressed by all hematopoietic cells
LFA-1 (leukocyte function antigen 1, CD11a/CD18 integrin)	Appears on all leukocytes; promotes intercellular adhesion
ICAM-1 (intracellular adhesion molecule 1, CD54)	Cell surface or matrix molecule; promotes binding to LFA-1
GFAP (glial fibrillary acidic protein)	Intermediate filament protein highly expressed by activated astrocytes
Phospholipase A2	Breaks down membrane lipids to form arachidonic acid
COX-1 (cyclooxygenase-1)	Converts arachidonic acid into prostaglandin H ₂ ; rate-limiting step in prostaglandin synthesis
COX-2 (cyclooxygenase-2)	Converts arachidonic acid into prostaglandin H ₂ ; rate-limiting step in prostaglandin synthesis; inducible expression
PGE2 (prostaglandin E2)	Member of prostaglandin family; may have both pro- and anti-apoptotic actions
FcγR-1 (immunoglobulin Fc region receptor 1)	Receptor on phagocytes for IgG bound to an antigen
HLA-DR (human leukocyte antigen DR)	Antigen-presenting surface molecule on immunocompetent cells
IL-6 (interleukin 6)	Inflammatory cytokine released by activated astrocytes and microglia
IL-1β (interleukin 1β)	Inflammatory cytokine cleaved by caspase-1 and induces COX-2 in CNS
MCP-1 (monocyte chemoattractant protein-1) (now known as CCL-2)	Chemokine that attracts monocytes and myeloid dendritic cells
M-CSF (macrophage-colony stimulating factor)	Growth factor cytokine that acts on monocytes and macrophages to cause them to differentiate
MMP-9 (matrix metalloproteinase-9)	Proteolytic enzyme that can degrade components of the extracellular matrix
C3d (complement C3 fragment d)	Degradation product of activated C3
C4d (complement C4 fragment d)	Degradation product of activated C4
CD-11b (complement receptor 3)	Phagocyte surface receptor recognizing an activated complement fragment from C3
g-Interferon	Inflammatory cytokine released by activated T cells; potent activator of microglia and leukocytes

enzyme that is released by leukocytes and reactive astrocytes to degrade proteins of the extracellular matrix and enhance the entry of leukocytes into areas of inflammation.

Prostaglandins represent inflammatory molecules with both pro-apoptotic and antiapoptotic actions (Bezzi et al. 1998). In the first step of prostaglandin synthesis, membrane lipids are broken down by phospholipase A2 to arachidonic acid. Interestingly, phospholipase A2 has been identified in activated glial cells in areas of neurodegeneration (Stephenson et al. 1999). Next, cyclooxygenase (COX) catalyzes the rate-limiting step of prostaglandin synthesis: the conversion of arachidonic acid to prostaglandin G₂ (PGG₂). Two distinct COX isoenzymes, COX-1 and COX-2, can mediate this reaction, and they are 65 % homologous. COX-1 is constitutively expressed in most tissues, and COX-2 is constitutively expressed in the kidney, stomach, and CNS. However, COX-2 expression is strongly inducible and can be significantly up regulated by several cellular factors, including multiple growth factors, interleukin-1β, tumor necrosis factor-α, lipopolysaccharide (LPS), phorbol ester, and elevated intracellular calcium concentration. A marked increase in COX-2 levels has been seen in ALS spinal cords, consistent with previous reports of increased levels of prostaglandin E2 (PGE₂), a further product of prostaglandin synthesis (Almer et al. 2002). Both mRNA and protein levels of COX-2 were elevated in ALS spinal cords, and only in pathologically affected areas (Almer et al. 2001).

32.3 Regulatory T Lymphocytes Influence ALS Disease Progression

To determine whether regulatory T lymphocytes play a neuroprotective role in ALS patients as they do in mSOD1 transgenic mice, we examined T lymphocytes in the peripheral blood of ALS patients (Beers et al. 2011a; Henkel et al. 2013). Both numbers of Tregs and their FoxP3 RNA expression were reduced in rapidly progressing ALS patients and inversely correlated with progression rates. In slowly progressing ALS patients, Tregs and FoxP3 expression were the same as healthy controls. The mRNA levels of FoxP3, TGF-β, IL4 and Gata3, a Th2 transcription factor, were reduced in rapidly progressing patients and were also inversely correlated with progression rates. No differences in IL10, Tbx21, a Th1 transcription factor or IFN-γ expression were found between slow and rapidly progressing patients. A 3.5-year prospective study with a second larger cohort revealed that early reduced FoxP3 levels were indicative of progression rates at collection and predictive of future rapid progression and attenuated survival. Collectively, these data suggest that Tregs and Th2 lymphocytes influence disease progression rates in ALS patients as well as in ALS mice. Importantly, early reduced FoxP3 levels could be used to identify rapidly progressing patients and serve as a biomarker of accelerating disease.

32.4 The Failure of Immunosuppression in ALS

Immunosuppression with steroids, cyclophosphamide, plasmapheresis, total lymphoid irradiation, and cyclosporine has been ineffective in halting the progression of ALS (Brown et al. 1986; Appel et al. 1988; Drachman et al. 1994). This failure of immunosuppressant therapy has been cited as evidence against an immune-mediated mechanism of motor neuron injury, but other possible explanations could exist. Immune suppression may be too little and too late. By the time symptoms and signs appear, extensive CNS damage may already have occurred. Further, such therapies may not adequately access the CNS and suppress the selective inflammatory reactions of activated microglia, T cells, and/or dendritic cells. Immunosuppression has similarly failed to ameliorate type-1 diabetes or other immune mediated endocrinopathies without invalidating the potential importance of immune mechanisms in disease pathogenesis. Finally, conventional immunotherapies target all populations of T lymphocytes, including Treg/Th2 and Th1/Th17 lymphocytes, and would simultaneously suppress both protective and cytotoxic populations. What is needed is not merely to decrease all T cells, leaving intact the ratio of neurotoxicity to neuroprotection. Potentially beneficial therapeutic efforts should be focused on increasing Tregs/Th2 and decreasing Th1/Th17 lymphocytes, thereby changing the ratio of neurotoxic to neuroprotective T lymphocytes. Similar therapeutic strategies targeting microglia or monocyte/macrophage populations would require changing the ratio of neurotoxic (M1) to neuroprotective (M2) activity by enhancing M2 and suppressing M1 microglia.

32.5 Summary

In the last two decades there has been a quantum leap in our understanding of what causes motor neuron injury and cell death and what factors mediate the ALS clinical phenotype. Foremost in these advances is the discovery of more than 20 gene mutations that cause ALS. Each mutation can cause multiple clinical phenotypes (pleiotropy), and many gene mutations can cause the same clinical phenotype. Thus factors other than the specific mutation are needed to explain phenotypic diversity, and accumulating evidence implicates the importance of neuroinflammation in modifying clinical phenotypes. In fALS and transgenic mouse models, motor neuron injury is initiated by specific mutations resulting in misfolded proteins.

Neuronal viability is initially sustained both by intracellular repair mechanisms as well as by anti-inflammatory responses from surrounding glia and adaptive immune T cells. As intracellular injury increases and intra- and extracellular factors can no longer compensate, “danger” signals are released from motor neurons that trigger microglial activation to release pro-inflammatory cytokines and free radicals and

promote a self-propagating neurotoxicity. Thus activated glia and peripheral immune cells, the hallmarks of neuroinflammation, are not simply a late response to motor neuron injury and degeneration, but contribute actively to the balance between neuroprotection and neurotoxicity. However, the extent of this neuroinflammatory response differs in different ALS patients: patients progressing rapidly have dramatically decreased regulatory T lymphocytic responses, while patients progressing slowly had normal to increased levels of Treg and FoxP3 expression. Similar differences in populations of neurotoxic M1 and neuroprotective M2 monocyte/macrophage/microglia may also contribute to phenotypic diversity.

Clearly both the initiating intracellular injury and the extracellular neuroinflammatory processes contribute to the broad clinical diversity and phenotypic expression of ALS, and both represent potential therapeutic targets. Shutting down the production of the specific misfolded proteins has the potential of dramatically curtailing the initiation of motor neuron injury. Efforts targeting neuroinflammation to suppress neurotoxicity and enhance neuroprotection have the potential of changing an acute rapidly progressive disease into a more slowly progressing disease and a more sustainable quality of life. The combination of both therapeutic approaches could help arrest the progressive and devastating nature of ALS and provide hope for our patients.

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32.6 Review Questions

- Clinical heterogeneity in ALS is readily explained by different gene disease-causing mutations.
 - True
 - False
- A single mutant gene can cause multiple clinical phenotypes, and many gene mutations can cause the same clinical phenotype. Answers
 - True
 - False
- The most common cause of familial ALS is
 - mutant SOD
 - mutant C9orf72
 - mutant FUS
 - mutant TDP-43
- Neuroinflammation in ALS is characterized by
 - Activated microglia
 - Activated T cells
 - Altered circulating T regulatory cells
 - All of above
 - None of above

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Abstract

Multiple lines of evidence have implicated neuroinflammation as both a cause and an effect of neurodegeneration in Huntington's disease (HD). Studies of post mortem human HD brains and HD mouse models have demonstrated that the huntingtin protein (mHTT) has neurotoxic effects due to cell-autonomous defects in neurons and through cell-cell interactions with dysfunctional astrocytes and microglia in the brain. Neurodegeneration has been linked to excitotoxicity caused by ion and neurotransmitter concentrations in the brain, supported by evidence that extracellular levels of these molecules are modulated by astrocyte-specific mechanisms which are impaired in HD. mHTT causes monocytes and microglia to be hyper-reactive in HD patients and mouse models, contributing to neurodegeneration. Key pro-inflammatory players NFkB, IL-6, and TNF- α are implicated with disease progression, and several lines of evidence suggest that these molecules are associated with more severe neurodegeneration. Dysregulation of the NFkB activation pathway in particular is seen in HD neurons, astrocytes, microglia, and monocytes and thus may be a major component of the cellular response to mHTT with therapeutic potential. Collectively, the immune response and neuroinflammation are seen as key aspects of pathogenesis in HD, and multiple lines of evidence suggest that the neuroimmune response is both a cause and effect of neurodegeneration.

Keywords

Huntingtin protein • Huntington's disease • Neurodegenerative disease • Neuroinflammation

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33.1 Introduction

33.1.1 Clinical Features

Huntington's disease (HD) is a dominantly transmitted neurodegenerative disorder, commonly of mid-life onset. The disease is characterized clinically by three cardinal features: (1) movement disorder, (2) cognitive changes, and (3) psychiatric features. While all individuals with HD experience movement disorder and some cognitive impairment, the severity of these can vary dramatically from one individual to the next. Consequently, the disease can present remarkably differently even among persons within the same family. Whereas some experience profound cognitive impairment or severe depression, others manifest very mild effects of these conditions. These varied presentations can make it difficult to anticipate the difficulties that an individual will encounter with HD, and make the recognition and diagnosis of onset difficult in the early stages of the disease.

Movement Disorder HD was originally defined by the dance-like nature of the movement disorder when George Huntington published his treatise "On Chorea" in 1872 (Huntington 1872). Chorea, Greek for dance, aptly describes the mid-stage characteristics of the adult presentation of the disease. However, because juvenile and adolescent onset cases often present with rigidity, and late stage adult disease may also include rigidity, chorea is not the only feature of the movement disorder worthy of mention. The nature of impaired movement early after onset is characterized by small subtle movements of extremities, face and tongue, which may not affect gait. In addition to the involuntary movement, there is also a slowing of voluntary movement.

Cognitive Changes The nature of cognitive impairment in HD has been the subject of studies and reviews (Paulsen 2011; van Duijn et al. 2007). Impairment of both short and long term memory is progressively involved, but varies significantly from one individual to the next. The relationship of cognitive impairment to cortical involvement on MRI has been found (Rosas et al. 2005) but the factors which are responsible for differential cortical versus subcortical neuropathological involvement are not known.

Psychiatric Features The most common behavioral feature of HD is depression, which may occur prior to onset of movement disorder and the severity of which may vary dramatically from one individual to the next. Other psychiatric diagnoses are also more common in HD, including psychosis, although this is not a common occurrence for this disease. However, a pattern of angry and aggressive out-

bursts, which may occur unexpectedly and out of proportion to the precipitating events is one of the most difficult for families with a loved one with HD to deal with, and can be challenging to manage medically. Because many HD patients are relatively young and robust, the threat of physically aggressive outbursts is unfortunately not uncommon and can alienate affected individuals from family and caretakers.

33.1.2 Genetics

Dominant Transmission The HD gene (*HTT*) is located on chromosome 4p16.3 (Gusella et al. 1983) and the mutation that causes the disease is an expansion in the number of repeats of the nucleotides cytosine, adenine and guanine (CAG) within the coding region of the first exon of the gene. A single copy of this alteration in the gene is sufficient to cause the disease and is responsible for the autosomal dominant mode of transmission (Group 1993). Descendants of HD gene carriers, regardless of the sex of the parent or the child, have a 50% chance of inheriting the gene. Because penetrance is high, with few exceptions, those who inherit the gene eventually develop the disease, unless they should die of another cause before onset.

CAG Repeat Size The nature of the genetic defect in the HD gene explains many of the genetic features of the disorder, including the variability in age at onset, tendency for juvenile disease to be inherited from fathers and sporadic appearance of new mutations to HD (Myers 2004). This CAG "triplet" is normally repeated about 20 times (normal range is 8–26 repeats), and an approximate doubling in the number of repeats to a threshold of 40 or more results in the expression of the disease (Group 1993; Duyao et al. 1993).

De Novo Mutation Repeats between 27 and 35, commonly known as intermediate repeats, can be meiotically unstable in paternal transmission whereby descendants of men with repeats in this range have been known to inherit disease associated repeats of 40 or more. In a sample of 1260 persons ascertained from HD families studied in the New England Huntington's Disease Research Center Without Walls, repeats between 27 and 35, represented about 3.2% of all repeats. The frequency in a non-HD sample of Parkinson disease cases allowed an estimation that the frequency of expansion of an intermediate repeat will expand to the clinical range as approximately 1/1000 (Hendricks et al. 2009). Thus while it is rare that intermediate allele carriers have progeny who inherit an allele in the clinical HD range, the frequency

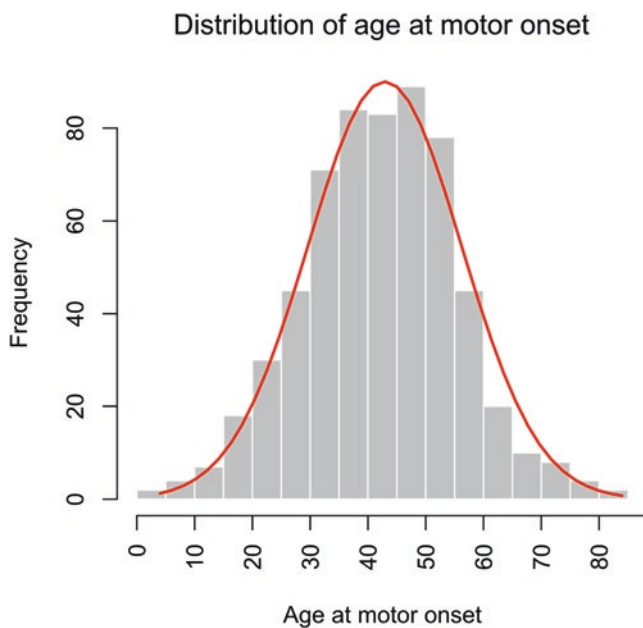


Fig. 33.1 The distribution of age at motor onset for N=600 manifest HD subjects whose brains are maintained by from the McLean Hospital Brain Tissue Resource Center. Average age at onset is 43

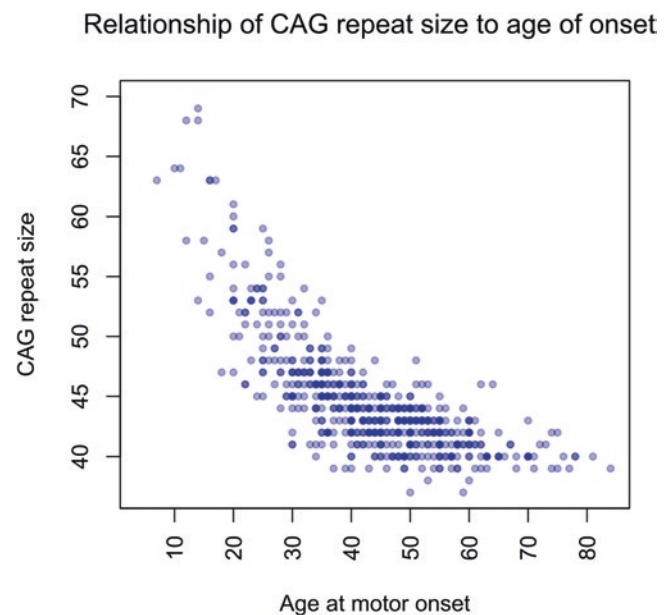


Fig. 33.2 Scatter plot relating age at motor onset to CAG repeat size for N=600 HD subjects. Age at motor onset and CAG repeat size is correlated at $r = -0.74$

of intermediate allele carriers in the general population means that the *de novo* occurrence of HD is not rare in HD clinic populations.

Reduced Penetrance Repeats between 36 and 39 are also rare (2.7% in the New England series), and are associated with reduced penetrance, whereby some persons with repeats in this range develop HD and others do not. The estimate of penetrance is weak due to the small numbers of observations and that most of those observed are persons who develop HD whereas those who do not manifest symptoms are likely to go undetected. Nonetheless, penetrance increases as the repeat size approaches 40, and may vary from as low as 25% among persons with 36 repeats to as high as 90% for persons with 39 repeats (Myers 2004).

Age at Onset HD typically strikes in mid-life but can occur as young as age 2 or 3 and as old as age 80 or more (Fig. 33.1). Survival from onset to death averages 17–20 years with some evidence that older onset is associated with slower progression, although survival may not be increased as elderly cases may die of competing causes.

HD is a disorder with highly variable clinical expression, as evidenced by the wide variation in onset age. The strong inverse relationship between age at onset and number of CAG repeats is unequivocal (Duyao et al. 1993) (Fig. 33.2). For HD cases derived from a series of HD brains from the McLean Hospital Brain Tissue Resource Center, the correlation between repeat size and onset age is $r = -0.74$ and

accounts for about 55% of the variance in onset age. Much of the strength of this correlation derives from the small sample of persons (about 5% of the sample) who have very large repeat sizes, and very young onset ages. Thus although the correlation between repeat size and onset age is strong, it is widely acknowledged that the repeat size is a poor predictor of onset age. The predictive shortcomings can be appreciated by examining the range of onset age for persons with a particular number of repeats. For example, persons in Fig. 33.2 with 44 CAG repeats exhibit onset ages as young as 28 and as old as 59 years of age. The 31 year span in onset demonstrates not only the poor predictive power for onset of the repeat size, but also the substantial variation in onset age that is not explained by the *HTT* repeat.

Significant familial aggregation for the age at onset in HD has been reported (Djousse et al. 2003; Rosenblatt et al. 2001). Using onset ages adjusted for the size of the HD repeat mutation, pairs of affected siblings were found to have remarkably similar onset ages, independent of the size of the HD repeat. Estimates of heritability of onset age after adjustment for the repeat size range from 56% to 65% (Djousse et al. 2003; Rosenblatt et al. 2001). Thus 56–65% of the variation in onset age, that is not attributable to the repeat size, can be attributed to modifier genes.

Sex of Parent Effect Merritt et al. (1969) first observed that a disproportionate number of cases with onset before the age of 21 had inherited the HD gene from affected fathers. The observation of earlier ages at onset in successive generations,

termed anticipation, is seen in several of the trinucleotide repeat disorders. Meiotic instability of the HD repeat in paternal transmission explains the observation of anticipation in HD. While meiotic instability may occur in both maternal and paternal transmission, in paternal transmission there is a propensity toward expansion of the repeat, with occasionally large expansions that are not seen in maternal transmission. For maternal transmission, nearly equal numbers of expansions and contractions are seen, and the shifts are small, ranging from 1 to 3 repeats. The observation that paternally transmitted repeats are prone to large increases in size (Duyao et al. 1993; Zühlke et al. 1993; Ranen et al. 1995) explains why most juvenile onset HD is inherited through the male germline.

33.1.3 Neuropathology

The neuropathological characteristics of HD involve a specific pattern of neuronal degeneration with important differences and similarities to other neurodegenerative diseases. A hallmark of the disease is the presence of intranuclear inclusions seen in neurons throughout the HD brain. These inclusions consist largely of the fragments of the huntingtin protein (HTT) containing the polyglutamine repeat as well as numerous other proteins to which it appears to be pathologically bound. Similar protein aggregates are seen in virtually every major neurodegenerative disease, including Alzheimer's disease, Parkinson's disease, Amyotrophic Lateral Sclerosis and others. Nonetheless, the location and composition of these aggregates varies significantly from one disease to the next. The inability to process protein fragments and their abnormal protein accumulation appears to be a driving neurotoxic mechanism for HD and other neurodegenerative diseases. Whether the aggregates themselves are neurotoxic or a protective process to sequester and remove these fragments from cytoplasmic cellular regions is not known.

Based upon MRI studies, evidence of neuropathological involvement precedes the onset of signs of the disease by many years (Aylward 2014). Atrophy of cortical grey and white matter as well as subcortical regions is readily quantifiable and increases with proximity to disease onset. These studies emphasize the effects of the disease on the whole brain, although there are clearly regions of focal involvement, mainly in the striatum which target the medium spiny neurons.

Striatal Involvement In HD the initial evidence of neurodegeneration is seen microscopically in the tail of the caudate nucleus and progresses in a rostral to caudal direction. In the head of the caudate nucleus, early neuronal loss is most evident in the medial region near the ependymal surface adjacent to the lateral ventricle (Vonsattel et al. 1985). Parallel involvement in the putamen is seen in the medial regions

adjacent to the internal capsule. Striatal involvement is associated with the motor aspects of the disease. Several MRI studies have identified a strong relationship between clinical features and the extent of brain atrophy in HD, including the relationship between cognitive performance and cortical atrophy (Rosas et al. 2005). Striatal involvement is strongly related to the size of the CAG expansion where larger repeats are associated with more severe involvement (Hadzi et al. 2012).

The extent of striatal involvement has been well quantified and characterized (Vonsattel et al. 1985; Hadzi et al. 2012) and does not correlate well the cortical atrophy (Hadzi et al. 2012). Variability in the relative involvement of cortical and subcortical regions likely influences the wide range of motor versus cognitive and psychiatric symptoms recognized among HD patients.

Cortical Involvement While both cortical and subcortical regions are involved in HD, the cortex is considerably less involved than the striatum. Atrophy occurs across all cortical regions (Vonsattel et al. 1985; Sotrel et al. 1991; Cudkowicz and Kowall 1990). As expected for progressive neurodegenerative disease, cortical atrophy increases over the course of the disease, but the extent of cortical involvement may vary dramatically from one individual to the next and is only weakly related to the CAG size (Hadzi et al. 2012).

White Matter Studies of both post-mortem brains (de la Monte et al. 1988) and by MRI show significant white matter involvement in HD (Rosas et al. 2005). White matter atrophy contributes to the loss of brain weight and overall brain atrophy at a level comparable to that of grey matter.

33.1.4 Molecular Pathogenesis of HD

The HD gene encodes a large, 348 kDa cytosolic protein (HTT) that is widely distributed throughout the body, with its highest levels in the brain (Sharp et al. 1995; Trottier et al. 1995). The protein structure of HTT suggests it is a multifunctional scaffolding protein, with multiple Huntington, elongation factor 3, protein phosphatase 2A, yeast kinase TOR1 (HEAT) repeats and hydrophobic alpha-helices that assist in protein-protein interactions, and a nuclear export signal (Bessert et al. 1995) which aids in nuclear transport. HTT directly associates with the microtubule motor dynein (Caviston et al. 2011), and indirectly binds kinesin-1 through Huntingtin-associated protein 1 (Li et al. 1998), modulating the bidirectional transport of endocytic and exocytic vesicles along cytoskeletal tracks (Colin et al. 2008). Within RNA transport granules, HTT colocalizes with Argonaute 2, a component of the RNA-induced silencing complex, indicating it may play a role in localized translational control

through the silencing of RNA transcription (Savas et al. 2010; Ma et al. 2010). HTT is critical for nervous system development (Tong et al. 2011) and embryonic lethal in knockout huntingtin mice (Nasir et al. 1995). HTT may also play a role in cytokine production in monocytes and macrophages under normal and HD settings (Träger et al. 2014b).

The HD mutation produces a neurotoxic, mutant HTT protein (mHTT). The polyglutamine tract (polyQ) expansion caused by the HD mutation exists within the N-terminal region of the HTT protein (Group 1993), which leads to the formation of intranuclear and cytoplasmic inclusions in the brains of HD patients (DiFiglia et al. 1997) as well as in HD *in vitro* models (Scherzinger et al. 1997). The N-terminal region is cleaved by caspases and calpains to produce N-terminal fragments in both normal and HD cells, where only the expanded polyQ fragments of HD self-associate and seed aggregates (Kim et al. 2001; Scherzinger et al. 1999; Wellington et al. 2002). Cytosolic and intranuclear inclusions containing mHTT fragments in neurons of the striatum and cerebral cortex are a histopathological hallmark of HD (Gutkunst et al. 1999). In post-mortem HD brains, extensive aggregation is observed in the striatum and in layers V and VI of the cerebral cortex, though smaller numbers of aggregates are observed in other brain regions not primarily affected by neurodegeneration in HD, including the basal ganglia, hippocampus, and cerebellum (Gutkunst et al. 1999). In particular, striatal medium spiny neurons and cortical interneurons exhibit extensive aggregation, where aggregates initially localize to the soma and dendritic processes but later are found in the nucleus as the disease progresses (Gutkunst et al. 1999).

Aberrant nuclear localization of mHTT fragments precedes cell death in neurons (Li and Li 2004). However, the process by which preferential death of striatal, medium spiny neurons occurs is not fully understood. HD pathogenesis is likely caused by a gain-of-function of mHTT, where the polyQ repeat expansion contributes to the misfolding and aggregate form of HTT, resulting in a protein with new, toxic function (Di Prospero and Tagle 2000). In a mouse model expressing a truncated mHTT protein, nuclear aggregation of mHTT fragments was determined to be a cell-autonomous process in striatal medium spiny and cortical interneurons, but no locomotor deficits or significant neuropathology were observed in contrast to the pan-neuronal model which exhibits these effects (Gu et al. 2007). This suggests that cell-cell interactions are critical to cause neurodegeneration in these models. mHTT may cause neurodegeneration through a toxic gain of function in its misfolded monomeric form, in its self-assembled oligomeric or sticky, aggregated forms, or by the loss of wildtype HTT function (through a loss-of-function or dominant-negative effect). Gain-of-function is the most well accepted hypothesis for HD pathogenesis, where the polyQ repeat expansion contributes to the misfolding and

aggregate form of HTT, resulting in a protein with new, toxic function (Di Prospero and Tagle 2000). Transcription factors, such as CREB-binding protein (CBP) (Steffan et al. 2000), specificity protein 1 (SP1) (Li et al. 2002), TATA-binding protein (TBP) (van Roon-Mom et al. 2002) and p53 (Steffan et al. 2000), and transcriptional repressors such as REST/NSRF (Zuccato et al. 2003), spuriously bind to mHTT intranuclear inclusions impairing their normal activity, leading to transcriptional dysregulation (Cha 2000). The transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (*NfκB*), which is particularly related to immune function, has shown altered activity in HD due to direct, aberrant interactions with soluble mHTT (Marcora and Kennedy 2010; Godavarthi et al. 2009).

HD is associated with the disruption of an assortment of cellular processes, impairing cell adhesion (Consortium 2012), mitochondrial energetics (Koroshetz et al. 1997), cellular transport (Cattaneo et al. 2001), autophagic processing (Ravikumar et al. 2004), ubiquitin-proteasome system (Wang et al. 2008), synaptic plasticity (Usdin et al. 1999) and enhancement of glutamate-related excitotoxicity (Liang et al. 2005). HTT cleavage is mediated by caspases to produce an amino-terminal polyQ containing fragment (Wellington et al. 1998), and mHTT is more susceptible to cleavage than its normal counterpart. Activation of caspases induces apoptosis; thus, overactive caspases have deleterious effects on already stressed neurons.

Evidence of increased DNA-damage has been observed in post-mortem caudate nucleus (Browne et al. 1997) and in mitochondrial DNA from the parietal cortex (Polidori et al. 1999) of individuals with HD and in R6/2 mice (Bogdanov et al. 2001). HTT has been shown to be phosphorylated by the cyclin-dependent kinase 5 (*CDK5*) after induction of DNA damage in rat, mouse, and human *in vitro* settings and *in vivo* in mice, where a loss of phosphorylation causes neurotoxicity in striatal neurons while increased phosphorylation protected against polyQ-induced toxicity (Anne et al. 2007). In a study using human embryonic stem cell derived neurons, DNA-damage was shown to enhance cleavage of wild type HTT in an IκB kinase (*IKK*)-dependent manner, and, in a striatal neuronal line from HD knock-in mice, induction of DNA-damage was shown to enhance mHTT cleavage as well (Khoshnan et al. 2009). Other studies have also shown increased HTT-related *IKK* activity of neurons in humans and transgenic mice, linking increased neurotoxicity to specific phosphorylation events on the full length HTT and mHTT proteins by *IKKβ* (Khoshnan et al. 2004; Thompson et al. 2009). This is significant for neuroinflammation since aggregation of N-terminal mHTT fragments correlates with neurodegeneration and an increase in mHTT cleavage mediated by *IKK* implicates the *NFκB* pathway in neurons.

Figure 33.3 illustrates the manifold cellular and molecular mechanisms that characterize HD pathology. Given the

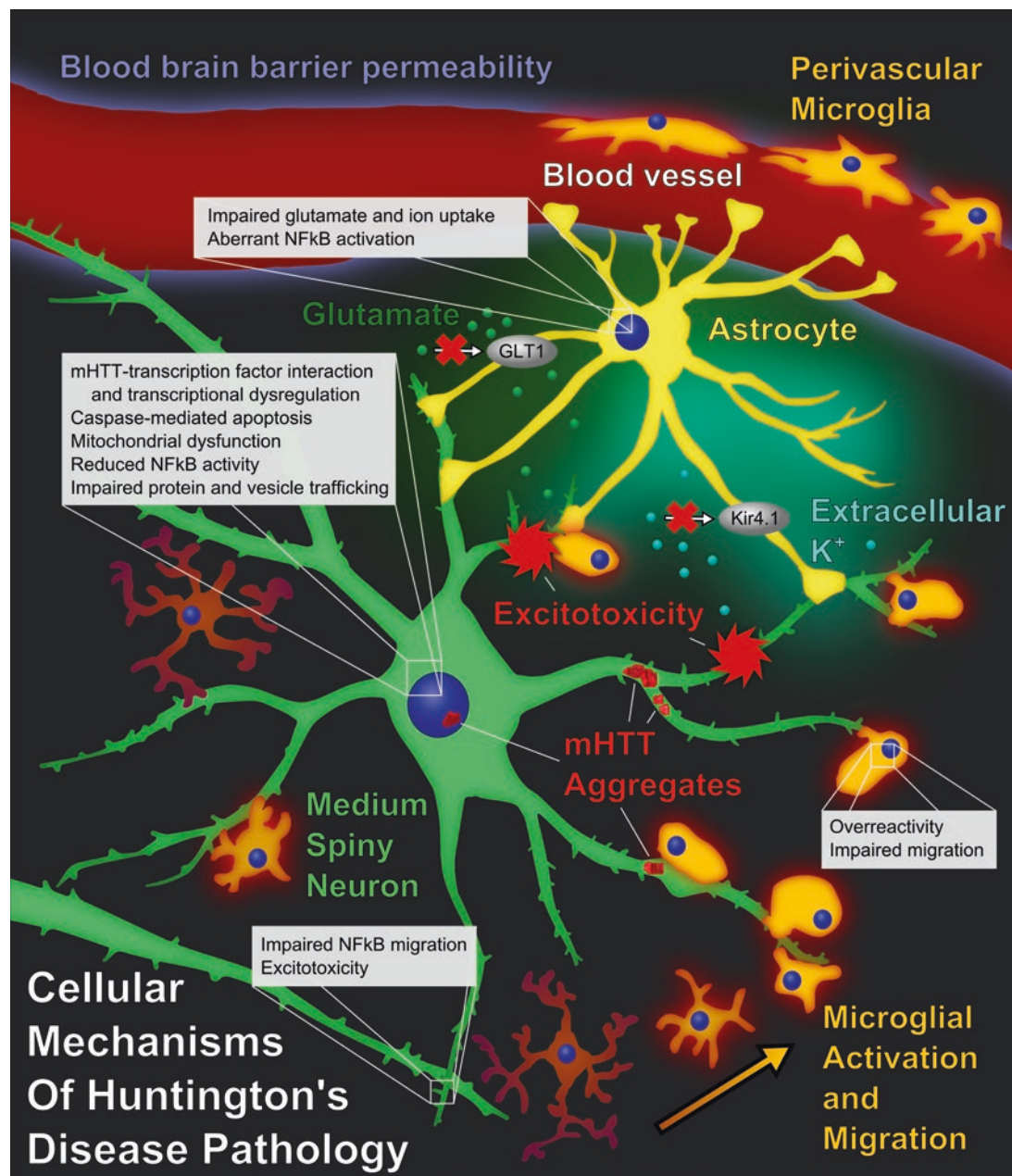


Fig. 33.3 Illustration of the primary cellular and molecular mechanisms that contribute to neurodegeneration in HD

complexity of HD pathogenesis and strain that HD neurons undergo, the role of the immune system the immune function in the brain may greatly impact the survival of stressed neurons. In the following sections, we will highlight the cellular and molecular roles of the immune system in HD.

33.2 Neuroinflammation in HD

In the central nervous system (CNS), both neurons and glia have been implicated in the neurodegenerative phenotype of HD. While neurons were initially thought to be the primary

affected brain cell type due to their selective degeneration, numerous studies have shown dysfunction in astrocytes and microglia due to mHTT expression. Studies focusing on peripheral tissues, especially in blood, also show signs of altered immune function in HD patients and mouse models.

33.2.1 The Neuroimmune Role of HTT in Neurons

Due to a number of factors including increased caspase-induced apoptosis, excitotoxicity, and transcriptional dysfunction, the

neurons affected by neurodegeneration in HD are stressed, suffer atrophy, and undergo apoptosis. As a result, damage associated molecular pattern molecules are released from severely challenged neurons, signaling for a large neuroimmune response. In a neuronal murine cell model of HD, increased expression of chemokines, such as murine chemokines KC (CXCL1), monocyte chemotactic protein 1 (*MCP-1*), as well as pro-inflammatory genes interleukin 6 (*IL-6*) and tissue inhibitor of metalloproteinases (*TIMP*) were observed in mHTT positive neurons as compared to controls, while the pro-inflammatory gene tumor necrosis factor alpha (*TNFalpha*) was significantly decreased (Godavarthi et al. 2009). Nuclear factor kappa B (*NFkB*) and proteasomal activity was observed to be significantly decreased in this study, suggesting these functions are cell-autonomous responses in the neurons of these mice.

Constitutive activation of *NFkB* in neurons has been shown to be neuroprotective and necessary for neuronal health (Bhakar et al. 2002; Fridmacher et al. 2003). *NFkB* normally resides in an inactive state outside the nucleus of neurons, localizing to the synapses, and is constitutively activated through glutamatergic transmission (Kaltschmidt et al. 1993, 1994; Wellmann et al. 2001; Meffert et al. 2003). Upon activation, *NFkB* is transported along dendritic spines in a HTT-dependent manner and imported into the nucleus. However, the transport of *NFkB* to the nucleus is impaired by mHTT (Marcora and Kennedy 2010), which may explain the observation that *NFkB* activity was decreased in an HD neuronal model derived from mice (Godavarthi et al. 2009).

33.2.2 The Neuroimmune Role of HTT in Glia

Considerable attention has been paid to the role of glia in mHTT mediated neurodegeneration. In particular, microglia and astrocytes have been directly linked to the severity of neurodegeneration in HD either through pro-inflammatory processes or increased excitotoxicity caused by dysregulated intercellular ionic and neurotransmitter concentration. No study to date has directly implicated oligodendrocytes with neurodegeneration in HD, though an increase in oligodendrocyte density was observed in the caudate nucleus of early-stage post-mortem human HD brains (Myers et al. 1991; Gomez-Tortosa et al. 2001).

33.2.2.1 HTT in Microglia

As the primary immune cells of the central nervous system, microglia are directly involved in many neurodegenerative diseases (Cunningham 2013) including HD and in response to brain injury in general (Hernandez-Ontiveros et al. 2013). Microglial activation is a hallmark of the symptomatic HD brain (Myers et al. 1991; Sapp et al. 2001; Pavese et al. 2006), and measures of the degree of activation have been used to predict onset of symptoms in presymptomatic HD

subjects (Tai et al. 2007; Politis et al. 2011). In symptomatic HD brains, the level of microglial activation is correlated with the amount of neurodegeneration, and the prefrontal cortex, a region not primarily affected by neurodegeneration, shows particularly strong evidence of activated microglia (Pavese et al. 2006).

mHTT is expressed in microglia (Kwan et al. 2012b). Studies in different HD mouse models indicate mHTT causes microglia to be more sensitive to acute inflammatory insult by lipopolysaccharide (LPS) injection than control mice (Franciosi et al. 2012; Hsiao et al. 2013; Crotti et al. 2014). Upon LPS injection, microglia in R6/2 mice show more nuclear localization of *NFkB* than LPS-treated wildtype (WT) mice, and the R6/2 mice show signs of chronic inflammation after acute inflammatory insult whereas WT mice do not (Hsiao et al. 2013). Microglia expressing the first exon of *HTT* containing the expanded polyQ tract (HDex1) transcribe more pro-inflammatory factors, in particular the cytokine *IL-6*, than microglia expressing WT *HTT*, and the genes associated with up-regulated mRNA species in these microglia compared with WT are enriched for innate immunity and inflammation overall (Crotti et al. 2014). The transcriptional mRNA profile of these HDex1 microglia appears to be controlled by the myeloid lineage-determining transcription factors PU.1, C/EBPalpha, and C/EBPbeta. Neurotoxic effects were observed when HDex1 microglia were cocultured with WT neurons, and this neurotoxicity increased when the cells were stimulated with LPS (Crotti et al. 2014). These studies suggest that microglia expressing HDex1 have a cell-autonomous, neurotoxic effect in the HD milieu.

The morphology and migration of microglia are also reported in HD. Microglia in the brain of full length mHTT mouse model YAC128 showed evidence of activation by enlarged cell bodies and decreased numbers as compared to WT mice (Franciosi et al. 2012). Expression of mHTT appears to impair microglial migration in humans and mice, possibly due to impaired actin remodeling as a result of aberrant mHTT-cofilin interaction (Kwan et al. 2012b), despite evidence of increased microglial localization to neurites in rat HDex1-cortico-striatal/microglial cell cultures (Kraft et al. 2012) and to the cerebrovasculature in the striatum in LPS-injected HD mice (Franciosi et al. 2012). The microglia that localize to neurites tend to cluster along thin processes and at points of intersection or overlap between neuronal processes, and particularly near process blebs and at the terminal ends of HDex1 striatal neurites with irregular morphology (Kraft et al. 2012). In this study, microglia were not observed to colocalize with neuronal mHTT fragment inclusions, suggesting cellular aggregates do not cause neurons to secrete microglia-activating signals at these locations. Localization of activated microglia to the cerebrovasculature seems to be due to evidence of blood brain barrier permeability upon induction of acute inflammation (Franciosi et al. 2012),

suggesting a part of the microglial response is due to defects in blood brain barrier integrity under pro-inflammatory conditions in HD. The correlation of microglial activation with severity of neurodegeneration, the apparent hyper-reactivity of microglia that express mHTT to pro-inflammatory insult, and altered migration capacity support a cell-autonomous mechanism in microglia that contributes to neurodegeneration in HD.

33.2.2.2 HTT in Astrocytes

Astrocytes are implicated in HD neuropathology in several ways. In a study of post-mortem HD human striatum, the number of glial fibrillary acidic protein (GFAP)-expressing astrocytes increased with grade of severity, and mHTT aggregates were co-localized with GFAP in grade 0 HD patients, suggesting astrocytic mHTT may be an early and progressive event that could be associated with the pathology of the disease (Faideau et al. 2010). Striatal astrocytes of R6/2 and human brain contain mHTT nuclear inclusions, though not all glia show evidence of aggregation (Shin et al. 2005; Tong et al. 2014). In astrocytes of R6/2 mice, nuclear inclusions were observed considerably later than those observed in neuronal cells, suggesting that astrocytes are more capable of clearing misfolded mHTT than neurons (Shin et al. 2005). The same study found no evidence that mHTT aggregation altered astrocyte morphology or directly killed cultured astrocytes *in vitro*, suggesting that mHTT fragments lead to glial dysfunction, rather than degeneration. In an HD mouse that selectively expressed either HTT or mHTT in astrocytes, HTT fragments were observed to be diffuse in the nucleus of cultured astrocytes, whereas mHTT showed nuclear aggregates (Bradford et al. 2009). The presence of mHTT aggregates in astrocytes, and the correlation of reactive astrocytes in human HD brain with disease severity, is strong evidence that these cells are affected by mutant HTT.

Astrocytes are responsible for maintaining neuronal health and function in part by controlling extracellular concentrations of ions and neurotransmitters, in particular potassium (K^+) and glutamate. One study reports astrocytes from symptomatic R6/2 mice were less able to maintain proper K^+ flux *in vivo* due to mHTT-driven inhibition of the Kir4.1 ion channel, resulting in altered excitability of nearby neurons (Tong et al. 2014). Exogenous introduction of Kir4.1 into these mHTT striatal astrocytes *in vivo* attenuated motor deficit and prolonged survival. Several studies report impaired glutamate uptake in astrocytes expressing mHTT (Bradford et al. 2009; Shin et al. 2005) and that the extent of this defect correlates with HD grade in human subjects (Faideau et al. 2010). Preliminary evidence showed that reduced expression of glutamate transporter 1 (*GLT-1*) was responsible for impaired glutamate uptake rather than altered protein function and that striatal neurons were more sensitive to glutamate toxicity than cortical neurons (Shin et al. 2005). The same study

showed that coculture of neurons expressing mHTT with WT astrocytes resulted in less neurodegeneration *in vitro*, and coculture of astrocytes expressing mHTT with WT neurons resulted in more neurodegeneration, which was linked to impaired glutamate uptake capacity in mHTT astrocytes. Oral administration of a kynurenine 3-monooxygenase inhibitor to R6/2 mice inhibits extracellular glutamate in the brain, prevents synaptic loss, reduces microglial activation, and extends survival (Zwilling et al. 2011). In astrocytes cultured from postnatal rat pup cortex, treatment with an autophagy inhibitor significantly increased mHTT aggregation, whereas treatment with an autophagy stimulator reduced aggregation, reversed changes in *GLT-1* expression, and glutamate uptake (Chen et al. 2012). The discovery methods to decrease neurotoxicity by rescuing ion channel dysfunction and glutamate uptake by astrocytes suggests a causal link from astrocytic dysfunction, glutamate concentration, and microglial activation to neurotoxicity.

33.2.2.3 HTT in Peripheral Cell Types

HTT is ubiquitously expressed throughout the body (Sharp et al. 1995) and several alterations in peripheral cell types have been observed in the presence of mHTT. mHTT fragments have been observed in monocytes, T-cells, and B-cells of presymptomatic and symptomatic HD patients and levels of mHTT transcripts correlated with disease progression in monocytes and T-cells (Weiss et al. 2012). Monocytes are of particular interest, since they are easily collected from HD patients and modifying migrating monocytes or precursors in the bone marrow may have therapeutic potential.

Blood monocytes originate in the bone marrow and derive from the same myeloid precursors that migrate to the brain to become microglia (Prinz and Priller 2014). mHTT has been detected in human monocytes (Weiss et al. 2012) and express increased levels of the cytokines IL-6, TNF α , and, upon differentiation into macrophages, IL-8 in presymptomatic HD subjects compared with controls, particularly after stimulation with LPS (Björkqvist et al. 2008; Godavarthi et al. 2009; Träger et al. 2014b). This hyper-reactivity of monocytes and macrophages in HD patients was attenuated when the levels of endogenous HTT was reduced using an anti-HTT short interfering RNAs (siRNA) (Träger et al. 2014a). In the same study, a reduction in cytokine production following anti-HTT treatment was also observed in control individuals, suggesting WT HTT plays a role in cytokine production in monocytes and macrophages under normal conditions. Hyper-reactivity was also observed in CD11b+ cells and myeloid cells derived from the spleen of R6/2 mice compared to WT mice after LPS treatment (Träger et al. 2014b). The increase in cytokine production seems to be due to increased interaction between HTT and IKK γ , leading to enhanced NF κ B activity (Träger et al. 2014a). These lines of evidence suggest that microglial hyperactivity

is influenced by mHTT via cell-autonomous changes that remain after differentiation from precursor myeloid cells.

Since microglia originate in the bone marrow and are affected by mHTT, bone marrow replacement may be a potential therapeutic for HD. Neither CD11b+ bone marrow myeloid cells nor macrophages derived from bone marrow cells exhibit increased pro-inflammatory cytokine production or phagocytosis as observed in more mature myeloid cells in R6/2 mice (Träger et al. 2014a). In a study where YAC128 and BACHD full-length mHTT mice were transplanted with WT bone marrow after lethal irradiation, the transplanted HD mice were more similar to WT mice in body weight and motor performance on hypokinetic assays (Kwan et al. 2012a). The untransplanted YAC128 mice demonstrated elevated levels of cytokines in the blood, consistent with previous studies (Björkqvist et al. 2008), and after transplantation with WT bone marrow these cytokine level differences between YAC128 and WT mice were abolished. The partial rescue of HD-like symptoms in two mouse models after bone marrow transplantation suggests that cell-autonomous effects caused by mHTT might be modified by manipulating the myeloid precursor cells in HD patients, but no studies testing this hypothesis have been performed to date.

Cannabinoid receptor 2 (*CB2*) has been linked to neuroprotection conferred through peripheral immune cells (Bouchard et al. 2012). In this study, genetic deletion of *CB2* accelerated onset of motor deficits and increased their severity in a slowly progressing HD mouse model. Treating normal BACHD mice with a *CB2* agonist ameliorated behavioral and neuropathological effects even at late stages, while coadministration with a *CB2* receptor antagonist that could not cross the blood brain barrier prevented the protective effects. This evidence is consistent with a model where neurodegeneration and *CB2* signaling are causally linked, and indeed *CB2* signaling dampens immune activation by decreasing activation of microglia after brain injury (Ashton and Glass 2007). Enhancing *CB2* activity in the periphery may therefore be a potential therapeutic for HD patients.

33.3 Molecular Players of Neuroinflammation in HD

Studies across HD human specimens, HD model organisms, and cell types reveal a consistent set of molecular players shown to be involved in HD. These include inflammation-related cytokines IL-6 and TNF α , the complement component system, genes within the NF κ B pathway, and extracellular glutamate in the brain. Multiple studies suggest that the activity of each of these groups of molecules is associated with neurodegeneration, and that modulating these systems can alter disease progression.

33.3.1 Cytokines and Chemokines

Pro-inflammatory cytokine levels are altered in human HD patients and mouse models. In humans, the cytokines *IL-6*, *IL-8*, *IL-4*, *IL-10*, and TNF α were increased in the blood and striatum of presymptomatic and manifest HD, where levels of the latter three were significantly associated with disease progression (Björkqvist et al. 2008; Godavarthi et al. 2009; Träger et al. 2014b). In addition to these, chemokine (C-C motif) ligand 2 (*CCL2*) and matrix metalloprotease 9 (*MMP9*) were increased in post-mortem HD striatum (Silvestroni 2009). A similar pattern is seen in mice (Kwan et al. 2012b; Kraft et al. 2012; Bouchard et al. 2012; Träger et al. 2014a; Crotti et al. 2014). In a neuronal murine cell model of HD, increased expression of chemokines murine chemokine (*KC*), monocyte chemoattractant protein 1 (*MCP-1*), and lipopolysaccharide-induced chemokine (*LIX*) were increased after acute induction of inflammation by LPS injection, whereas TNF α receptors were significantly decreased (Godavarthi et al. 2009).

33.3.2 Complement Cascade

Levels of the complement cascade proteins are elevated in HD patient blood. In particular, C3, C4, C7, and C9 were found in two studies of symptomatic human HD blood (Leblhuber et al. 1998; Dalrymple et al. 2007) and in neurons, myelin, and astrocytes of human HD striatum (Singhrao et al. 1999). The Singhrao et al. (1999) study found that complement mRNA levels were two to fivefold higher in human HD striatum compared to controls and showed C3 and C9 to be expressed in reactive microglia on the membranes of neurons, contributing to neuronal necrosis and proinflammatory activity. Genetic deletion of C3 from an HD mouse model did not show alteration of disease progression, however, suggesting this complement component is not critical for neurodegeneration (Larkin and Muchowski 2012).

33.3.3 NF κ B Pathway

Dysregulation of NF κ B by mutant HTT is a central theme in studies of human HD and HD mouse models, where mHTT affects the NF κ B pathway in two principle ways delineated by cell type. In neurons, WT HTT has been shown to directly interact with glutamate-activated NF κ B by transporting it from the synapse along dendritic spines to the nucleus (Kaltschmidt et al. 1993, 1994; Wellmann et al. 2001; Meffert et al. 2003). In HDex1 mice, mHTT seems to impair this transport, causing decreased nuclear concentration of NF κ B and likely NF κ B pathway dysfunction (Marcora and Kennedy 2010). Constitutive activation of NF κ B in neurons

has been shown to be neuroprotective and necessary for neuronal health (Bhakar et al. 2002; Fridmacher et al. 2003), suggesting this dysfunction may be a critical aspect of neurodegeneration. In contrast, R6/2 astroglia and human HD monocytes exhibit increased NF κ B activation due to aberrant interactions between mHTT and the IKK (Khoshnan et al. 2004; Hsiao et al. 2013; Träger et al. 2014a). Specifically, multiple lines of evidence suggest that mHTT exon 1 associates with IKK γ and activates the IKK complex, which in turn increases IKK β activity and subsequent NF κ B activation (Khoshnan and Patterson 2011). Blocking IKK β , inhibiting NF κ B, or inducing degradation of I κ B appears to reduce mHTT toxicity in these contexts. Each of these studies found an association between increased NF κ B activity and more severe degeneration. These studies suggest that NF κ B dysregulation in neurons and astrocytes is a major component of neurodegeneration in HD.

33.4 Outlook

Interventions in the immune response show potential as therapeutic avenues in attenuating the severity of neurodegeneration in HD. Dysregulated glutamate levels can be at least partially restored by rescuing astrocytic dysfunction in ion and neurotransmitter uptake pathways using mechanistically-linked compounds. Since the primary affected cell types are in the brain, identifying compounds that cross the blood brain barrier remains a major challenge in administering treatments in this context. However, administering treatments that affect immune cells in the periphery which can then migrate to the brain may have therapeutic potential. The hyper-reactive monocyte and microglia response can be dampened by modulating specific monocyte receptors in the periphery and by replacing bone marrow with myeloid precursors that lack mutant HTT. Studies have not suggested that manipulating peripheral monocytes in this way is curative, but delaying or ameliorating symptoms caused by immune-related neurodegeneration may be an important aspect of a combined therapeutic regimen. Anti-inflammatory therapeutics have shown promise in HD, so a first avenue of treatment might involve modifications of lifestyle to promote anti-inflammatory processes.

Clearly, challenges to our understanding of HD remain as well to how best to develop effective therapies. The widespread cell-specific changes in response to mHTT stand in stark contrast to the deceptively simple monogenic nature of the disease. For this reason, “silver bullet” treatments likely do not exist for this disease using currently available pharmacological tools. Successful therapies may therefore require multiple agents that specifically target the different sources of neurotoxicity. Neuroinflammation plays a central role in neurodegeneration in HD and thus may be fundamentally important to the development of future treatments.

33.5 Review Questions

- The age at onset of Huntington’s disease is highly variable. Studies suggest which of the following is the most likely sources of this variability?
 - Exposure to neurotoxins such as pesticides or organic solvents have been shown to be associated with earlier onset age.
 - Analyses of pairs of affected siblings show heritability of onset age suggesting other genes influence onset age.*
 - Maternal transmission of HD is strongly associated with early onset in HD.
 - The size of the CAG repeat expansion has been shown definitively to explain all of the variance in onset age of HD.
 - There are no known factors influencing onset age in HD.
- Which of the following cell types have been implicated in the neuroimmune response in HD?
 - Neuron
 - Microglia
 - Astrocytes
 - All of the above*
- Complement cascade proteins are one of the molecular mechanisms implicated in HD. What did studies of genetic deletion of C3 show in terms of clinical disease progression?
 - HD mouse models with C3 deletion showed protection against HTT protein accumulation and longer survival.
 - HD mouse models with C3 deletion showed more rapid disease progression and shorter survival.
 - HD mouse models with C3 deletion showed a reduced inflammatory response which was neuroprotective.
 - C3 deletion did not alter disease progression in HD mouse models.*
- DNA damage has been implicated in human HD post-mortem brain, and this has been associated with increased cleavage of HTT. This pattern is related to neuroinflammation in HD by which pathway?
 - Htt cleavage mediated by an I κ B kinase (IKK) implicates the NF κ B pathway in neurons.*
 - The aberrant cytoplasmic localization of mHTT fragments.
 - Dramatic increase in the pro-inflammatory gene tumor necrosis factor alpha (TNF α)
 - Facilitation and increase in NF κ B transport by mutant HTT.
- Which of the following is true regarding the role of microglia in HD?
 - Mutant HTT is not expressed in microglia.
 - Microglial activation is a hallmark of HD neuropathological involvement.*

- c. Microglial activation is only seen in the striatum in HD brain.
- d. Microglial activation occurs in HD brains but is not related to the extent of pathological involvement in the brain.

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Qingzhong Kong and Richard A. Bessen

Abstract

The human prion diseases are a diverse group of neurodegenerative diseases that are classified by several criteria including disease etiology, *PRNP* polymorphisms at codon 129, PrP^{Sc} types and subtypes, prion incubation period and disease duration, clinical presentation and neuropathology. Creutzfeldt-Jakob disease (CJD) is unique in that it can have a sporadic, inherited, and infectious etiology, and all three forms can subsequently be transmitted following experimental inoculation of brain tissue. PrP^{Sc} is an unusual pathogen that fails to induce a prion-specific host immune response, but PrP^{Sc}-induced damage to the brain likely causes a proinflammatory response that contributes to neurodegeneration. The human prion diseases are primarily localized to the nervous system, but variant CJD (vCJD) also has a peripheral distribution in secondary lymphoid tissues that is consistent with oral exposure to bovine spongiform encephalopathy (BSE). The prion agent uses the immune system for targeting to lymphoid organs, agent replication, and neuroinvasion through fibers that innervate lymphoid tissues. In chronic inflammatory conditions in which lymphoid organogenesis can occur in non-lymphoid tissues, ectopic peripheral prion infection can be established and could play a role in prion transmission.

Keywords

Creutzfeldt-Jakob disease • Fatal insomnia • Gerstmann-Straussler-Scheinker disease • Kuru • Neuroinvasion • Prion • Protein misfolding • Protein-only hypothesis

34.1 Introduction

The modern history of the prion diseases is one of novel microbes, anthropological intrigue, and food safety mishaps. The prion diseases, also called the transmissible spongiform

We are very saddened that Dr. Richard Bessen passed away recently. Richard is a well-known scientist in the prion field. He has made significant contributions to many aspects of prions and prion diseases, including the etiology, strains, transmission, shedding and neuroinvasion of various prions. He will be missed.

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encephalopathies, are fatal neurodegenerative diseases that can be sporadic, inherited, or infectious. These multiple origins are unique among human disease. The basis of all prion diseases is the misfolding of the host prion protein into the disease-specific prion protein conformation, called PrP^{Sc}. Transmission of PrP^{Sc} into naïve hosts can lead to additional prion protein misfolding and induction of neurodegenerative disease. Hence, prion diseases are transmissible but are caused by a novel pathogen that lacks a prion-specific nucleic acid genome.

In the 1950s Carleton Gajdusek, an American physician, was attracted to the highlands of New Guinea by reports of a mysterious condition called kuru that was ravaging the Fore stone-age tribes. Kuru primarily afflicts woman and young children and victims succumb to neurological illness that includes shivering and twitching but rapidly progresses to ataxia, paralysis, and eventually death. Gajdusek was perplexed

as to the cause of kuru and it was the observation of William Hadlow, a veterinary pathologist, which led to the recognition of the human prion diseases. Hadlow observed the striking similarity in neuropathology between scrapie, a transmissible disease in sheep, and kuru and reasoned that if kuru and scrapie are similar diseases, then kuru should be transmissible to non-human primates (Hadlow 1959). Gajdusek inoculated kuru into chimpanzees and transmitted neurological disease (Gajdusek et al. 1966). Once the infectious etiology of kuru was established, transmission of kuru was linked to ritualistic endocannibalism among the Fore people. Shortly afterwards, Gajdusek reported transmission of sporadic Creutzfeldt-Jakob disease (CJD), a neurological disease first described in the 1920s, to chimpanzees and they developed neuropathology characteristic of the prion diseases, which includes neuronal loss, spongiform changes, and astrogliosis. These pioneering studies established that kuru and CJD, which were thought to be unrelated diseases of unknown etiology, belong to the same group of human transmissible spongiform encephalopathies. Gajdusek received the Nobel Prize in Physiology or Medicine in 1976 for his work on human prion diseases.

Although the human and animal prion diseases were known to be transmitted by an infectious agent, the unusual resistance of these agents to chemical and physical inactivation was perplexing and led to theories that they were caused by an unconventional slow virus. In 1982, Stanley Prusiner reported that scrapie in sheep was caused by “prions”, which were defined as infectious proteinaceous particles that are devoid of a nucleic acid (Prusiner 1982). This hypothesis was met with immense skepticism from the scientific community since it challenged the central principle of molecular biology that genetic information flows from DNA to RNA to protein. However, Prusiner, and others, were able to build

upon this theory and his pioneering work was also recognized with a Nobel Prize in Physiology or Medicine in 1997.

Another bizarre chapter in prion diseases began in the mid-1980s with the identification of bovine spongiform encephalopathy (BSE) in cattle. This prion disease was transmitted by industrial cannibalism in which livestock feed was supplemented with meat and bone meal that was unknowingly derived from prion-infected sources, possibly from scrapie-infected sheep. BSE peaked at over 35,000 cases a year in the United Kingdom in the early 1990s but the prevalence has been greatly reduced by the removal of ruminant-derived protein sources in livestock feed. However, the BSE agent is a highly pathogenic strain of prion and is the causative agent for a new human prion disease called variant CJD (vCJD) (Will et al. 1996).

The human prion diseases, which include CJD, kuru, Gerstmann-Straussler-Scheinker disease (GSS), fatal insomnia, and the recently identified variably protease sensitive prionopathy (VPSPr), can be classified into three groups based on etiology (Table 34.1).

34.2 Prion Protein Gene and Gene Products

34.2.1 Prion Protein Gene and PrP^C

The prion protein is a normal cellular membrane protein that is expressed at high levels in the nervous system, testis, muscle, some hematopoietic cells, and follicular dendritic cells (Makrinou et al. 2002). In humans the prion protein is encoded by a single copy gene, which is called *PRNP* and is located on chromosome 20. As illustrated in Fig. 34.1a, *PRNP* has two exons and a single intron that span about 15 kilobases; exon 2

Table 34.1 Origin and prevalence of the human prion diseases

Human prion disease (percent)	Prevalence	Origin (distribution)
Sporadic (85–90 %)		
Creutzfeldt-Jakob disease	1 in 10 ⁶ /year	Spontaneous (worldwide)
Fatal insomnia	≥10 cases	Spontaneous
Variably protease sensitive prionopathy	≥21 cases	Spontaneous (US, UK, Europe)
Familial (10–15 %)		
Creutzfeldt-Jakob Disease		
E200K-129M	3–5 % ^a	Germline mutation in <i>PRNP</i>
All other families	>97 families	Germline mutations/insertions in <i>PRNP</i>
Fatal familial insomnia	≥44 families	Germline mutation in <i>PRNP</i>
Gerstmann-Straussler-Scheinker	>72 families	Germline mutations/insertions in <i>PRNP</i>
Acquired (~1 %)		
Variant CJD	229 cases	BSE infection (UK, Europe, others)
Iatrogenic CJD	≥470 cases	Medical practices (France, Japan, others)
Kuru	~2700 cases	Cannibalism (Papua New Guinea)

^aPercent of all human prion diseases based upon epidemiological data from Italy and Japan

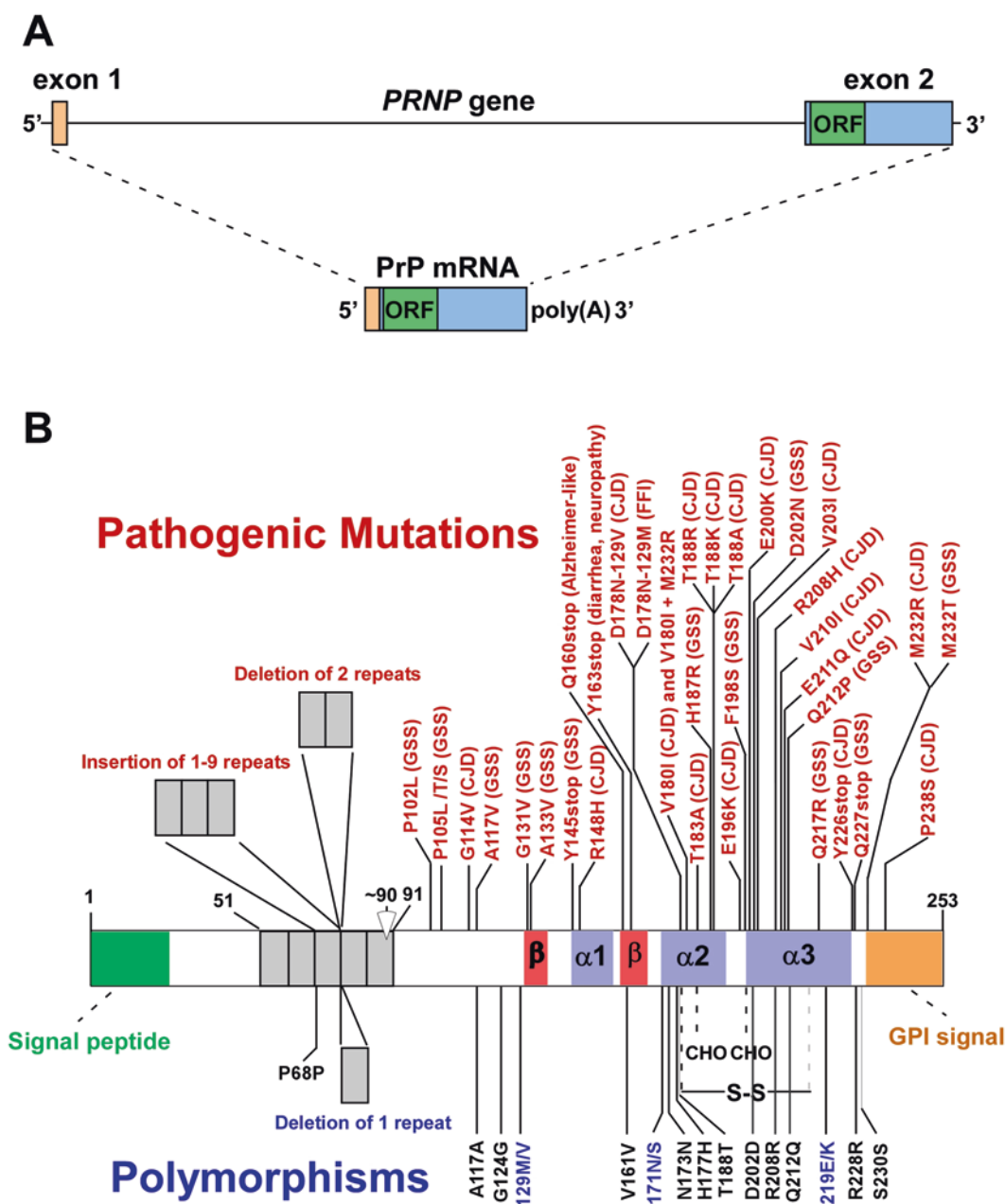


Fig. 34.1 Diagram of the *PRNP* gene and the PrP protein. (a) The prion protein gene and mRNA. The PrP ORF is located within exon 2 and is highlighted in green. (b) The PrP protein linear map with post-translational modifications, polymorphisms, and pathogenic mutations (modified from Fig. 2 in Kong et al. 2003). The numbers indicate the amino acid residue position in the prion protein and the single letter designation for amino acids is used to denote polymorphisms and mutations. The signal peptide at the amino terminus and the glycosylphosphatidylinositol (GPI) signal peptide at the carboxyl terminus

are indicated in green and orange, respectively. The octapeptide repeats are indicated by gray boxes, while the three α -helices and two short β -sheets are indicated by pale blue and red boxes, respectively. Mutations linked to the human prion diseases are illustrated in red type above the PrP map while normal polymorphisms are indicated below. The arrowhead at amino acid 90 indicates the major cleavage site for proteinase K, which degrades the N-terminal portion of PrP^{Sc}. The two N-linked carbohydrate groups (CHO) and disulfide linkage (-S-S-) are also illustrated.

contains the prion protein coding sequence (Makrinou et al. 2002). In the *PRNP* coding region there are a large number of pathogenic mutations that are linked to, or have been associated with, the familial prion diseases (Fig. 34.1b) (Kong et al. 2003). Polymorphisms are also present and the one at codon 129 (methionine or valine) plays an important role in deter-

mining susceptibility and/or the clinical phenotype/disease duration in the prion diseases.

The cellular form of the prion protein, called PrP^C, is synthesized as a 253 amino acid precursor protein in humans (Fig. 34.1b) (Prusiner 1991). The N-terminal 22 amino acid residues serve as the signal peptide that allows insertion of

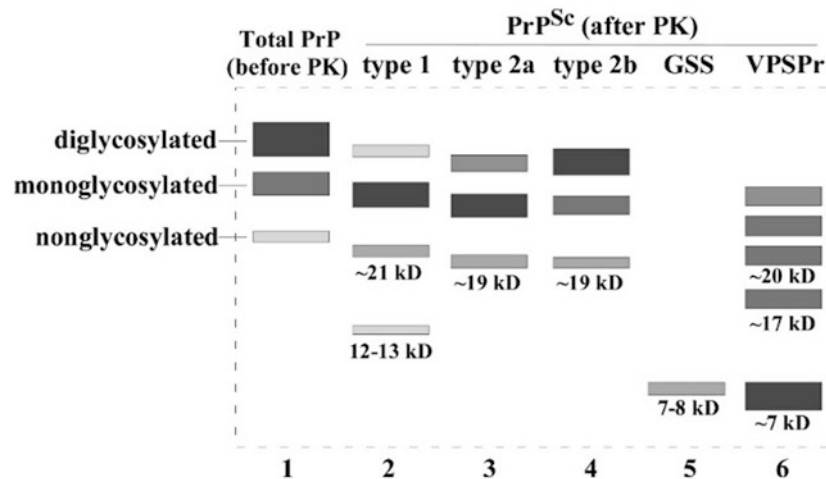


Fig. 34.2 Schematic diagram of PrP^{Sc} types found in human prion diseases. The three major prion protein polypeptide glycoforms are illustrated on SDS-PAGE (*lane 1*). The relative amount of each PrP polypeptide in relation to the other glycoforms is indicated by the shading intensity of each band. The molecular weight of the major nonglycosylated PrP^{Sc} polypeptide band is indicated in kilodaltons (kD). PrP^{Sc}

types are defined after limited digestion with proteinase K (PK), which removes the N-terminal portion of the prion protein (*lanes 2–6*). PrP^{Sc} subtypes are defined by the molecular weight of the nonglycosylated PrP^{Sc} polypeptide, the ratio of the three PrP^{Sc} glycoforms, as well as the identity of codon 129. This is one criterion that is used for the molecular classification of the human prion diseases

the nascent PrP^C peptide into the secretory pathway during biosynthesis. The C-terminal 22 amino acid residues are involved in the covalent addition of the glycosylphosphatidylinositol (GPI) moiety to serine at residue 231 (i.e., Ser²³¹). The GPI anchor tethers PrP^C to the extracellular side of the plasma membrane. PrP^C has two N-linked glycosylation sites (Asn¹⁸¹ and Asn¹⁹⁷) that are the sites of attachment of complex carbohydrates. There are three major PrP^C glycoforms that are defined by the number of N-linked carbohydrate moieties; these are referred to as diglycosylated, monoglycosylated, and nonglycosylated forms (Fig. 34.2). The N-terminal region of PrP^C has five octapeptide repeats that bind metal ions (e.g., copper, zinc) but the structure of this domain is undefined, while the C-terminal portion consists of three α -helical and two short β -sheet domains (Fig. 34.1b). The cellular function(s) of PrP^C is diverse. It has been implicated in neurogenesis and differentiation (Steele et al. 2006), astrocyte maturation (Arantes et al. 2009; Hartmann et al. 2013), differentiation of pluripotent progenitors cells toward the neural lineage (Peralta et al. 2011), proliferation of oligodendrocyte precursor cells (Bribian et al. 2012), proliferation and self-renewal of neural progenitor cells (Santos et al. 2011; Prodromidou et al. 2014), hematopoietic stem cell self renewal (Zhang et al. 2006), cell-cell adhesion (Solis et al. 2013), microtubular cytoskeleton disassembly (Niezanski et al. 2006) and microtubule-dependent transport (Niezanska et al. 2012), metal ion transportation (Wong et al. 2001), antioxidant

activity, signal transduction, apoptosis, and sleep regulation (Tobler et al. 1996; Lasmezas 2003).

34.2.2 The Protein-Only Hypothesis for the Prion Diseases

Identification of the infectious agent causing prion diseases has been controversial due to its small size and resistance to procedures that inactivate most microorganisms as well as nucleic acids. Extensive efforts to detect a prion-specific small virus or nucleic acid genome have been unsuccessful (Safar et al. 2005). In contrast, procedures that destroy proteins also inactivate prion infectivity. Despite much debate, it is generally agreed that prion diseases are caused by prions, which are defined as proteinaceous infectious particles that lack a nucleic acid genome (Prusiner 1991). The protein-only hypothesis states that the infectious prion agent is a misfolded form of PrP^C devoid of specific nucleic acids (called PrP^{Sc} where “Sc” refers to scrapie). PrP^{Sc} has distinct biochemical properties from PrP^C that include partial resistance to degradation by proteolytic enzymes, insolubility in detergents, and aggregation into linear fibrils. The basis for these unusual features of PrP^{Sc} is a conformational conversion of α -helical to β -sheet conformation. This protein misfolding, or prion replication, has been postulated to occur by a protein self-assembly mechanism by which the abnormal PrP^{Sc} polymer or fibril can convert the monomeric PrP^C into a subunit of the growing PrP^{Sc} polymer (Jarrett and Lansbury 1993).

Although the scientific community was initially skeptical of the prion hypothesis, in the past 30 years several key experiments have demonstrated support for this novel concept. PrP null mice, in which the endogenous prion protein gene has been removed, do not make PrP^{Sc}, prion infectivity or develop prion disease when inoculated with the scrapie agent (Sailer et al. 1994). Additional studies used transgenic mouse models in which the prion protein transgene corresponds to a mutation common in Gerstmann-Straussler-Scheinker disease (GSS), a proline to leucine mutation at codon 102 (i.e. P102L). These transgenic P102L mice, when overexpressing the mutant PrP protein, spontaneously develop prion disease and the neuropathology is characterized by PrP^{Sc} amyloid plaques, which are similar to plaques found in GSS patients (Hsiao et al. 1990). This classic study demonstrated that mutations in *PRNP* are directly linked to the human prion diseases. More definitive proof of the prion hypothesis is the *in vitro* generation of infectious prions from recombinant prion protein (Legname et al. 2004; Deleault et al. 2007; Kim et al. 2010; Wang et al. 2010). Additional studies indicate that host cofactors also play a role in prion replication (reviewed by Ma and Wang 2014).

34.2.3 Molecular Classification of PrP^{Sc} in Human Prion Diseases

Biochemical characterization of PrP^{Sc} reveals heterogeneity in the polypeptide patterns among the human prion diseases and this has been useful for the molecular classification of PrP^{Sc} (Gambetti et al. 2003). Limited proteinase K digestion of PrP^{Sc} removes a ~7 kilodalton N-terminal fragment but leaves intact a protease-resistant PrP^{Sc} core. Typically, there are three protease-resistant polypeptide fragments, referred to as glycoforms, which differ in molecular weight by the addition of zero, one, or two N-linked carbohydrate groups (Fig. 34.2). Among the human prion diseases, PrP^{Sc} polypeptide fragments can vary in number (typically 1–3), molecular weight, and the ratio of the three glycoforms. Based on these criteria, PrP^{Sc} is classified into four operational groups called type 1, type 2 (a and b), GSS-type, and variably protease sensitive prionopathy (VPSPr)-type (Table 34.2, Fig. 34.2). Type 1 PrP^{Sc} is found in the majority of sporadic CJD (sCJD)

cases, some familial CJD (fCJD) cases, and at small amounts in all variant CJD (vCJD) cases (Yull et al. 2006). The type 2a PrP^{Sc} is associated with a minority of sCJD cases, sporadic fatal insomnia (sFI) and some fCJD cases. Many sCJD patients (20 % or more) possess both type 1 and type 2 PrP^{Sc}. In vCJD, PrP^{Sc} polypeptides have a similar molecular weight as found in type 2a PrP^{Sc} but the ratio of the three PrP^{Sc} glycoforms are different. Therefore, vCJD is referred to as type 2b PrP^{Sc} (Fig. 34.2). In GSS, a 7–8 kilodalton (kD) PrP^{Sc} polypeptide is referred to as GSS-type PrP^{Sc} and it is only found in GSS cases. In VPSPr, PrP^{Sc} consists of multiple PrP fragments [~7kD (GSS-like), ~17kD, ~20kD, ~23kD and ~26kD] (Table 34.2, Fig. 34.2) (Zou et al. 2010, 2013). The significance of the PK-resistant PrP-CTF12/13 fragments (Fig. 34.2) is not clear (Zou et al. 2003).

34.3 Epidemiology and Clinical Features of Human Prion Diseases

The human prion diseases are rare but fatal neurodegenerative diseases that have long incubation periods ranging from years to decades. The clinical course is progressive and exhibits a complex neurological pattern that also can last from months to years. Presenting symptoms vary depending on the type of human prion disease, but most will display cognitive, motor, and behavioral deficits during the clinical phase. Some of the prion diseases target to specific brain regions while others have a widespread distribution in the brain. The incubation time, age at onset of clinical symptoms, and disease duration is variable among the human prion diseases and there is often overlap in these features among the major types of human prion diseases (Table 34.1).

34.3.1 Sporadic Human Prion Diseases

Sporadic CJD is the most common human prion disease and accounts for 85–90 % of all human cases (Gambetti et al. 2003). Sporadic prion diseases are associated with type 1, type 2a (sCJD), or a mixture of type 1 and type 2 PrP^{Sc}. sCJD can be further classified into five subtypes based on a combination of molecular, clinical and pathological features

Table 34.2 Classification of PrP^{Sc} types in human prion diseases

PrP ^{Sc} type ^a	N-terminus, amino acid	C-terminus, amino acid	Nonglycosylated PrP ^{Sc} , kilodalton
Type 1	~82	231	~21
Type 2	~97	231	~19
GSS-type	74–90	147–153	7–8
VPSPr	~93	NA	~7, 17, 20

^aAfter limited proteolytic digestion with proteinase K

including 1) the genotype at codon 129 of *PRNP* (Methionine or Valine) and 2) the type of protease resistant PrP^{Sc} fragments (1 or 2). These subtypes are designated sCJMM1/sCJDMV1, sCJMM2, sCJDVV1, sCJDVV2, and sCJDMV2. These five subtypes of sCJD are associated with distinct clinical and/or pathological characteristics (Parchi et al. 1999; Gambetti et al. 2003). In addition to clinical symptoms, periodic sharp wave (PSW) complexes on electroencephalogram (EEG), MRI (hyperintense signal), and the presence of 14-3-3 protein in cerebral spinal fluid (CSF) are useful diagnostic markers for CJD.

The sCJMM1/sCJDMV1 subtype displays either MM or MV at PrP codon 129 and type 1 PrP^{Sc}. It is the most common subtype and accounts for 60–70 % of all sporadic human prion disease. This sCJD subtype has a mean age at clinical onset of 65 years of age and the mean duration of clinical symptoms prior to death is approximately 4 months. The symptoms at clinical presentation can include cognitive impairment, widened gait or ataxia, behavioral signs (including depression, anxiety, psychosis), and vision defects. Myoclonus and pyramidal signs develop at later stages of clinical disease. PSW complexes on EEG are present in the majority (~80 %) of subjects within 3 months of clinical onset. Protein 14-3-3 in CSF is found in 95 % of cases. Therefore, concurrent PSW and 14-3-3 positive CSF are often used as a diagnostic marker for sCJD.

The sCJDVV2 subtype has VV at PrP codon 129 and type 2 PrP^{Sc}. It is the second most common form of sCJD and accounts for ~16 % of all sporadic human prion disease. The mean age at onset is 60 years and the mean duration of the clinical phase is 6 months. Ataxia is the most common presenting symptom for this subtype. At later stages, dementia almost always develops and is accompanied by myoclonus and pyramidal signs. PSW on EEG is rare and CSF 14-3-3 is positive in ~80 % of cases.

The sCJDMV2 subtype has MV at PrP codon 129 and type 2 PrP^{Sc}, and it represents ~9 % of the sporadic cases. This subtype has a phenotype very similar to that of sCJDVV2 except for a longer clinical phase before death (mean of 17 months), greater involvement of cognitive and mental symptoms at earlier stages, and frequent aphasia and apraxia at later stages.

The sCJMM2 subtype has MM at PrP codon 129 and type 2 PrP^{Sc}, and it accounts for 2–8 % of the sporadic cases. The mean age at onset is 65 years and the mean clinical duration is 16 months. Cognitive impairment is the universal presenting symptom and is sometimes accompanied by aphasia. At later stages, myoclonus and pyramidal signs, and sometimes Parkinsonism, apraxia, and seizures, develop. There is no PSW on EEG while CSF 14-3-3 is positive in most cases.

The sCJDVV1 subtype has VV at PrP codon 129 and type 1 PrP^{Sc}, and it accounts for 1 % of the sporadic cases. The mean age at onset is 39 years and the mean clinical duration is

15 months. This younger age of clinical onset is unusual for a sporadic human prion disease and more closely resembles the age of onset that is characteristic in vCJD. This subtype shows dementia at the early clinical stages that is followed by myoclonus and pyramidal signs. There is no PSW on EEG and the CSF 14-3-3 is positive in all cases tested.

In sporadic fatal insomnia (sFI) there is MM at PrP codon 129 and type 2 PrP^{Sc}. sFI accounts for ~2 % of the sporadic cases and the phenotype is similar to that of inheritable FFI, which is characterized by insomnia, but visual signs, cognitive impairment, and motor signs are also common. The mean age at onset is 50 years, but has been observed as early as 13 years, with a mean clinical duration of 24 months.

In variably protease sensitive prionopathy (VPSPr) (Gambetti et al. 2008, Zou et al. 2010, 2013; Head et al. 2013) the PrP^{Sc} molecular properties and clinical symptoms are similar among patients with polymorphisms at codon 129 of *PRNP*, but there are some distinctions. In 129VV subjects, the mean age at onset was 72 years and mean clinical duration was 45 months; the symptoms included psychiatric signs, behavior and mood changes, and often language deficits and cognitive impairment. In 129MV subjects, the mean age at onset was 65 years and mean clinical duration was 23 months; the symptoms included psychiatric signs, Parkinsonism, and often ataxia and myoclonus. In the two 129MM subjects, the age of onset was 64 and 78 and clinical duration was 41 and 50 months; the symptoms included Parkinsonism, ataxia, progressive diffuse cognitive impairment and myoclonus.

34.3.2 Familial Human Prion Diseases

The familial prion diseases are inherited in an autosomal dominant manner and account for 10–15 % of all human prion disease. They include familial CJD (fCJD), GSS, and fatal familial insomnia (FFI) (Gambetti et al. 2003; Kong et al. 2003). The familial prion diseases are caused by pathogenic mutations in the *PRNP* coding sequence. To date, these include 24 mis-sense mutations that result in amino acid changes, 2 nonsense mutations that result in premature stop codons, and about 30 insertion or deletions that lead to changes in the octapeptide repeat number (Fig. 34.1b). There are 16 polymorphic sites in *PRNP* (Fig. 34.1b). The most important polymorphism is at codon 129, which affects the clinical symptoms in *cis* to the mutant allele and modifies the age of onset and disease duration in *trans* to the mutant allele (Kong et al. 2003).

There are 17 mutations in *PRNP* that have been linked to fCJD: G114V, R148H, D178N-129V, V180I, T183A, T188A/K/R, E196K, E200K, V203I, H208R, V210I, E211Q, Y226stop, M232R and P238S (Fig. 34.1b). In fCJD the disease phenotype is often similar to that found in sCJD, but there can be heterogeneity between individuals carrying dif-

ferent or even the same *PRNP* mutation. This is illustrated in the most common fCJD cases, which is caused by an E200K amino acid substitution in the prion protein. Some individuals with the E200K genotype have a methionine at codon 129 on the mutant allele (i.e. fCJD^{E200K-129M}) and a type 1 PrP^{Sc}, while others with the same E200K mutation have a valine at codon 129 (i.e., fCJD^{E200K-129V}) and a type 2 PrP^{Sc}. In fCJD^{E200K-129M}, the mean age at onset is 58 years and the average clinical duration is 6 months. Symptoms at clinical onset include cognitive and mental impairment that is often accompanied by cerebellar signs and sometimes by visual symptoms and myoclonus. Later in the disease course, seizures and motor and sensory peripheral neuropathy can also be present. These later signs are rare in sCJD. There is PSW on EEG and CSF 14-3-3 is also positive in the majority of affected subjects. In contrast, fCJD^{E200K-129V} individuals have a phenotype similar to that of sCJDVV2, which presents primarily as ataxia followed by myoclonus with PSW on EEG at late stages. This phenotypic difference between fCJD^{E200K-129V} and fCJD^{E200K-129M} illustrates the dramatic influence of the codon 129 polymorphisms on disease phenotype and PrP^{Sc} type. Other common fCJD mutations include D178N-129V and V210I-129M or insertions of one to four octapeptide repeats.

GSS is an inheritable prion disease characterized by PrP^{Sc} deposits in the form of amyloid plaques in the cerebral cortex, degeneration of pyramidal tracts, and a long clinical duration. In GSS there is a highly variable age at onset (between 20 and 73 years) and duration of the clinical phase (mean of 5 years, but range between 5 months and 21 years). The symptoms usually include a slowly progressive cerebellar syndrome, pyramidal signs, and cognitive decline that often develop into dementia. GSS could be mistaken for other common neurodegenerative diseases due to the similarity in clinical signs. These include olivopontocerebellar atrophy, spinocerebellar ataxia, Parkinson disease, amyotrophic lateral sclerosis, Huntington's disease, and Alzheimer disease (Kong et al. 2003). Mutations in *PRNP* that have been linked to GSS include P102L, P105L/T/S, A117V, G131V, A133V, Y145stop, H187R, F198S, D202N, Q212P, Q217R, Q227stop and M232T.

PrP^{P102L-129M} is the most common GSS genotype and is the first reported *PRNP* mutation linked to human prion disease. It has been reported in at least 28 families around the world and it may have occurred independently more than a single time since it is detected in families of different ethnicity. In GSS^{P102L-129M}, a protease-resistant PrP^{Sc} polypeptide of 7–8 kilodaltons is the major component of amyloid plaques in the brain. The age at onset is between 30 and 63 years with clinical duration of 1–10 years. However, some subjects have a CJD-like phenotype accompanied by a short clinical duration of 5–9 months. The initial symptoms include progressive cerebellar signs with ataxia, dysarthria, incoordination of saccadic movements, and occasional pyramidal and pseudobulbar signs. Dementia and akinetic mutism develop at

late stages of the disease. Polymorphic variants of the PrP^{P102L-129M} allele include PrP^{P102L-129V} and PrP^{P102L-129M-219K} and these have distinct disease phenotypes.

Fatal familial insomnia is the second most common inherited prion disease and is associated with a D178N-129M mutant allele (Kong et al. 2003; Montagna et al. 2003). The average age at onset is 49 years but can vary between 20 and 72 years. The duration of the clinical phase also is highly variable with an average of 11 months for 129MM subjects and 23 months for 129MV subjects. Severe insomnia is the most prominent symptom and is likely due to prion targeting to the thalamus, which is reflected in abnormal brain activities in this region on positron emission tomography (PET) and single-photon emission computed tomography (SPECT) scans. Other symptoms can include episodes of hallucinations and confusion, myoclonus, spasticity, and seizures. EEG is usually slowed and PSW complexes are often present in patients with long duration. There is a characteristic shortened sleep time and irregular transition between sleep stages on polysomnography. The same D178N mutation is found in fCJD but these individuals have a 129V genotype on the mutant allele and, as a result, the disease phenotype is different and does not include insomnia.

In addition, two recently detected *PRNP* mutations are not associated with conventional prion diseases. Patients with a PrP Q160stop mutation present with a Alzheimer's disease phenotype that is accompanied by widespread PrP deposition in the frontal cortex (Guerreiro et al. 2014). Patients with the PrP Y163stop mutation exhibit chronic diarrhea and an autonomic neuropathy associated with widespread PrP amyloid deposition in peripheral tissues, spinal cord and cortical regions of the brain, but prion transmission to mice was not observed (Mead et al. 2013).

34.3.3 Acquired Human Prion Diseases

The acquired prion diseases account for less than 1 % of all human prion disease cases and these include variant CJD, iatrogenic CJD (iCJD), and kuru (Will 2003). In these cases, prion infection is either orally acquired or associated with accidental transmission via medical practices. Many of these latter cases involve contamination with brain tissue, which contains the highest amount of prion infectivity, from the donor host.

Variant CJD was discovered in 1996 and is most likely due to ingestion of BSE-contaminated food products, but direct proof is lacking due to the long interval between exposure and onset of disease. Removal of meat and bone meal supplements from livestock feed in the late 1980's has greatly reduced the prevalence of BSE in the United Kingdom from over 34,000 cases in 1992 to less than 1000 cases in 2005. As a result, human exposure to BSE has also been

greatly reduced. To date, there are 229 cases of vCJD primarily in the United Kingdom and France (Maheshwari et al. 2014). The annual number of vCJD cases reached a peak at 28 in 1999, but this number of cases is relatively low considering that approximately 1,000,000 BSE-infected cattle have entered the human food chain in the United Kingdom (UK) during the 1980s and 1990s. Due to the long prion incubation period, it is difficult to predict the size of the vCJD epidemic. A retrospective survey of tonsils and appendix specimens (i.e. sites of prion agent replication) removed during routine surgery in the UK revealed only three PrP^{Sc} positive samples in >18,000 cases (Hilton et al. 2004), while another study of 63,007 archived tonsil samples using two PrP^{Sc} immunoassays was equivocal (Clewley et al. 2009). However, a recent screening of 32,441 fixed appendix samples in the UK detected 16 PrP^{Sc} positive samples, predicting an overall prevalence of 1 in 2000 people with subclinical vCJD infection in the UK population (Gill et al. 2013). How many of these subclinical carriers of vCJD will develop clinical disease is uncertain.

The clinical and pathological phenotype of vCJD is distinct from most of the other human prion diseases and these unusual features were important in the identification of vCJD (Ironsides et al. 2005). Unlike the common sporadic forms of human prion disease that mostly afflict individuals in their sixth decade, vCJD is found in younger individuals with mean age of onset at 28 years, ranging between 12 and 74 years of age (Will 2003). The length of the incubation period is uncertain but is estimated to be ~11 years (Cooper and Bird 2003). The mean clinical duration is ~14 months, which is longer than the 4 months for sCJDMM1 but comparable to that of 16 months for sCJDMM2. With the exception of one vCJD case that had PrP^{129MV}, all other vCJD subjects had PrP^{129MM}. The PrP^{129MM} genotype has a prevalence of 39% in the UK population so the higher distribution in vCJD cases indicates a stronger preference for younger people with a PrP^{129MM} genotype. There could be an age-related exposure or age-dependence for vCJD but none has been demonstrated. The type 2b PrP^{Sc} is characteristic of vCJD (Table 34.2, Fig. 34.2) and PrP^{Sc} deposits are present in florid amyloid plaques in the brain. In contrast to the diverse clinical symptoms among sCJD cases, the symptoms of vCJD subjects are more uniform. The presenting symptoms include psychiatric signs and occasionally neurological symptoms (persistent pain, memory impairment). After about 6 months, signs of ataxia, cognitive impairment, and involuntary movements become apparent. Bilateral pulvinar high signal in the thalamus on MRI is prominent in the majority of cases (~78%) (Zeidler et al. 2000). There is general slowing but no PSW complexes on EEG.

Iatrogenic CJD is the second most common acquired human prion disease and these cases are the result of accidental infection due to contact with prion contaminated tis-

ues or instruments during medical procedures (Table 34.1). The mode of prion infection include surgical equipment (e.g., surgical instruments, depth electrodes), transplantation of human tissues (corneal, dura mater), intramuscular injections with growth hormone or gonadotrophin extracted from human pituitary tissues, or blood transfusion (Will 2003). The most likely source of infection is from donors with subclinical sCJD, except for the two transfusion-related cases that have been linked to blood donors who developed vCJD several years later (Ironsides 2006). The incubation period in these transfusion related cases was 5–6 years, which is shorter than primary vCJD infection in humans.

The most common cases of iCJD are in recipients of dura mater graft or human growth hormone therapy that was used to treat children with growth deficits (reviewed by Will et al. 2013 and Brown 2013). There have been 226 iCJD cases reported in the USA, UK, France, and New Zealand and 5 other countries that have been linked to growth hormone extracted from cadaveric pituitaries. The clinical and pathological features of growth hormone therapy-related iCJD are distinct from sCJD. The mean incubation period is 17 years (range between 4.5 and 42 years) and the presenting symptoms include progressive cerebellar signs that are sometimes followed by dementia at later stages. A total of 228 iCJD cases have been found in recipients of dura mater homograft, mostly in Japan, Spain, France, Germany, Italy and UK. Afflicted individuals show typical sCJD symptoms with an incubation period of 12 years (range between 1.5 and 30 years).

Kuru is an acquired human prion disease of indigenous tribes in Papua New Guinea that is linked to ritualistic endocannibalism (Alpers 1979). More than 2700 cases have been recorded since the 1950s, but this disease has gradually disappeared since the cessation of cannibalism in the late 1950s. The mean incubation period for kuru is about 12 years, but the longest on record is over 50 years (Collinge et al. 2008). The clinical duration is 6–36 months in adults and the main presenting symptom is cerebellar signs, which are often followed by severe dysarthria at late stages in the absence of dementia.

34.4 Neuropathology of Human Prion Diseases

The three principal neuropathological features in human prion diseases are neuronal loss, gliosis, and spongiform changes primarily in grey matter (Mikol 1999). There is considerable diversity among the human prion diseases with respect to the location of pathology in the brain and type of neurons affected. Pathology is not confined to a single brain structure but is widely distributed in the CNS. For each of the human prion diseases, there are typical distinguishing features of neuropathology that are partially defined by the dis-

tribution of pathology in the cerebral cortex, subcortical grey matter, and brainstem. Spongiform changes can vary in density and size and range from microvacuolation to the fusion of vacuoles to produce status spongiosis. The degree of spongiform change does not appear to correlate with neuronal loss. Amyloid plaques are also present in the brain in some of the human prion diseases, most notably kuru, vCJD and GSS. These amyloid plaques exhibit birefringence when stained with Congo red and viewed under polarized light.

PrP^{Sc} deposition in the central nervous system is also a hallmark feature of prion disease (Prusiner 1991). The type of PrP^{Sc} deposit and its location varies among the human prion diseases. PrP^{Sc} is also a component of amyloid plaques. A characteristic of vCJD and kuru is florid amyloid plaques, which is a large cluster of PrP^{Sc} aggregates interspersed with vacuolar pathology. PrP^{Sc} deposition is also present in peripheral tissues of patients with vCJD, primarily in secondary lymphoid tissues. However, no histological changes are apparent in peripheral organs of prion-infected hosts.

In prion diseases there is a notable absence of encephalitis or inflammation in the brain. The lack of adaptive immune response to PrP^{Sc} is likely due to host tolerance to PrP^C. However, there is an increase in the numbers of microglia, activation of astrocytes, and an upregulation of inflammatory cytokines, chemokines and receptors. The complement pathways is also activated as evidenced by binding of C1q and C3b components to PrP^{Sc}, and complement membrane attack complexes associated with neurons in the brain (Burwinkel et al. 2004; Kovacs et al. 2004; Mabbott 2004). The precise role of these host responses in neuroprotection and/or neurodegeneration during prion infection is not clear.

The neuropathology among sCJD cases is not uniform and is variable in nature, severity and location in the brain. In most cases spongiform change is found in the cerebral cortex, cerebellar cortex, and/or the subcortical grey matter (Ironsides et al. 2005). This is accompanied by reactive gliosis and neuronal loss but there is not a consistent relationship between spongiform change and cell loss. In approximately 10 % of sCJD, PrP^{Sc} amyloid plaques are present in the cerebral cortex but most cases have a diffuse synaptic PrP^{Sc} distribution. In contrast to the clinical and pathological diversity present in sCJD, the pathology in vCJD is more uniform. The central pathological features in vCJD are the presence of florid PrP^{Sc} plaques in the cerebral and cerebellar cortex, severe spongiform change in the caudate and putamen, and neuronal loss in the thalamus and midbrain. PrP^{Sc} is also present in affected brain regions. The neuropathology of vCJD is distinct from the other human prion diseases.

In the familial human prion diseases there are also variable clinical and neuropathological features. GSS is characterized by unicentric and multicentric PrP^{Sc} amyloid plaques that are primarily found in the cerebellum, but can also be present in the cerebral cortex (Ghetti et al. 1995). Spongiform

change and neuronal loss can be focal but some GSS patients have no spongiform changes, while in other cases with a short duration, there is widespread spongiform change. In some GSS families, severe neurofibrillary tangle degeneration is prominent. A feature of FFI is a thalamic degeneration characterized by severe spongiform change, neuronal loss, and astrocytosis in thalamic nuclei (Gambetti et al. 1995).

In VPSPr patients there is brain atrophy and diffuse slowing of cerebral electrical activity (Zou et al. 2010). Spongiform change is modest in the cerebral cortex and focal spongiform vacuoles are occasionally found in the molecular layer of the cerebellum. The neuropathological lesions are usually more severe in the 129VV and 129MV than in the 129MM cases. The pattern of PrP^{Sc} immunostaining is slightly different among the three PrP genotypes.

34.5 Peripheral Prion Replication and Neuroinvasion

The sites of prion agent replication and spread are largely dictated by whether the etiology of prion disease is sporadic, familial, or acquired. For example, in sCJD and fCJD, disease initiates in, and is largely confined to, the nervous system. In vCJD, which is acquired via oral ingestion, infection likely begins in secondary lymphoid tissues prior to neuroinvasion and spread to the CNS.

34.5.1 vCJD and Oral Routes of Animal Prion Transmission

Variant CJD is BSE infection in humans and the most probable route of infection is by ingestion of BSE-contaminated food products. The spread of the vCJD agent following oral ingestion has not been directly investigated but evidence suggests it follows a similar pathway to that described for oral ingestion in the animal prion diseases. These pathways have been well described for scrapie in sheep and in rodent models and have three phases: agent entry and replication in secondary lymphoid tissues, spread from secondary lymphoid tissues to the CNS along peripheral nerves, and dissemination and degeneration in the CNS (Fig. 34.3). Following oral prion ingestion, the prion agent is initially found in the distal ileum (Heggebo et al. 2000). Entry is likely across the intestinal epithelium via the M cells in the Peyer's patches. Prions then spread to the draining gut-associated lymphoid tissue (GALT) where prion agent is found within months after ingestion (van Keulen et al. 1999). In the GALT, the prion agent is primarily associated with follicular dendritic cells and macrophages in the germinal center of lymphoid follicles. From the GALT the prion agent spreads to other secondary lymphoid tissues including lymph nodes and the spleen over the next several

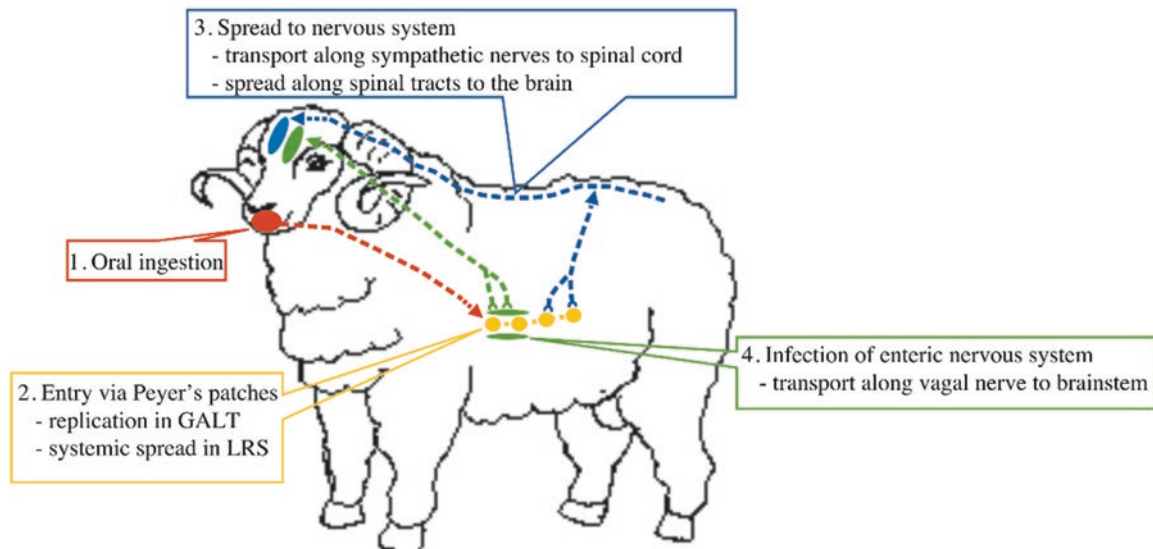


Fig. 34.3 The stages of prion agent infection and neuroinvasion following oral ingestion of scrapie in sheep

months (van Keulen et al. 2000). The mechanism of prion agent dissemination in secondary lymphoid tissues has not been determined but is thought to be via prion-infected migratory cells that travel in the lymph. A blood borne route is also possible since low levels of prion infectivity have been found in the blood of sheep with scrapie and humans with vCJD (Ironsides 2006).

In scrapie-infected sheep, spread of the prion agent from the gut to the CNS occurs via peripheral nerve fibers. From the GALT, the prion agent enters the sympathetic nerves that innervate secondary lymphoid tissues and spreads to the spinal cord via the autonomic nervous system (McBride et al. 2001). The prion agent then ascends the spinal cord along spinal tracts into the brain (Fig. 34.3) (van Keulen et al. 2000). In a second pathway, after prion infection of the GALT, there is a delay before agent replication is found in the enteric nervous system. From these sites, the prion agent enters the vagal nerve and spreads to the brainstem where initial agent deposition is found in the dorsal motor nucleus of the vagus (van Keulen et al. 2000; Beekes et al. 1998). Early PrP^{Sc} deposition in this nucleus is indicative of neuroinvasion following oral ingestion. In vCJD, spread from secondary lymphoid tissues to the CNS is likely to take months to years.

After prions spread to the central nervous system they can spread away from the CNS along nerve fibers to additional peripheral sites. Recent advances in prion detection have led to the discovery that vCJD infection is found at low levels in some endocrine and reproductive tissues, muscle, kidney, and skin as well as in blood and urine (Peden et al. 2006; Notari et al. 2010; Moda et al. 2014). The ability to detect PrP^{Sc} in blood has resulted in more effective screening of patients with undiagnosed neurological diseases and patients at high risk for prion disease.

Evidence for oral ingestion in vCJD is provided by the peripheral PrP^{Sc} distribution. In humans with vCJD, PrP^{Sc} is present in the ileum and the sympathetic ganglia that provide autonomic innervation to the gut (Hilton 2006). Variant CJD infection is also present in FDCs in secondary lymphoid tissues including the Peyer's patches, tonsil, spleen, lymph nodes, and appendix. This distribution of the prion agent suggests that agent entry via oral ingestion results in the spread of vCJD infection to distal secondary lymphoid tissues (Hilton 2006). This peripheral distribution of the prion agent in lymphoid tissue and autonomic nervous system of the gut is not found in sCJD or the familial human prion diseases suggesting that vCJD is acquired by peripheral exposure.

34.6 CNS Pathogenesis of Sporadic, Familial, and Iatrogenic Prion Diseases

34.6.1 Sporadic CJD

In sCJD the process or events that lead to PrP^{Sc} formation and accumulation are thought to be limited to the CNS. Until recently PrP^{Sc} has not been found in the spleen, lymph nodes, tonsil or appendix in patients with sCJD (Head et al. 2004), and the apparent absence of prion infection in secondary lymphoid tissues has been cited as evidence against acquisition of prion infection from peripheral exposure. With improved detection methods PrP^{Sc} has now been found in secondary lymphoid tissue in sCJD (Rubenstein et al. 2013). However, there is an absence of infection in the sympathetic ganglia that provide autonomic innervation to the gut, indicating that it is unlikely that there is infection of the enteric nervous sys-

tem or acquisition of sCJD following ingestion of an unrecognized source of prion contamination. Prion infection also is detected in the peripheral nervous system of patients with sCJD. PrP^{Sc} deposition is found in retina, trigeminal ganglion, the olfactory nerves and epithelium, dorsal root ganglia and an occasional peripheral nerve (Head et al. 2004). This distribution is consistent with spread of the prion agent away from the CNS along cranial or spinal nerve fibers. PrP^{Sc} has also been found in skeletal muscle of patients with sCJD (Glatzel et al. 2003).

34.6.2 Familial Prion Disease

In familial prion diseases germline mutations in the prion protein gene leads to PrP^{Sc} deposition in the brain and nervous system. Prion agent has not been found in peripheral tissues in the limited number of familial prion disease cases that have been examined.

34.6.3 Iatrogenic CJD

Iatrogenic CJD can be acquired by either peripheral or CNS exposure to the prion agent from donor tissues or contaminated surgical equipment. The length of time to development of prion disease appears to be related to the site of prion inoculation. Shorter incubation periods are typically found following direct prion exposure to the brain (e.g., contact with stereotactic electrodes or instruments during neurosurgery) while longer incubation periods are seen following peripheral exposure (e.g., intramuscular injection of growth hormone) to the prion agent. In the latter cases, the incubation period can be up to several decades in duration (Will 2003). Due to the low number of iCJD cases, there are few studies examining the peripheral distribution of prion agent. One study reported the presence of PrP^{Sc} in the dorsal root ganglia and a few peripheral nerve fibers of two patients with dura mater related iCJD, while another study found PrP^{Sc} in small nerve fibers in muscle (Ishida et al. 2005). The pathway of neuroinvasion in iCJD associated with growth hormone therapy is less clear and prion infection was not found in secondary lymphoid tissues of a single patient, but the trigeminal ganglion and dorsal root ganglion were found to be sites of prion infection (Head et al. 2004). This limited amount of data would suggest that the prion agent is restricted to the CNS or PNS in iCJD.

34.7 Prion Agent Interaction with the Immune System

The immune system plays a central role in the pathogenesis of prion infection. In the acquired human prion diseases, it is the initial site of agent replication prior to entry into the ner-

vous system but the immune system does not appear to be essential in the pathogenesis of the familial and sporadic human prion diseases. Following peripheral exposure to the prion agent, the immune system can amplify prion infection but, interestingly, the adaptive immune response does not play a role in the clearance of the prion agent.

34.7.1 Prion Infection of Secondary Lymphoid Tissues

In the 1960s it was first described that the spleen is an early site of prion agent replication following peripheral scrapie infection of mice (Eklund and Hadlow 1969). Prion replication in the spleen is present shortly after peripheral exposure and reaches a plateau level prior to detection of prion agent in the brain. Mice that receive immunosuppressive therapy or splenectomy prior to peripheral inoculation experience a delay in prion replication in the periphery and spread to the CNS (Kimberlin and Walker 1989). Mice that are deficient in B cells also are resistant to peripheral prion infection, but B cells do not appear to be a target for prion infection (Mabbott and Turner 2005; Aguzzi and Heikenwalder 2005). One site of PrP^{Sc} deposition in the spleen and lymph nodes is in follicular dendritic cells (FDCs), and PrP^C expression on FDCs is required for prion infection of these cells (Mabbott and Turner 2005; Aguzzi and Heikenwalder 2005). FDCs are located in the germinal center of secondary lymphoid tissues and play a role in the presentation of antigen to B cells. B cells are important in FDC maturation and they secrete lymphotoxins (LT) that bind to LT receptors on FDCs. In the absence of B cells or LT, FDCs do not mature and are not susceptible to prion infection.

Several lines of evidence indicate that FDCs are important in prion agent replication in secondary lymphoid tissue (Mabbott and Turner 2005; Aguzzi and Heikenwalder 2005). Temporary de-differentiation of FDCs by injection of antibodies to LT receptors causes a loss of mature FDCs, a disorganization of the cytoarchitecture of the germinal center, and a delay or complete block in peripheral scrapie replication and neuroinvasion. A permanent loss of mature FDCs and disorganization of lymphoid structure is also found in null mice that lack LT or LT receptor genes. When these mice are peripherally challenged with the prion agent, they also demonstrate a greatly reduced capacity for agent replication and, in some cases, a complete block in peripheral prion pathogenesis. These studies indicate that FDCs are important sites of prion agent replication in lymphoid tissues and provide a critical step in the early pathogenesis of prion diseases.

34.7.2 Trafficking of the Prion Agent to Secondary Lymphoid Tissues

The complement system plays a role in the transport of the prion agent from peripheral sites of entry to FDCs in second-

ary lymphoid tissue (Mabbott 2004). Mice that lack the C3 complement receptor have a delay in prion replication in the spleen and in the onset of neurological disease. Complement component C3 plays a pivotal role in activation of both the classical and alternative complement pathways. Mice that lack the C1qa component or factor B and C2 complement components are deficient in the classical and alternative complement pathways, respectively, and these mice have a delay in prion agent replication in the spleen. As a result, they survive longer than wild type mice following intraperitoneal prion infection. If low doses of the prion agent are inoculated into the periphery of these complement-deficient mice, they do not develop prion disease while wild type mice remain susceptible to prion disease following peripheral infection. Mice that lack the C1qa component are the most resistant to peripheral prion challenge and binding of C1q to PrP^{Sc} can activate the classical complement pathway, and result in the targeting of PrP^{Sc}-complement complexes to CD21/35 complement receptors on FDCs (Mitchell et al. 2007; Zabel et al. 2007).

34.7.3 Trafficking of the Prion Agent to the Central Nervous System

The transfer of the prion agent from FDCs to peripheral nerves in the secondary lymphoid tissues is necessary for neuroinvasion. The FDCs are physically separated from the sympathetic nerve fibers but in chemokine receptor CXCR5 null mice there is a reorganization of the germinal center and the sympathetic nerves are now in close proximity to FDCs. Peripheral prion infection in these mice resulted in faster neuroinvasion into the spinal cord indicating that there is accelerated transfer of prion agent from the FDCs to the peripheral nervous system (Prinz et al. 2003). In transgenic mice that overexpress nerve growth factor, there is hyperinnervation of the sympathetic nervous system; these mice have a faster onset of prion agent replication in the spinal cord after peripheral challenge even though the onset of replication in the spleen is similar to wild type mice (Glatzel et al. 2001). Inhibition of sympathetic innervation in newborn mice with antibodies to nerve growth factor or chemical sympathectomy causes a delay in prion agent replication in the CNS (Glatzel et al. 2001). The transfer of the prion agent from FDCs to sympathetic nerves is crucial for prion neuroinvasion into the CNS.

34.7.4 Role of Inflammation in the Peripheral Distribution and Transmission of the Prion Agent

In a healthy host peripheral prion infection primarily involves infection of secondary lymphoid tissues such as the lymph

nodes and spleen. In mice with chronic inflammation in a peripheral organ that normally does not contain secondary lymphoid tissue, prion agent replication can also be found. In rodent models of nephritis, pancreatitis and hepatitis in which there is upregulation of lymphotoxins in the kidney, pancreas or liver, respectively, lymphoid tissue containing FDCs can develop in the inflamed organ. Following peripheral inoculation, prion infection can be established in these organs since transient secondary lymphoid tissues are present (Heikenwalder et al. 2005). In one model of prion infection in the kidney of mice with glomerulonephritis, prions could also be found in urine (Seeger et al. 2005). These findings indicate that chronic inflammation can lead to an altered organ tropism of the prion agent and induce excretion of the prion agent from a secretory organ.

In sheep with mastitis, a chronic inflammation of the mammary gland, and concurrent scrapie, prion infection is present in FDCs and macrophages in lymphoid follicles that develop adjacent to mammary ducts (Ligios et al. 2005). Secretion of the scrapie-infected macrophages in colostrum of sheep with mastitis could potentially enhance vertical transmission from ewe to lambs since milk is a natural source of scrapie infectivity (Konold et al. 2008; Lacroux et al. 2008).

34.8 A Broader Definition of Prions That Encompasses Neurodegenerative Diseases of Protein Misfolding

There is an emerging recognition that key events related to the misfolding of normal proteins in prion diseases are also common to many late-onset, human neurodegenerative diseases. As described above, a central feature of prion disease involves the misfolding of the monomeric cellular prion protein to form PrP^{Sc} polymers and, in some cases, PrP^{Sc} plaques. In related neurodegenerative diseases, normal host proteins that misfold and accumulate into altered pathogenic conformations include A β in Alzheimer's disease, α -synuclein in Parkinson's disease, tau in the tauopathies, superoxide dismutase-1 in amyotrophic lateral sclerosis, and likely nuclear RNA binding proteins in amyotrophic lateral sclerosis (reviewed in Prusiner 2013). In all these diseases the general mechanism of protein misfolding is mediated by the pathogenic protein polymer, which can serve as a template to further direct misfolding of the normal host protein. These prion-like human diseases are also similar to prion diseases in that they have genetic (10–20% of cases) and sporadic (80–90% of cases) etiologies, but epidemiological evidence suggests that they are not transmissible diseases. Interestingly, in transgenic mouse models of Alzheimer's disease and tauopathy, experimental inoculation of diseased human brain can accelerate protein misfolding of A β and tau, respectively,

and lead to earlier onset neurodegeneration (Meyer-Luehmann et al. 2006; Clavaguera et al. 2009). As these pathogenic proteins misfold within the CNS they follow defined neuroanatomical circuits and spread across neural synapses, which is also a common feature of PrP^{Sc} dissemination in the nervous system. In the future our understanding of prion diseases and neurodegenerative diseases with prion-like features may include common pathogenic events that initiate protein misfolding and induction of neurological damage.

34.9 Review Questions

1. What is a prion disease?
2. What is the “Protein-only hypothesis”?
3. What are the major types of human prion diseases based on clinical phenotypes?
4. What are the major PrP^{Sc} types in human prion diseases?
5. After peripheral infection, prion neuroinvasion is mediated through what cell type?
6. What is the role of B cells in prion neuroinvasion?
7. What are protein misfolding diseases?

34.10 Answers

1. Prion disease is a family of diverse transmissible neurodegenerative diseases that requires the prion protein for prion agent replication and pathogenesis.
2. The “Protein-only Hypothesis” proposes that the infectious prion agent is a misfolded form of PrP^C devoid of specific nucleic acids (called PrP^{Sc} where “Sc” refers to scrapie) that is capable of self-perpetrating replication using PrP^C as the substrate.
3. The major types of human prion diseases based on clinical phenotypes include CJD, GSS, Kuru, Fatal Insomnia, and VPSPr.
4. The major PrP^{Sc} types in human prion diseases include type 1, type 2 (a and b), the GSS type, and the VPSPr type.
5. Prion neuroinvasion is mediated by FDCs.
6. B cells are required for prion neuroinvasion because they generate lymphotoxins that are required for the maturation of FDCs.
7. Diseases characterized by a post-translational change in the conformation of a normal host protein which results in an altered protein structure and protein polymerization. The protein polymer accumulates in the CNS and acts as a template for protein mediated misfolding of additional host precursor protein.

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Abstract

Glaucoma is defined as a disturbance of the structural or functional integrity of the optic nerve that causes characteristic changes in the optic nerve leading to permanent defects in the visual field. If left untreated, it can lead to blindness. Glaucoma is the leading cause of irreversible blindness throughout the world, and is second only to macular degeneration in the US. Amongst the risk factors, elevated intraocular pressure (IOP), thin corneal thickness (CCT), increasing age, and large cup to disc ratio are established. Concerning its pathogenesis, mechanical and vascular theories are fundamental. In addition, many other mechanisms contribute; such as immune factors, apoptosis, glutamate-induced excitotoxicity, free radicals, nitric acid synthase, and genetic mutations. To assess glaucoma; tonometry, gonioscopy, examination of optic disc, and visual field testing are essential. Animal models for glaucoma have been used to study the pathophysiological basis of glaucoma, aqueous humor dynamics, the trabecular meshwork, the ciliary muscle, and retinal ganglion cells. Animal experiments have provided a worthwhile means of evaluating diagnostic modalities and treatments, both medical and surgical.

Keywords

Glaucoma • Intraocular pressure • Optic nerve • Retinal ganglion cell

35.1 Introduction

The word glaucoma has its origin in ancient Greek (Hippocrates, approximately 400 BC), meaning clouded or blue-green hue, most likely describing a patient having a cloudy cornea or advanced cataract precipitated by chronic elevated pressure. Over the last 100 years, further understanding of the concept of glaucoma has accelerated.

Primary open-angle glaucoma (POAG) is a multifactorial chronic optic neuropathy with a characteristic acquired loss of optic nerve fibers. Cupping and atrophy of the optic disc occur in the absence of other known causes. Such damage develops in the presence of open anterior chamber angles and produces characteristic visual field abnormalities. Some

subsets of patients can exhibit the characteristic optic nerve damage and visual field defects while having an intraocular pressure (IOP) within the normal range (normal tension glaucoma). IOP is the only risk factor that can be currently modified in POAG. Elevated IOP in the absence of any clinically apparent optic nerve damage is referred to as ocular hypertension (OHT).

A neurodegenerative disease has loss of specific neuronal populations with axonal loss and a characteristic clinical syndrome which is progressive in nature. Glaucoma is a degenerative disorder of the retinal ganglion cells (RGC) leading to characteristic optic nerve morphology and visual field defects. This loss of axons continues in the lateral geniculate nucleus (Chen et al. 2013; Gupta et al. 2009) and in the visual cortex (Yu et al. 2013; Yucel and Gupta 2008). Untreated glaucoma progresses due to continued loss of RGC and visual field constriction.

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Other forms of glaucoma include angle closure glaucoma, in which the outflow of aqueous humor is physically obstructed by the iris. Secondary glaucomas have obstruction of the aqueous humor pathways secondary to another disease process such as inflammation, trauma, bleeding, pigment, pseudoexfoliation and others. The only glaucoma that fits the definition for a neurodegenerative disorder is POAG and the rest of the chapter will primarily refer to POAG.

POAG is a major worldwide health concern because of its usually asymptomatic but progressive nature, and because it is the leading cause of irreversible blindness in the world. With appropriate screening and treatment, glaucoma usually can be identified and treated to prevent and slow significant visual loss.

35.2 Epidemiology

35.2.1 Prevalence

In the United States (US), multiple population studies [e.g., Framingham (Leibowitz et al. 1980), Baltimore (Tielsch et al. 1991), and Barbados (Leske et al. 1997)] have been performed to estimate the prevalence of eye diseases, including POAG and OHT.

Estimates of the prevalence of glaucoma [(Friedman et al. 2004a); Table 35.1] and blindness [(Congdon et al. 2004); Fig. 35.1] in the US demonstrate the following: glaucoma is a leading cause of irreversible blindness, second only to macular degeneration; only one half of the people who have glaucoma may be aware that they have the disease; and more than 2.25 million Americans aged 40 years and older have POAG.

More than 1.6 million people have significant visual impairment from glaucoma, with 150,000 bilaterally blind in the US alone (Tielsch et al. 1991). These statistics emphasize the need to identify and closely monitor those at risk for glaucomatous damage.

Approximately 6.7 million people are bilaterally blind from POAG worldwide, and more than 2 million people will develop POAG each year (Quigley 1996).

35.2.2 Prognosis

Several studies (Gordon et al. 2002) have shown the incidence of new onset of glaucomatous damage in previously unaffected patients to be approximately 2.6–3 % in patients with IOPs 21–25 mm Hg, 12–26 % with IOPs 26–30 mm Hg, and approximately 42 % with IOP higher than 30 mm Hg. The ocular hypertension treatment study (OHTS) found that

Table 35.1 Prevalence of glaucoma^a by age, gender, and race

Age, years	Prevalence/100 population (95 % CI)		
	White subjects	Black subjects	Hispanic subjects
Women			
40–49	0.83 (0.65–1.06)	1.51 (0.94–2.41)	0.34 (0.15–0.72)
50–54	0.89 (0.78–1.02)	2.24 (1.59–3.14)	0.65 (0.37–1.15)
55–59	1.02 (0.89–1.16)	2.86 (2.16–3.78)	0.98 (0.61–1.58)
60–64	1.23 (1.07–1.41)	3.65 (2.83–4.69)	1.49 (0.97–2.28)
65–69	1.58 (1.37–1.82)	4.64 (3.54–6.05)	2.24 (1.43–3.49)
70–74	2.16 (1.87–2.49)	5.89 (4.28–8.05)	3.36 (2.00–5.60)
75–79	3.12 (2.68–3.63)	7.45 (5.06–10.84)	5.01 (2.68–9.15)
≥80	6.94 (5.40–8.88)	9.82 (6.08–15.48)	10.05 (4.35–21.52)
Men			
40–49	0.36 (0.27–0.47)	0.55 (0.31–0.95)	0.39 (0.18–0.85)
50–54	0.61 (0.50–0.74)	1.71 (1.25–2.32)	0.69 (0.39–1.25)
55–59	0.85 (0.72–1.00)	3.06 (2.30–4.04)	1.00 (0.61–1.64)
60–64	1.18 (1.02–1.37)	4.94 (3.69–6.59)	1.44 (0.92–2.24)
65–69	1.64 (1.40–1.91)	7.24 (5.40–9.63)	2.07 (1.32–3.23)
70–74	2.27 (1.90–2.72)	9.62 (7.29–12.59)	2.97 (1.79–4.89)
75–79	3.14 (2.53–3.90)	11.65 (8.81–15.25)	4.23 (2.32–7.60)
≥80	5.58 (4.15–7.47)	13.21 (7.85–21.38)	7.91 (3.53–16.77)

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CI confidence interval

^aGlaucoma indicates primary open-angle glaucoma

Fig. 35.1 Causes of blindness (best-corrected visual acuity <6/60 [$<20/200$] in the better-seeing eye) by race/ethnicity. AMD indicates age-related macular degeneration; DR, diabetic retinopathy. Reproduced with permission from: Congdon N, O'Colmain B, Klaver CC, Klein R, Munoz B, Friedman DS, Kempen J, Taylor HR, Mitchell P, Eye Diseases Prevalence Research Group (2004) Causes and prevalence of visual impairment among adults in the United States. Arch Ophthalmol 122:477–485

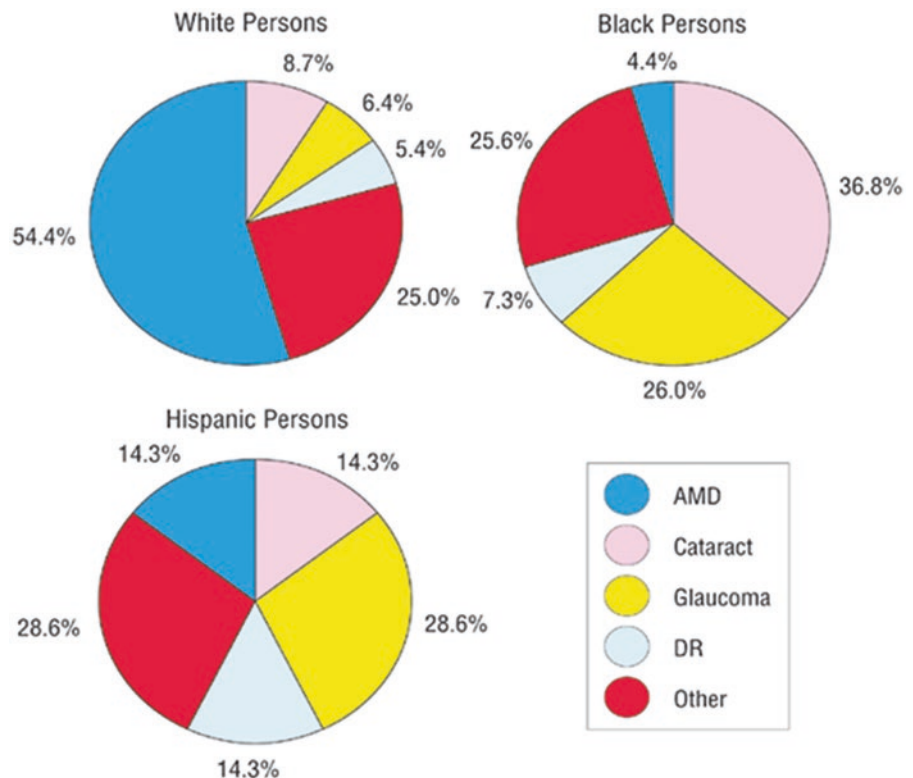


Table 35.2 Risk factors for glaucoma

Established	Highly probable	Possible
Elevated IOP	Race (African descent ^a)	Presence (or absence) of diabetes
Thin CCT	Positive family history	Hypertension
Increasing age	Low diastolic perfusion pressure	
Large cup to disc	Migraines	
Ratio	IOP fluctuations	
	Sleep apnea	
	Immunological disorders	
	Myopia	

IOP intraocular pressure, CCT central corneal thickness

^aIn US, West Africa, Caribbean

approximately 10 % of patients with IOPs ranging from 24 to 31 mm Hg but with no clinical signs of glaucoma at baseline develop glaucoma over 5 years, with conversion to glaucoma reduced by 50 % if patients are preemptively started on IOP-lowering therapy (Kass et al. 2002). Significant subsets of higher and lower risk exist when central corneal thickness (CCT) is taken into account.

35.2.3 Risk Factors

35.2.3.1 Intraocular Pressure

In eyes suspected of being affected with glaucoma, loss of visual field develops at a rate of approximately 1–3 % per year of observation (Gordon et al. 2002). High IOP is the most important risk factor (Table 35.2). The relative impor-

tance of other risk factors also is indicated in Table 35.2. In population-based surveys, between 25 and 50 % of those with glaucomatous damage to the optic nerve have IOP in the normal range. However, the probability of injury increases exponentially with higher IOP (Heijl et al. 2002). Large diurnal fluctuation in IOP is another independent risk factor for glaucoma (Nouri-Mahdavi et al. 2004).

35.2.3.2 Race

Prevalence of POAG is 3–4 times higher in blacks than in whites in the US (Friedman et al. 2004b; Tielsch et al. 1991). Glaucoma is the most common cause of irreversible blindness in the US among people of African or Hispanic descent [(Congdon et al. 2004); Fig. 35.1]. Blacks in the US are more likely to develop glaucoma early in life, and they tend to have a more aggressive form of the disease.

The Barbados Eye Study (Leske et al. 1997) over 4 years showed a 5 times higher incidence of developing glaucoma in a group of black ocular hypertensives as compared with a predominantly white population. Some population studies have found the mean IOP in blacks to be higher than Caucasian controls. Other studies (Tielsch et al. 1991) found no difference. Consequently, further studies are required to clarify this issue.

The OHTS (Brandt et al. 2001) demonstrated that black patients in the US overall may have reduced CCT, thereby leading to underestimation of true IOP. The reduced CCT was associated with a higher risk of developing glaucoma. Therefore, CCT measurement is particularly important in African-American patients who are glaucoma suspects. The OHTS also demonstrated a larger cup to disc ratio in Blacks, which was an independent risk factor for the development of glaucoma [(Feuer et al. 2002; Gordon et al. 2002); Table 35.2]. These two confounding variables discovered in the multivariate analysis appeared to account for the apparent increased risk in blacks demonstrated in the univariate analysis (Gordon et al. 2002).

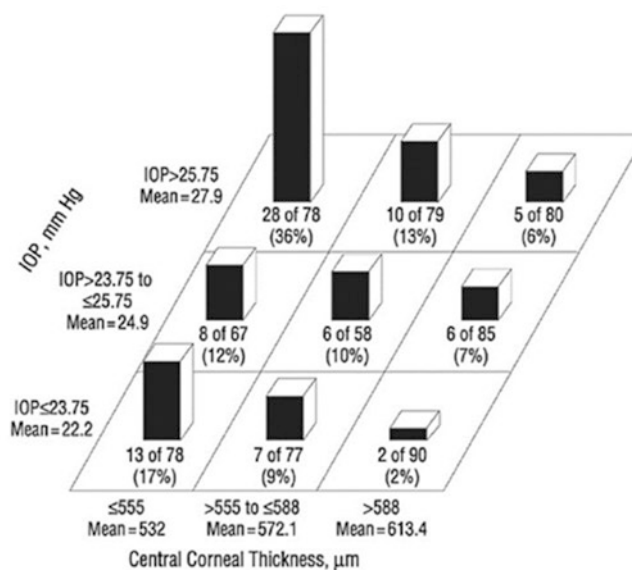
35.2.3.3 Age

Age older than 40 years is a risk factor for the development of POAG. Up to 15 % of African-American men are affected

by the ninth decade of life [(Friedman et al. 2004a); Table 35.1]. Consequently, glaucoma is found to be more prevalent in the aging population, even after compensating for the mean rise in IOP with increasing age in the US. However, the disease itself is not limited to only middle-aged and elderly individuals.

35.2.3.4 Central Corneal Thickness

CCT is an important risk factor for the development of glaucoma [(Gordon et al. 2002); Fig. 35.2]. CCT likely influences the measurement of IOP with many tonometers, including applanation techniques (Brandt et al. 2001). Increased CCT beyond the mean of 545 μm causes overestimation of IOP; lower CCT translates into underestimation of the IOP. A thin cornea (for example, 480 μm) may occur with glaucomatous visual field loss despite 'normal' applanation IOP because the measurements are fallaciously low. Conversely, a thick cornea (for example, 620 μm) might occur in an eye with high IOP, normal visual fields and a normal optic nerve because a thicker cornea results in false overestimation of true IOP. Ehlers et al (Ehlers et al. 1975) extrapolated that applanation tonometry is overestimated or underestimated by approximately 5 mm Hg for every 70 μm difference in measured CCT from mean thickness. It is also possible that CCT may itself constitute an intrinsic risk (or



IOP= Intraocular pressure

Fig. 35.2 Risk for developing primary open glaucoma varies with central corneal thickness. The numbers and percentage of participants in the observation group who developed primary open-angle glaucoma (median follow-up, 72 months) are indicated below each bar. Participants are grouped by baseline intraocular pressure of ≤ 23.75 mm Hg, > 23.75 mm Hg to ≤ 25.75 mm Hg, and > 25.75 mm Hg and by central corneal thickness measurements of ≤ 555 μm , > 555 μm to

≤ 588 μm , and > 588 μm . These percentages are not adjusted for length of follow-up. Reproduced with permission from: Gordon MO, Beiser JA, Brandt JD, Heuer DK, Higginbotham EJ, Johnson CA, Keltner JL, Miller JP, Parrish RK 2nd, Wilson MR, Kass MA (2002) The Ocular Hypertension Treatment Study: baseline factors that predict the onset of primary open-angle glaucoma. Arch Ophthalmol 120: 714–720

protective) factor for glaucomatous optic nerve damage or a marker of changes in aqueous humor dynamics (Gulati et al. 2011), independent of its ability to affect the IOP measurement.

35.2.3.5 Family History

Relatives of those with glaucoma are more likely to have the disease (Drance et al. 1981). However, there is no clear Mendelian pattern of inheritance for glaucoma. The Rotterdam Eye Study (Wolfs et al. 2000) concluded that the prevalence of glaucoma was 10.4% in siblings of patients with glaucoma and the relative risk of having POAG increased 9.2-fold for individuals with a first-degree relative with POAG. High IOP may be the inherited feature of glaucoma, or inherited risk factors independent of IOP may be involved. The genetics of glaucoma will be discussed further in section 3.

35.2.3.6 Systemic Risk factors

The association between diabetes mellitus (DM) and glaucoma has been unclear. Epidemiology studies in different populations have given conflicting results. The Blue Mountain Eye Study in Australia (Mitchell et al. 1997), The Rotterdam Study (Dielemans et al. 1996) and the Beaver Dam Study (Klein et al. 1994) in the USA have all shown DM to be associated with glaucoma. Other studies such as the Baltimore Eye Study (Tielsch et al. 1995) in the US or the Diabetes Audit and Research in Tayside Study in the United Kingdom (Ellis et al. 2000) have failed to show an association of POAG with DM. Since DM is a disease of small vessels of the body, it is plausible that it has an association with glaucoma.

Some reports have suggested an association between high blood pressure and glaucoma, but this relation is not fully understood and is not uniformly observed. There appears to be a subset of patients with low diastolic perfusion pressures who are at higher risk for POAG (Wilson et al. 1987). Some studies indicate that patients with DM have higher IOPs and a higher rate of glaucoma than people without DM. However, other studies fail to demonstrate this relationship. In a study in which the presence of DM was self-reported, diabetes protected against the development of glaucoma (Gordon et al. 2002). Other abnormalities in vascular function, including migraine headache (Cursiefen et al. 2000) and vasospasm in the extremities (Broadway and Drance 1998), appear to be associated with glaucoma as demonstrated in some studies.

Sleep apnea has been shown to be a risk factor for glaucoma in some studies (Mojon 1999). Myopia may be another risk factor for glaucoma (Daubs and Crick 1981). The relatively thin eye wall and large globe in severely nearsighted people may account for a possible susceptibility to optic nerve damage under the influence of IOP. The association between factors such as concurrent cardiovascular disease

(Tielsch et al. 1991) has not been demonstrated consistently.

In a retrospective study from the Mayo clinic, patients with POAG were found to have a lower intracranial pressure as compared to patients without POAG (Berdahl et al. 2008). This link between cerebrospinal fluid (CSF) pressure and glaucoma may be related to the mechanical effects of the intracranial pressure on the lamina cribrosa of the optic nerve.

35.3 Pathophysiology

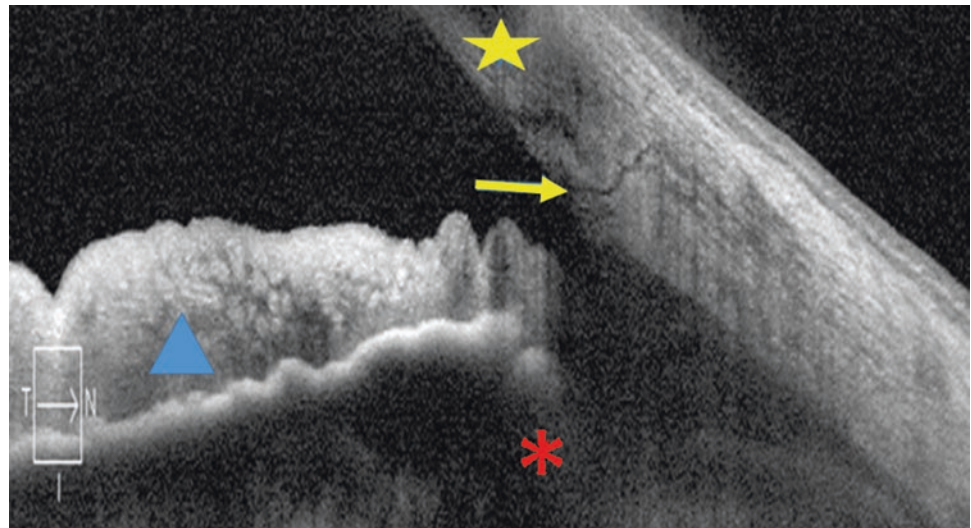
As introduced at the beginning of this chapter, POAG is an optic neuropathy wherein IOP is the best characterized risk factor. Therefore, the pathophysiology can be viewed at two levels: factors leading to elevated IOP and neuronal injury and death consequent to elevated IOP.

35.3.1 Elevated Intraocular Pressure

The physiologic processes related to maintenance of physiologic IOP and the probable causes of its dysregulation are largely limited to the anterior segment of the eye. IOP is maintained by a balance between aqueous inflow and outflow. Aqueous humor in the eye is produced by the ciliary body in the posterior chamber, from where it passes through the pupil to the anterior chamber and exits the eye through structures in the anterior chamber angle, a point of confluence of outer corneoscleral layer and middle uveal layer of the eye (Fig. 35.3).

The outflow pathways for the aqueous can broadly be divided into 2 categories: conventional outflow and uveoscleral outflow. Conventional outflow is believed to be through the trabecular meshwork to the Schlemm's canal, to collector channels and aqueous veins, to the episcleral network of vascular channels called the episcleral veins. The trabecular meshwork and Schlemm's canal complex occupy the scleral sulcus. The site of most resistance in this complex primary determinant of IOP is believed to be the region along the inner wall of the Schlemm's canal and juxtacanalicular tissue (Johnson 2006; Rosenquist et al. 1989). The outflow through this pathway is believed to be pressure dependent and is driven by the pressure differential between the IOP and the pressure in the episcleral vessels. Outflow facility is a measure of the ease with which aqueous can flow through this system and is the inverse of the resistance in the pathways involved. Removal of the trabecular meshwork and the inner wall of the Schlemm's canal can eliminate approximately 75% of the resistance in the system with the remaining 25% believed to be contributed by more distal structures

Fig. 35.3 Optical coherence tomography of a normal anterior chamber angle. *Star*=cornea, *asterisk*=ciliary body, *arrowhead*=iris, *arrow*=Schlemm's canal with collector channel



(Grant 1963; Rosenquist et al. 1989). The second, unconventional, egress path of aqueous from the eye is less well defined and is believed to be routed through the root of the iris, to the interstitial spaces of the ciliary body and into the suprachoroidal space (Alm and Nilsson 2009). This uveoscleral outflow is pressure independent in the physiological range of IOP.

The relationship between these inflow and outflow parameters of the eye can be summarized by the modified Goldmann equation as follows:

$$\text{IOP} = (\text{Aqueous inflow} - \text{uveoscleral outflow}) / \text{Outflow facility} + \text{Episcleral venous pressure.}$$

The daytime aqueous inflow rate has not been found to be elevated in primary and secondary glaucomas (Brown and Brubaker 1989; Larsson et al. 1993; Larsson et al. 1995; Levene et al. 1976; Ziai et al. 1993). A slightly higher nighttime flow rate has been reported in patients with primary open angle glaucoma (Larsson et al. 1995). The primary pathology involved in elevated IOP has been an increased resistance in both the conventional pathway and reduced flow through the uveoscleral pathway (Grant 1951; Grant 1958; Johnson et al. 2008; Johnson et al. 2008; Toris et al. 2002; Toris et al. 2010). Elevated episcleral venous pressure has not been found in cases of POAG or OHT (Podos et al. 1968) but plays a role in some forms of secondary glaucoma such as that found in association with Sturge-Weber Syndrome (Cibis et al. 1984; Phelps 1978; Shiau et al. 2012).

Several anatomic correlates of increased resistance in the trabecular pathways have been described. Some of these are thickening and fusion of trabecular lamellae secondary to trabecular endothelial cell loss, collapse of trabecular beams,

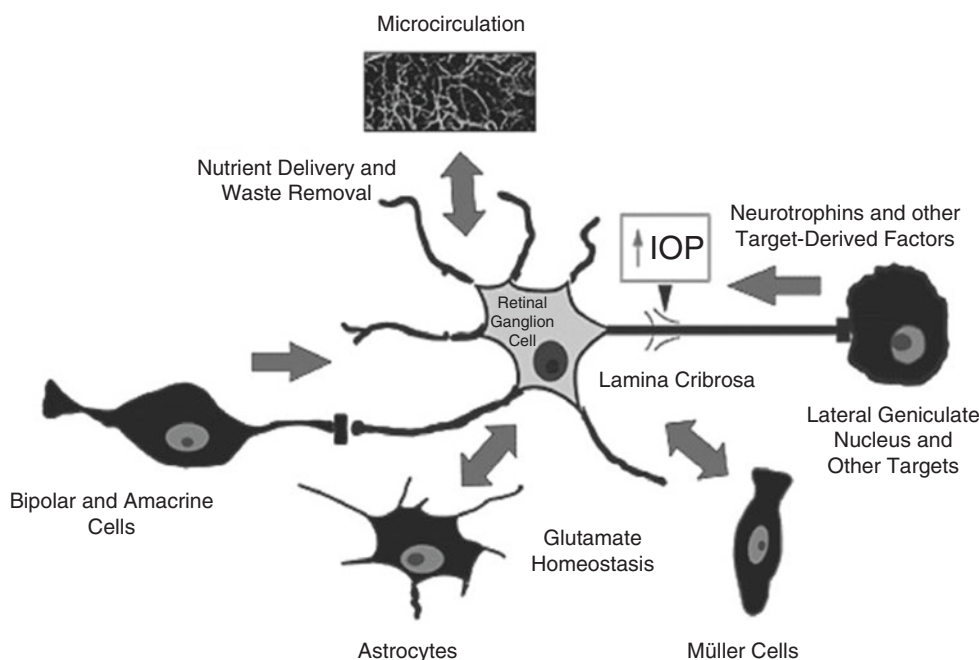
increase in electron dense plaques and possible changes in the glycosaminoglycan composition of the basement membranes and stroma of trabecular meshwork (Tektaş and Lutjen-Drecoll 2009). The anatomic correlates of reduced uveoscleral outflow in OHT and POAG are not well defined at this time. Uveoscleral outflow has been shown to decrease with age (Toris et al. 1999) and administration of cholinergic agents (Crawford and Kaufman 1987) and increased with clinically used doses of prostaglandin analogs (Crawford et al. 1987; Gulati et al. 2012; Hayashi et al. 1987; Nilsson et al. 1989). An effect on matrix metalloproteinases and tissue inhibitors of metalloproteinases is believed to be the mechanism involved in the improvement of uveoscleral outflow with prostaglandins (Alexander et al. 1991; Brew et al. 2000; Murphy and Docherty 1992).

35.3.2 Optic Nerve Damage

Despite significantly elevated intraocular pressure, only a small percentage of subjects develop clinically manifest glaucomatous damage. At the same time glaucomatous optic nerve damage and consequent visual field loss has been shown to progress despite substantial IOP lowering. There is no clear explanation of this individual variability in the susceptibility of the optic nerve to varying levels of IOP. In general, the cause of glaucomatous optic neuropathy is unknown.

A unifying pathogenic mechanism, among differing hypothesis regarding the preceding mediator, appears to be stasis of axoplasmic flow at the level of the optic nerve head (ONH). The obstruction to the flow involves both the slow and the fast phases and both retrograde and anterograde

Fig. 35.4 Several mechanisms have been hypothesized to have a role in causing retinal ganglion cell death in glaucoma. IOP indicates intraocular pressure. Reproduced with permission from: Weinreb RN, Levin LA (1999) Is neuroprotection a viable therapy for glaucoma? *Arch Ophthalmol* 117:1540–1544



transport (Minckler et al. 1976; Minckler et al. 1977; Minckler et al. 1978; Quigley et al. 1979). In a non-human primate model of chronic IOP elevation, the flow obstruction preferentially affected the magnocellular layers of the lateral geniculate nucleus (Dandona et al. 1991).

The RGC death occurs as a consequence of triggering of apoptotic pathways, or programmed cell death. This could result from loss of essential trophic stimuli as a consequence of disruption of axoplasmic flow. Loss of axons involves early loss of axons by Wallerian degeneration and subsequent somal death after an abortive attempt at regeneration (Buckingham et al. 2008). Besides damage to the primary ganglion cells injured by the glaucomatous process, the apoptotic cells create a toxic environment leading to injury and death of surrounding cells by mechanisms that may involve glutamate excitotoxicity or free radical mediated injury (Hare et al. 2001). Glaucomatous damage appearing as loss of discrete groups of nerve fiber bundles may be reflective of this proximity of cells being injured or a primary pathological process at the lamina cribrosa where these axons come together.

Many pathophysiological mediators common to all neurodegenerative diseases, including glaucoma, have been identified, such as increase in glutamate, conformationally altered self-proteins, increase in inflammation-associated factors [cyclooxygenase-2 (COX-2), tumor necrosis factor (TNF- α), nitric oxide (NO)], increase in extracellular matrix proteins and growth-associated inhibitors (myelin-related proteins), oxidative stress, and malfunctioning of local immune cells (microglia). These mediators evoke a response for which the tolerance of the neural tissue is minimal.

Multiple theories exist concerning how IOP can be one of the factors that initiates glaucomatous damage in a patient (Fig. 35.4). Two of the fundamental theories include the mechanical and vascular theory.

The mechanical theory proposes that elevated IOP causes a backward bowing of the lamina cribrosa, resulting in kinking of the axons as they exit through the lamina pores (Müller 1858). Damaged RGC axons are affected by neurotrophin deprivation leading to subsequent apoptosis and cell death. The vascular theory proposes that cell death is triggered by ischemia, whether induced by elevated IOP or as a primary insult (Jaeger 1858). It is possible that in any given patient one or both factors may play out to varying degrees. Intuitively, the mechanical damage hypothesis may play a larger role when the IOP is significantly elevated above normal levels and the vascular predispositions may play a prominent role in cases where glaucoma continues to progress despite adequate IOP lowering.

35.3.3 Role of Intracranial Pressure

Recently, there has been an interest in the role that CSF pressure may have in the pathophysiology of glaucoma (Jonas et al. 2015). This is based on the theory that higher CSF pressure may provide some sort of tectonic support to lamina cribrosa faced with elevated IOP, and glaucomatous damage at the ONH may be consequent to an imbalance between IOP and CSF pressure (Ren et al. 2011). Experimental reduction of CSF pressure in monkeys has been shown to produce nerve fiber and optic nerve damage similar to glaucoma (Yang et al.

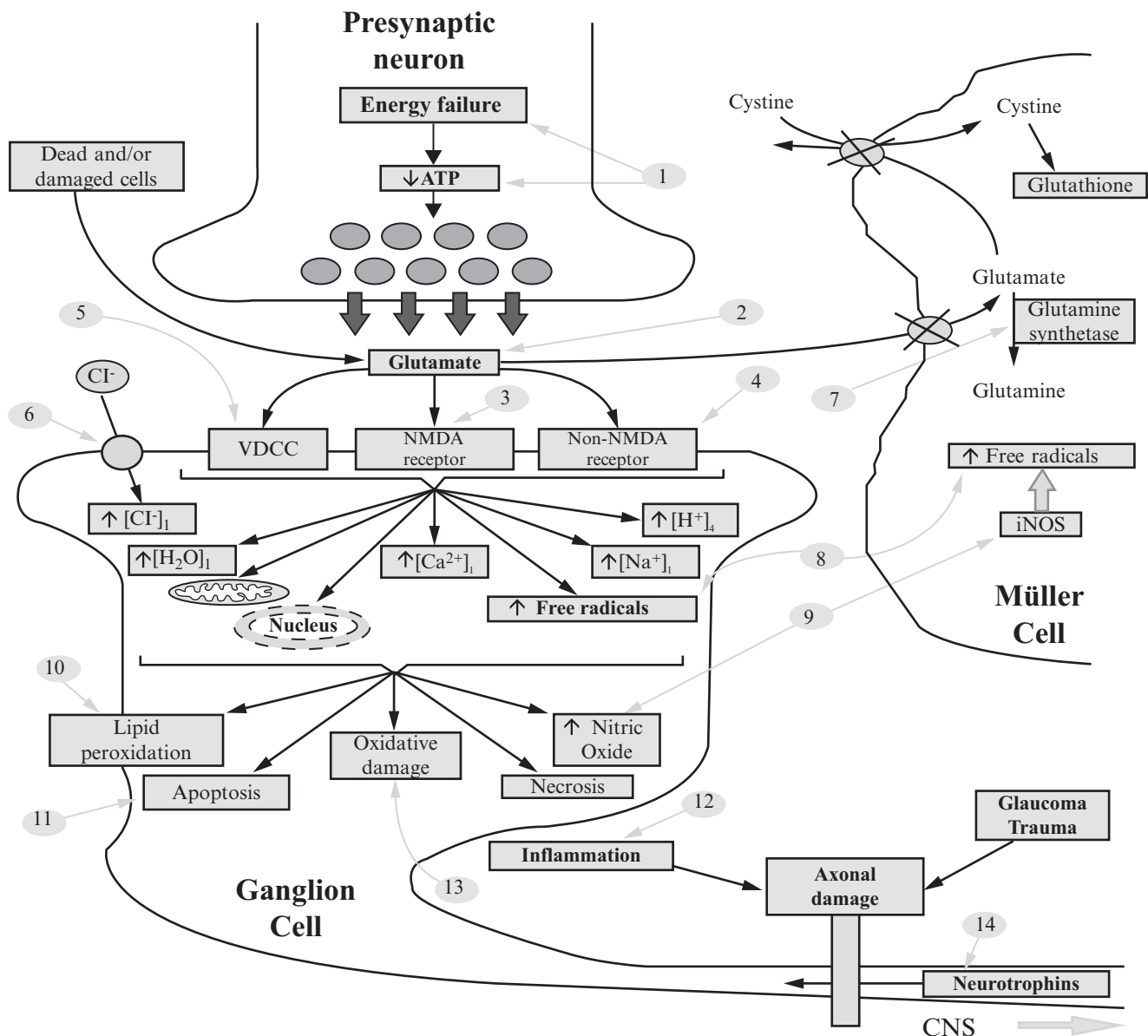


Fig. 35.5 Diagrammatic summary of potential targets for intervention in ganglion cell death. (1) Prevent energy loss, (2) reduce extracellular glutamate, (3) block ionotropic NMDA receptors, (4) block non-NMDA ionotropic glutamate receptors, (5) block excessive influx of Ca^{2+} through voltage-gated channels, (6) prevent influx of chloride and water, (7) enhance intracellular glutamate catabolism, (8) prevent excessive production of free radicals, (9) prevent detrimental effects of

nitric oxide, (10) prevent lipid peroxidation, (11) prevent induction of apoptosis, (12) reduce any inflammation, (13) stop oxidative damage, and (14) maintain neurotrophin support. Reproduced with permission from: Osborne NN, Ugarte M, Chao M, Chidlow G, Bae JH, Wood JP, Nash MS (1999) Neuroprotection in relation to retinal ischemia and relevance to glaucoma. *Surv Ophthalmol* 43 Suppl 1:S102–S128

2014). Intracranial pressure has been found to be significantly lower in POAG and normal tension glaucoma as compared to control subjects with no glaucoma (Berdahl et al. 2008).

35.3.4 Glaucoma Therapy

Based on the above pathophysiology there are broadly speaking two therapeutic targets in glaucoma management. One is

to lower the IOP. The other is to facilitate ganglion cell survival independent of IOP lowering, also referred to as neuroprotection (Fig. 35.5). Almost all of clinical glaucoma management at this time targets IOP lowering. Numerous well designed clinical trials have proven the benefit of IOP lowering in the preservation of visual function in glaucoma patients (AGIS Investigators 2000; Collaborative Normal-Tension Glaucoma Study Group 1998; Gordon et al. 2002; Leske et al. 2003; Lichter et al. 2001). Despite tremendous interest in

neuroprotection there are currently no well proven neuroprotective strategies available for glaucoma management. Various agents have shown promise in preclinical studies. Some of these are *N*-methyl-D-aspartate (NMDA) antagonists (memantine), alpha-2 agonists, calcium channel blockers, antioxidants and nitric oxide synthetase inhibitors. Memantine has been the subject of 2 phase III clinical trials, the results from these have not been published. Even though a clinical trial comparing the efficacy of timolol and brimonidine did report better visual field preservation with brimonidine, the study is limited by several potential biases involved, as detailed in a recent Cochrane review (Krupin et al. 2011). The review concluded that the current evidence in support of neuroprotective strategies in the management of open angle glaucoma to be inconclusive (Sena and Lindsley 2013). The review also comments on the current lack of availability of adequately sensitive outcome measures to be able to detect a neuroprotective effect of agents, if there was one.

35.3.5 Potential Therapeutic Targets for Neuroprotection

Figure 35.5 is a diagrammatic representation of some of the potential neuroprotective targets characterized at this time.

35.3.5.1 Immune Mechanisms

Neural injury has long been considered self destructive and progressive primarily due to the autoimmunity to self antigen. The anti-self antigen response has been shown in these experiments to have a coincidental beneficial and modulating effect (Schwartz 2005). The autoimmune T cells limit the activity of the microglial framework and blood-borne macrophages, affecting their ability to deal with a local excitatory situation. Such a controlling effect has a beneficial impact on both survival and regrowth. Boosting the beneficial effect by the use of a safe antigen that can cross-react with self-antigens at the site under stress might provide an effective therapeutic approach that influences both rescue and regeneration (Schwartz 2005). Copolymer 1 (Cop-1), an approved drug for the treatment of multiple sclerosis, can be used as a treatment for autoimmune diseases and as a therapeutic vaccine for neurodegenerative diseases (Kipnis and Schwartz 2002). These results have special implications for glaucoma therapy since glaucoma is the most common cause of optic neuropathy.

35.3.5.2 Apoptosis

Apoptosis is classically defined by an orderly pattern of internucleosomal DNA fragmentation, chromosome clumping, cell shrinkage, and membrane blebbing (Wyllie et al. 1980). This is followed by disassembly of the cell into multiple membrane-enclosed vesicles that are engulfed by neighboring cells with-

out inciting inflammation. Apoptosis has often been labeled as '*programmed or physiological cell death*'.

Several gene families have been identified that play either positive or negative roles in determining whether a cell (in glaucoma, the RGC) will undergo apoptosis. Caspases are cysteine proteases that play a central role in both propagating apoptotic signals and carrying out disassembly of the cell (Hutchins and Barger 1998). Many triggers activate caspases, including increased intracellular calcium, changes in cellular energy balance, and adenosine 3'5' cyclic phosphate.

The main inhibitors of apoptosis are Bcl-2 and related proteins. They have multiple complex functions, such as inhibiting intermediate proteins that activate caspases, gauging intracellular damage and maintaining organelle integrity in ways that are not fully understood. For example, over expression of Bcl-2 inhibits both apoptotic and necrotic cell death by suppressing lipid peroxidation (Adams and Cory 1998).

35.3.5.3 Glutamate-Induced Excitotoxicity

The excitatory amino acid glutamate is known to cause neurotoxicity via binding of glutamate to the NMDA receptor and the kainate receptors. This can be secondary to many types of cellular damage, including other final common pathways of damage such as oxidative stress or free radical damage (Osborne et al. 1999). Activation of these receptors allows entry of excessive amounts of calcium. Abnormally high concentrations of calcium lead to inappropriate activation of complex cascades of proteases, nucleases, and lipases. These directly damage cellular constituents and lead to ganglion cell death.

One neuroprotective strategy, therefore, is to interrupt the resultant excitotoxic cascade by blocking the NMDA receptor with drugs such as memantine (Lipton 2003).

35.3.5.4 Free Radicals

Free radicals are generated not only through activation of glutamate receptors but also as the inevitable by-product of normal oxidative metabolism. This is especially true in the retina and the RGCs, which have an extremely high metabolic rate. This triggers further changes including calcium release that causes cell damage (as summarized in section 3.5.3. above). Endogenous antioxidants such as vitamins E and C, superoxide dismutase (Greenlund et al. 1995), and glutathione normally inactivate free radicals.

35.3.5.5 Nitric Oxide Synthase

Nitric Oxide Synthase (NOS) is postulated to have a role in RGC axonal toxicity. Inducible NOS is known to be up regulated in the optic nerve in glaucoma (Liu and Neufeld 2001). Inhibitors of NOS, such as 3-aminoguanidine, decreased the RGC loss by 70 % in rat eyes with raised IOP (Neufeld et al. 1999).

35.3.5.6 Dopamine Deficiency

Dopamine has been recognized to have neuroprotective actions in glaucoma. Retinal dopaminergic cells can be detected by the immunohistochemical staining of tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis. It has been observed that NMDA induced RGC toxicity dramatically decreased tyrosine hydroxylase immunostaining at the junction between the inner nuclear layer and inner plexiform layer in glaucoma (Kitaoka and Kumai 2004).

35.3.5.7 Heat-Shock Proteins

Heat-shock proteins (Hsp) are known to be an intrinsic protection mechanism associated with an early response against physiologic and environmental stress (Wax and Tezel 2002). Certain Hsp have special effects upon neurons, including Hsp 72, 27 and 60. Hsp 27 and 60 have been demonstrated to be upregulated in RGCs of glaucoma (Tezel et al. 2000). Recent work has shown that geranylgeranylacetone can induce Hsp 72 in RGCs and protect them from glaucomatous damage in a rat glaucoma model (Ishii et al. 2003).

35.3.5.8 Calcium Channel Blockers

Calcium channel blockers may reduce the ischemic apoptotic stimulus by improving perfusion through their vasodilatory effects (Osborne et al. 2004a; b). In addition calcium channel blockers may also interfere with the calcium influx mentioned in the glutamate induced cytotoxicity above (Choi 1988; Schurr and Rigor 1992; Siesjo and Bengtsson 1989). The vascular dilation effects associate with beta 1 agonists may also be mediated through inhibition of calcium influx of the cell membrane through the voltage gated calcium channels (Bessho et al. 1991; Hester et al. 1994; Setoguchi et al. 1995). At this time there is some limited evidence to support the effect of calcium channel blockers in preservation of visual fields in glaucoma patients (Koseki et al. 1999; Sawada et al. 1996).

35.3.5.9 Genetic Mutations

Myocilin (MYOC) is one gene that has been associated with early- and late-onset POAG. The MYOC gene was mapped to the open-angle glaucoma locus (GLC1A) region of chromosome 1q25, and mutations were discovered in patients with adult and juvenile open-angle glaucoma. Mutations in this gene are found in 3–5 % of adult-onset POAG patients and in 8–20 % of juvenile-onset POAG patients (Wiggs et al. 1998). Different mutations are associated with juvenile and adult glaucoma. In particular, the most common mutation associated with adult glaucoma is a nonsense mutation, whereas all the mutations associated with juvenile glaucoma are missense mutations.

Mutation studies and genetic linkage analyses have shown a causative effect of the CYP1B1 gene in the development of primary congenital glaucoma. The CYP1B1 locus has also

been implicated as a modifier gene in the development of POAG, and in some cases as the causative gene in POAG and other anterior segment dysgeneses (Vasiliou and Gonzalez 2008).

Using single large pedigrees affected by POAG, seven genetic loci have been described (GLC1A-G), and glaucoma-predisposing genes have been identified in three of these loci: GLC1A, myocilin (Wiggs et al. 1998); GLC1E, optineurin (Rezaie et al. 2002); and GLC1G, WDR36 (Monemi et al. 2005). Each of these genes is only responsible for a small fraction of cases of POAG, reflecting the small percentage of POAG that is inherited as a Mendelian trait rather than as a complex trait.

The optineurin protein may function to protect the optic nerve from TNF-alpha-mediated apoptosis, and the loss of function of this protein may decrease the threshold for RGC apoptosis in patients with glaucoma. Surprisingly, mutations in this gene do not appear to contribute significantly to the optic nerve degeneration that is a component of typical high-pressure POAG (Wiggs et al. 2003). Optineurin has been associated with subjects that experience progressive glaucomatous optic nerve changes at low or normal pressures, so called “normal pressure glaucoma.” Optineurin has also been implicated in sporadic amyotrophic lateral sclerosis with or without superoxide dismutase-1 mutations, confirming optineurin’s role in a separate progressive degenerative neuropathy (Maruyama and Kawakami 2013; Deng et al. 2011).

More recent genome wide association studies have demonstrated a strong POAG association at the SIX1/SIX6 locus (Wiggs et al. 2012). Deletions of SIX1 and SIX4 have been associated with bilateral anophthalmia and pituitary abnormalities (Bennett et al. 1991). In animal models, mutations at the SIX1/SIX6 locus result in early exit from the cell cycle among RGC progenitors. A common risk variant at the SIX6 locus, His141, is present in approximately 40 % of subjects of European descent, and 99 % of subjects of African descent. In POAG and non-glaucomatous eyes that are homozygous for the risk allele there is a significantly thinner retinal nerve fiber layer at the superior and inferior poles of the optic nerve, the site of the earliest nerve fiber loss in glaucoma (Carnes et al. 2014; Cheng et al. 2014; Kuo et al. 2015).

The exact role that IOP plays in combination with these other factors and the latter’s significance in the initiation and progression of subsequent glaucomatous neuronal damage and cell death requires further studies to elucidate.

35.4 Clinical Features

35.4.1 History

The initial patient interview is important in the evaluation for glaucoma. Because of the “silent” or asymptomatic nature of

POAG, patients usually will not present with any visual complaints until late in the course of the disease. A history should include the following:

1. Past ocular and medical history including:
 - Eye pain
 - Redness
 - Multicolored halos (indicative of intermittent IOP elevation and related corneal edema)
 - Headache
 - Previous ocular disease such as cataracts, uveitis, diabetic retinopathy or vascular occlusions
 - Previous ocular laser or surgery
 - Ocular or head trauma
 - Pertinent vasculopathic systemic illnesses, including Raynaud's phenomenon or migraine
2. Current medications, including any anti-hypertensive medications or corticosteroids used topically or systemically. Systemic and topical medications can effect IOP and ONH perfusion. Sulfa-derivative medications can result in a rare idiosyncratic reaction that causes ciliary body effusion and secondary angle closure related IOP elevation.
3. Family history of glaucoma in first-degree relatives.

35.4.2 Physical Exam

Screening the general population for POAG is most effective if targeted at those at higher risk, such as African Americans, those with a family history of glaucoma, and elderly individuals, especially if the screening includes IOP measurements, assessment of the optic nerve and screening visual fields.

For individuals of age 65 years or above, recommended frequency for a comprehensive adult medical eye evaluation is 6–12 months when risk factors for glaucoma are present and 1–2 years in the absence of risk factors. For glaucoma suspect individuals under 40 years, the frequency of the eye evaluation should be 2–4 years and 5–10 years in the presence and absence of risk factors for glaucoma, respectively (American Academy of Ophthalmology Glaucoma Panel 2010a).

A standard comprehensive eye examination, including gonioscopy, is performed at the initial visit. If any visual field or optic nerve changes consistent with glaucoma are present, additional testing should be done to establish the diagnosis, help in defining target (or safe) levels of IOP, and developing a treatment and follow-up plan.

In patients with glaucoma, factors that determine frequency of evaluations include the severity of damage, the stage of the disease, rate of progression, extent to which the IOP exceeds the target pressure, and the number and significance of other risk factors for damage to the optic nerve. If the target IOP has been achieved and there is no progression

of damage, it is recommended to follow up with ONH exam and visual field testing at 6 to 18-month intervals in those with mild to moderate glaucoma, and as frequently as every 4 months in patient with severe stage glaucoma (American Academy of Ophthalmology Glaucoma Panel 2010b). On the contrary, if there is evidence of progression of damage and the target IOP has not been achieved, more frequent testing is required until stability is confirmed.

35.4.2.1 Tonometry

When checking IOP, record measurements for both eyes, the method used (Goldmann applanation is most commonly used), and the time of the measurement. IOP varies from hour-to-hour in any individual. Many studies indicate that the circadian rhythm of IOP peaks in the early hours of the morning just after waking. Intraocular pressure measurements should be made at different times of the day to check for diurnal variation. A diurnal variation of more than 5–6 mm Hg indicates an increased risk for glaucoma.

IOP can also change with body position, rising transiently when changing from an upright to a supine position. The positional change in IOP is greater in people with OHT and glaucoma (Prata et al. 2010). Aqueous humor outflow facility does not change with the body position, implying other ocular parameters are causing the IOP change observed (Selvadurai et al. 2010).

A difference between contralateral eyes of 3 mm Hg or more indicates greater suspicion of glaucoma. An average of a 10% difference between individual measurements is expected. In most circumstances, the measurements should be repeated on at least 2–3 occasions before deciding on a treatment plan.

35.4.2.2 Gonioscopy

The angle of the eye is examined by gonioscopy, which requires the use of special lenses. Gonioscopy is performed to rule out angle-closure or secondary causes of IOP elevation, such as angle recession, pigmentary glaucoma, exfoliation syndrome, and trabeculitis. The peripheral contour of the iris is examined for plateau iris, and the trabecular meshwork for peripheral anterior synechiae, as well as for neovascular or inflammatory membranes and cell aggregates. Schlemm's canal may be seen if blood refluxes into the canal. This might indicate elevated episcleral venous pressure caused by conditions such as a carotid-cavernous fistula, Graves orbitopathy, idiopathic elevated episcleral venous pressure, or Sturge-Weber syndrome.

Ultrasound biomicroscopy (Fig. 35.6) and anterior segment optical coherence tomography (Fig. 35.3) are also helpful in assessing the angle, iris, and processes of the ciliary body to rule out anatomical pathology and secondary causes of elevated IOP.

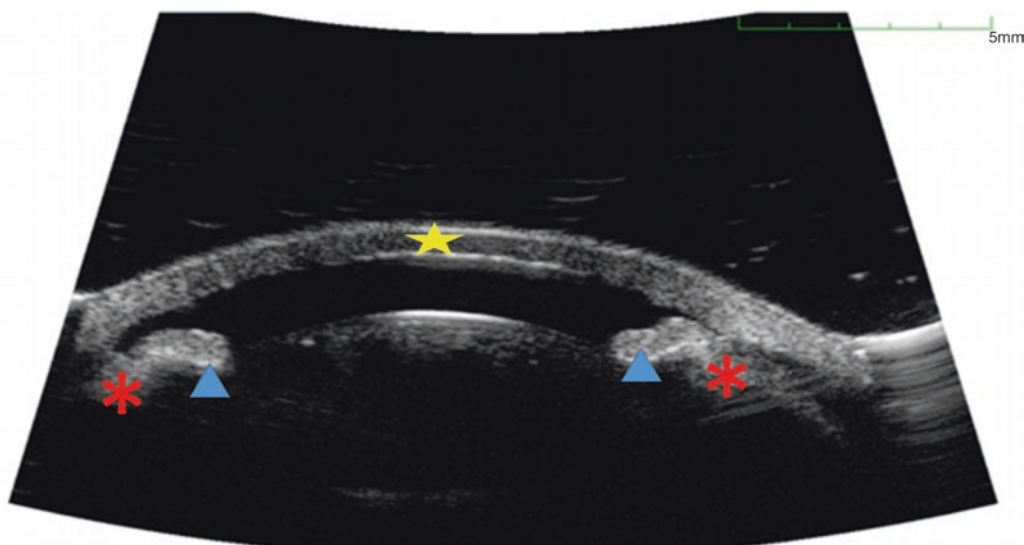


Fig. 35.6 Ultrasound biomicroscopy in an eye with narrow angle and bombé configuration of the iris. *Star*=cornea, *asterisk*=ciliary body, *arrowhead*=iris

Fig. 35.7 Stereo photographs representing normal optic nerve head with small central cup (*left*) and typical glaucomatous changes with advanced cup and visualization of temporal lamina cribrosa pores and peripapillary atrophy (*right*)



35.4.2.3 Optic Disc and Nerve Fiber Layer

The optic disc is examined to estimate the cup-to-disc ratio and to identify excavation and enlargement typical of glaucoma. The optic disc is a complex, three-dimensional anatomic structure. Stereoscopic viewing during slit-lamp examination improves the accuracy of disc evaluation. Single cutoff values for the cup to disc ratio designed to distinguish between normal subjects and those with glaucoma are imperfect for screening, since the normal ratio varies considerably with optic disc size. When the cup to disc ratio equals or exceeds 0.6, the probability of glaucoma increases dramatically. In screening for glaucoma, however, even combined criteria that includes the level of IOP and cup to disc ratio identifies only two thirds of patients with glaucoma.

The disc should be viewed stereoscopically to assess for evidence of glaucomatous damage. The following characteristics should be noted: cup-to-disc ratio in the horizontal and vertical meridians; color and slope of the cup; appearance of the disc; progressive enlargement of the cup (comparison to prior photos); evidence of nerve fiber layer damage using a red-free filter; notching or thinning of disc rim, particularly at the superior and inferior poles; pallor; presence of nerve fiber layer hemorrhage; asymmetry between cups; peripapillary atrophy (association with the development of glaucoma when Beta-zone atrophy is present); or congenital nerve abnormalities. Stereo fundus photographs are acquired as a baseline for future comparisons (Fig. 35.7).

Other imaging techniques that utilize different physical properties of light for optical analysis can document the status of the optic nerve and the nerve fiber layer. Furthermore, they can be used to detect changes over time (Medeiros et al. 2004). The value of these technologies for diagnosing glaucoma and for determining progression over time has been demonstrated in early to moderate glaucoma but their utility in advanced glaucoma is diminished with severe loss of the retinal nerve fiber layer making serial measurements difficult and less predictable of glaucoma progression.

Confocal scanning laser ophthalmoscopy can evaluate the optic disc and peripapillary retina in 3 dimensions and provide quantitative information about the cup, neuroretinal rim, and contour of the nerve fiber layer. Scanning laser polarimetry measures the change in the polarization state of an incident laser light passing through the naturally birefringent nerve fiber layer to provide indirect estimates of peripapillary nerve fiber layer thickness. Optical coherence tomography uses reflected light in a manner analogous to the use of sound waves in ultrasonography to create computerized cross-sectional images of the retina and optic disc, and also gives quantitative information about the peripapillary retinal nerve fiber layer thickness that can be compared to age matched controls (Fig. 35.8).

Fluorescein angiography, ocular blood flow analysis via laser Doppler flowmetry, color vision measurements, contrast sensitivity testing, and electrophysiological tests (e.g., pattern electroretinography, visual evoked potentials) are used as research tools in the evaluation of POAG patients. Routine clinical use is not advocated at this time.

35.4.2.4 Visual Field Testing

Automated threshold testing (e.g., Humphrey 24-2) is performed to evaluate for glaucomatous visual field defects. If the patient is unable to perform automated testing, Goldmann perimetry may be substituted. New onset glaucomatous defects are found most commonly as an early nasal step, temporal wedge, or paracentral scotoma (more frequent superiorly). Generalized depression also may occur, but is less specific for glaucomatous damage.

Swedish Interactive Threshold Algorithm (SITA)-based software (Fig. 35.9) decreases testing time and boosts reliability, especially in older patients (Schimiti et al. 2002). Short wavelength automated perimetry (SWAP) or blue-yellow perimetry may provide a more sensitive method of detecting visual field deficits, especially in patients with OHT without defects demonstrated by standard testing. If the Humphrey standard automated perimetry results are normal, SWAP can be utilized in detecting visual field loss earlier. Prior studies suggest SWAP may detect visual loss or progression up to 3–5 years earlier than conventional perimetry, as well as in 12–42 % of patients previously diagnosed

with only OHT (Racette and Sample 2003). Because the testing time may be lengthened, SWAP testing may be strenuous for some patients. However, more recent SITA-SWAP algorithm software may speed up the testing time and improve reliability (Bengtsson 2003).

The pupil size should be documented at each testing session since pupillary constriction can reduce retinal sensitivity and mimic progressive visual field loss. This is especially important in patients that are using miotic therapy with pilocarpine.

To establish a reliable baseline, visual fields may have to be repeated several times on successive visits, especially if initial testing shows low reliability indices. Newer glaucoma progression analysis software can help identify reliable perimetric baselines, and probability-based analyses of subsequent fields can assist in distinguishing true progression over time from fluctuation or artifact. In follow-up, if the patient is at low risk for onset of glaucomatous damage, then repeat testing may be performed once a year. If a high risk of impending glaucomatous damage is present, then testing frequency is adjusted accordingly (as frequent as every few months).

Frequency doubling perimetry (also termed frequency doubling technology or FDT) is a relatively new technology that may detect functional visual loss at an earlier stage in the glaucomatous disease process, thereby detecting more patients who currently are misdiagnosed as having OHT instead of early POAG. Current sensitivities and specificities are continuing to improve, but more baseline data are needed to determine under what circumstances these new techniques will prove to be most useful.

35.5 Differential Diagnosis

Several disorders may mimic glaucomatous ONH and/or visual field changes. Those that resemble structural glaucomatous optic neuropathy or interfere with assessment of glaucomatous cupping include optic disc anomalies, tilted disc syndrome, optic nerve drusen, optic nerve pits, optic nerve colobomas, myelinated nerve fibers, ischemic optic neuropathy and optic atrophy.

Factors that mimic glaucomatous visual field loss include: branch retinal artery and vein occlusions; chorioretinal scars; retinal areas treated by photocoagulation or cryotherapy; demyelinating disorders; cerebrovascular accidents, tumors, or other lesions affecting the optic nerve, chiasm, optic tract, optic radiation, and/or the remaining course of nerve fibers to the occipital cortex. Other abnormalities that could account for “pseudo-glaucomatous” visual field defects or vision loss include vitreous hemorrhage, proliferative retinopathy or other retinal disorders.

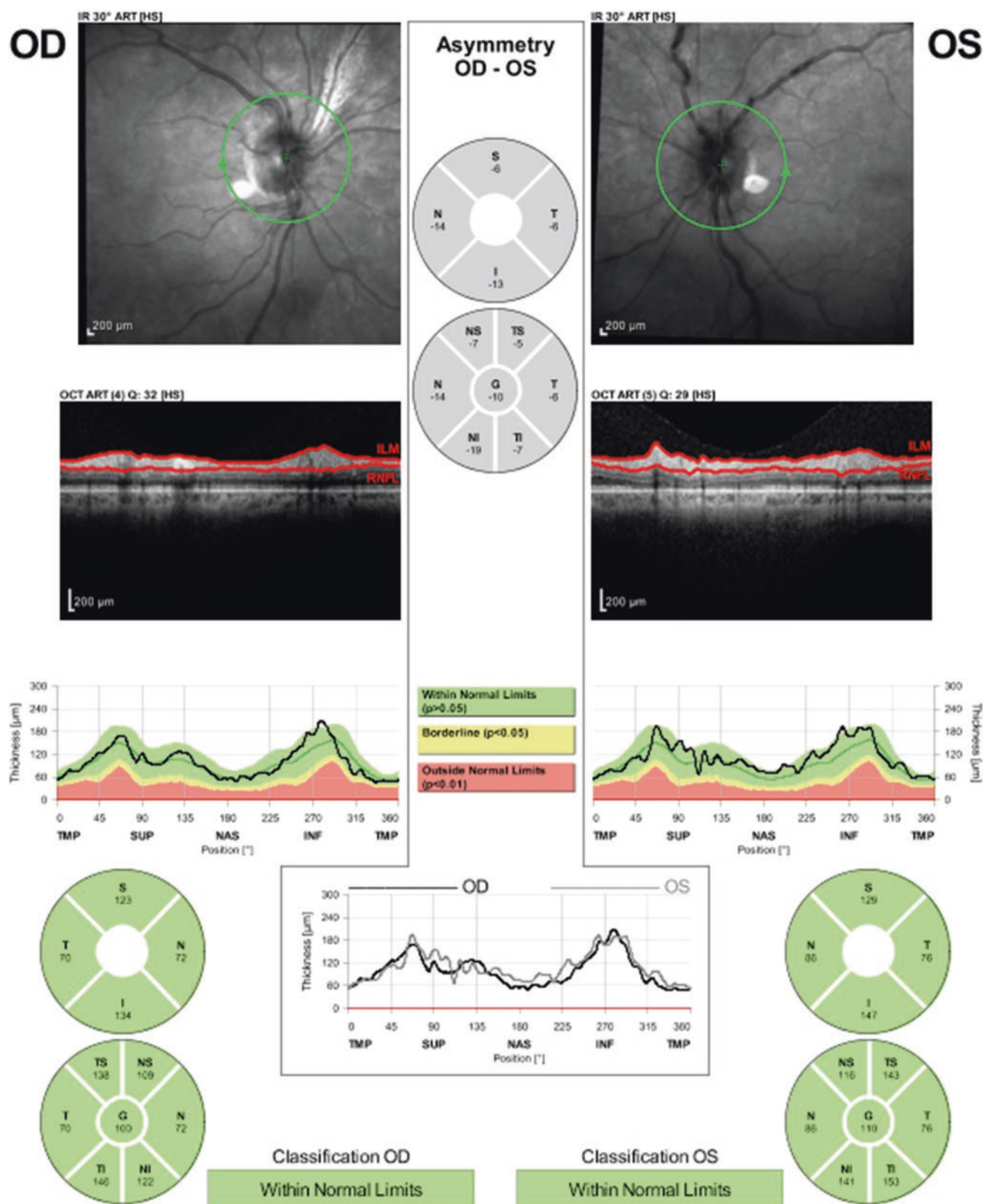


Fig. 35.8 Optic nerve optical coherence tomography in a patient with a normal optic nerve. *Top row*—red free optic nerve photo. *Second row*—cross sectional identification of the retinal nerve fiber layer (outlined in red). *Bottom section*—comparison with age-matched control population

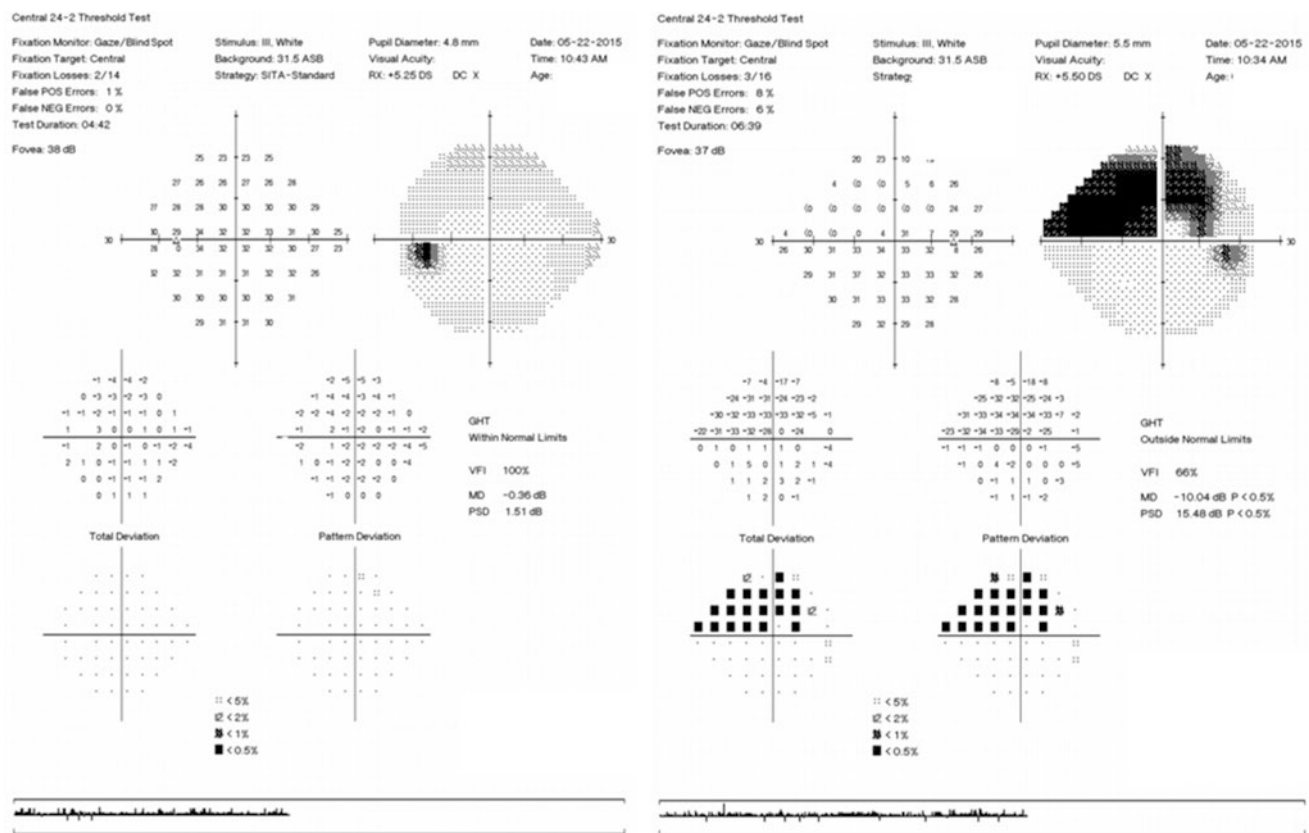


Fig. 35.9 Humphrey 24-2 SITA standard Visual Field Test. Normal right eye visual field. Left eye demonstrating a superior arcuate scotoma of severe stage glaucoma related visual field loss in a patient with exfoliative glaucoma

35.6 Animal Models

Animal models for glaucoma have been used to study the pathophysiological basis of glaucoma. Animal based experiments are more representative than cell culture models particularly in the study of aqueous humor dynamics, the trabecular meshwork-ciliary muscle complex, RGC, ONH axons, ONH framework, and vascular flow dynamics in glaucoma subtypes (Weinreb and Lindsey 2005).

Conducting studies in animal models often are challenging and occasionally require anesthesia, which might affect the parameter being assessed. Conditioned reflex training may help elicit cooperation. Use of remote controlled implants and newer drug delivery options represent advances made on this front.

Animal experiments have provided excellent means to study medical and surgical treatments for glaucoma prior to human trials. Animal models are required to study *de novo* drugs and their structural and functional influence on ocular tissues.

Genetic modulation of animal models is another area of research. Mice are genetically engineered to suit experimen-

tal needs. New models are required and will be developed to advance our understanding of the glaucomatous disease process. Mouse mutants with glaucoma provide models for human glaucoma. From the clinical standpoint, some mouse model share occasional similarities with various aspects of glaucoma occurring in humans. The life span and replication time (one mouse year is equal to 30 human years) allow developmental and invasive studies and make it possible to follow glaucoma progression in a reasonable span of time (Chang et al. 2005). Some of the new mouse glaucoma models used are: Gpmb [glycoprotein (transmembrane)] with mutation on chromosome 6 to study iris pigment dispersion (Anderson et al. 2002); Trypl with chromosome 4 mutation to study iris stromal atrophy (Anderson et al. 2002); Nm2702 with chromosome 11 mutation to study inner retina loss and optic nerve cupping (Chang et al. 2005); CALB/Rk and si/si model to study early enlargement of optic cup or coloboma (Chang et al. 2005); AKXD-28 for ganglion cell loss and optic nerve cupping (Anderson et al. 2001); E50K optineurin transgenic mouse for normal tension glaucoma (Tseng et al. 2015); and nm1839-model for Axenfeld's syndrome, anterior segment dysgenesis or buphthalmos (Chang et al. 2005). Due to complex regulatory gene interactions and a still

Table 35.3 Advantages and limitations of several animal models of glaucoma

	Monkey	Rabbit	Rat	Mouse
General similarity to human eye	++++	++	++	++
Similarity to human aqueous dynamics	++++	+	+++	++++
Experimental IOP elevation	+++	++	+++	+++
Spontaneous IOP elevation	–	+	–	+++
Cupping, optic axon loss with IOP	++++	–	+++	+++
Genome sequenced	– ^a	–	–	+++
Many transgenic strains available	–	–	+	++++
Readily available, low cost	–	+	+++	++++
Ease of maintenance and handling	–	++	+++	+++
Ease of testing	+	+++	++	+

Reproduced with permission from: Weinreb RN, Lindsey JD (2005) The importance of models in glaucoma research. *J Glaucoma* 14:302–304

^aThe human and monkey genomes are sufficiently similar that data from the human genome project often can be applied to experiments using non-human primates

incomplete understanding of the effects of SIX6, no mouse model yet exists. The effect of glaucoma on the electroretinogram response has been evaluated in mice and is currently being evaluated for its role in diagnosis and monitoring glaucoma in humans.

The pig as a model for glaucoma shares many phylogenetic similarities with that of the human and is more readily available compared to the non-human primate model (Ruiz-Ederra et al. 2005). The porcine retina is more similar to human than other large mammals such as dog, goat or cow. Techniques used for glaucoma diagnosis in humans such as optical coherence tomography or multi-focal electroretinography can be reliably studied in pig eyes. Studies of the pig aqueous outflow system demonstrate it to be an acceptable model for specific glaucomas (McMenamin and Steptoe 1991).

The non-human primate model of OHT with its resultant optic neuropathy closely reflects the optic neurodegeneration associated with human glaucoma. Utilization of the glaucoma model in monkeys has helped to further the understanding of aqueous humor dynamics, glaucomatous damage in the RGCs and other structures along the visual pathway, and the effects of pharmacological agents on ocular tissues (Rasmussen and Kaufman 2005). Fundus photographs of glaucomatous monkeys show the morphologic changes consistent with human OHT with the advantage of observing progressive changes over a shorter period of time by withholding treatment despite very high IOPs (Gaasterland and Kupfer 1974). The ability to correlate histologic changes in glaucomatous monkey eyes with functional and imaging data is a major advantage of the monkey model. Humphrey visual field defects can be performed by trained glaucomatous monkeys (Harwerth et al. 2002). The types of visual field defects are similar to those in glaucomatous humans.

Utilization of an experimental glaucoma model in non-human primates has led to advances in the understanding of glaucomatous changes in the visual pathways from photoreceptors to the visual cortex (Rasmussen and Kaufman 2005).

Newer investigative modalities such as contemporary perimetry techniques have been studied in primate models. Electroretinography can be performed in monkeys to evaluate changes produced by glaucomatous optic neuropathy.

The choice of animal is governed by the objectives and requirements of the study (Table 35.3). Size of the animal eye, similarity with human eyes, cost and availability are factors that influence the selection of the animal type.

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35.7 Review Questions

1. The estimated world population that is bilaterally blind from primary open angle glaucoma is:
 - a. 2–3 million
 - b. 3–4 million
 - c. 4–5 million
 - d. 5–6 million
 - e. 6–7 million
2. The primary pathology in open angle glaucoma is believed to be
 - a. Increased aqueous production
 - b. *Increased resistance in conventional outflow*
 - c. Increased episcleral venous pressure
 - d. Decreased intra cranial pressure
3. The pathway followed by aqueous in eye sequentially involves
 - a. Episcleral veins, anterior chamber, trabecular meshwork, Schlemm's canal, ciliary body
 - b. Anterior chamber, trabecular meshwork, Schlemm's canal, ciliary body, episcleral veins
 - c. *Ciliary body, anterior chamber, trabecular meshwork, Schlemm's canal, episcleral veins*

- d. Ciliary body, anterior chamber, Schlemm's canal, trabecular meshwork, episcleral veins
4. Mechanisms that might contribute to optic nerve damage in glaucoma include:
 - a. Apoptosis
 - b. Glutamate-induced excitotoxicity
 - c. Nitric oxide synthase
 - d. Free radicals
 - e. *All of the above*
5. Mutations in which of the following genes have been shown to be associated with the pathogenesis of glaucoma?
 - a. GLC1A
 - b. Myocilin
 - c. Optineurin
 - d. GLC1G
 - e. *All of the above*
6. Which gene product is implicated is associated with normal pressure glaucoma and amyotrophic lateral sclerosis?
 - a. GLC1A
 - b. Myocilin
 - c. *Optineurin*
 - d. GLC1G
 - e. SIX6
7. Which of the following are characteristic changes that might be observed in the optic nerve head in primary open angle glaucoma except:
 - a. Increased cup to disc ratio
 - b. Notching of the neural rim
 - c. Disc hemorrhage
 - d. New vessels within the disc
 - e. *All of the above are changes associated with glaucoma*
8. Which racial background portends the greatest risk for glaucoma and glaucoma related blindness
 - a. Caucasian
 - b. *African*
 - c. Latino
 - d. Asian
9. All of the following are examples of tests to evaluate the anterior chamber angle, EXCEPT:
 - a. Gonioscopy
 - b. Anterior segment OCT
 - c. Ultrasound Biomicroscopy (UBM)
 - d. *A-scan Ultrasound*
10. All of the following are methods to assess for visual field loss when glaucoma is suspected, EXCEPT:
 - a. Humphrey Automated Perimeter
 - b. Goldmann Perimeter
 - c. *Full field Electroretinogram (ERG)*
 - d. Frequency doubling technology (FDT)

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Abstract

Ocular manifestations may be the initial, presenting features of various neuro-immune and neuro-degenerative diseases. Ophthalmic involvement may present as orbital, intraocular or adnexal inflammation or as discrete lesions involving different components of the eye and may serve as the barometer for the severity of the underlying systemic diseases. Often, the lesions may present as an ophthalmic emergency, requiring aggressive therapy before the diagnosis can be confirmed. The clinician scientist must be vigilant and maintain a high level of suspicion in order to diagnose the condition early and institute prompt therapy. The ocular signs and symptoms may be associated with a constellation of neurological complaints. Therefore, an integrated approach with close collaboration among ophthalmologists, neurologists, internists and pathologists is required to ensure optimal patient care, preserving not only the vision but also the lives of the patients.

Keywords

Adamantiades-Behçet's disease • Cornea • Optic nerve • Retina • Uveitis • Vogt-Koyanagi-Harada disease

36.1 Introduction

The eye has been recognized as an immune-privileged organ due to the presence of non-permeable blood-ocular barriers. However, over the years, a number of systemic disorders have been associated with characteristic ophthalmologic manifestations, which may often lead to severe visual morbidity. Recognition of such pathological changes in the eye may provide valuable clues leading to the diagnosis of the associated systemic immunologic dysfunction. Typically,

systemic immunologic conditions affect musculoskeletal, gastrointestinal, renal and neurological systems. Identification of the constellation of ocular and systemic signs may allow the clinician to consider a definitive diagnosis and plan appropriate management.

Intraocular inflammation of the uvea (uveitis) is the hallmark of ophthalmic involvement associated with systemic autoimmune diseases. In addition, there may be concomitant involvement of extraocular structures and the periorbital. Pathological changes such as inflammation of the retinal and/or choroidal vasculature, optic nerve swelling, macular edema, exudative retinal detachment, corneal ulceration and granulomatous inflammation of the uveal tissue are common causes of visual loss in these patients (Vodopivec et al. 2014).

Autoimmune diseases may also present initially only with ophthalmologic manifestations. The diagnosis of the underlying systemic disease, which may be life-threatening, may be delayed, unless the clinician scientist maintains a high level of suspicion. With appropriate screening, comprehensive examination and laboratory evaluation, the underlying

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systemic immunologic dysfunction can be identified and treated to prevent further ophthalmic, neurologic and systemic morbidity. Similarly, detailed evaluation of ocular symptoms and periodic, routine eye examination of patients with autoimmune diseases may allow early detection, treatment and prevention of significant visual loss.

36.2 Pathophysiological Basis of Ocular Disease

The immune privilege of the eye is maintained due to interplay of several complex mechanisms that include both innate and adaptive arms of immunity and by contribution of both, local and systemic factors that decrease inflammatory responses (Stein-Streilein and Caspi 2014). Anatomical barriers in the eye, such as the blood-ocular-barrier, avascularity of structures such as the cornea and sclera, lack of lymphatic channels, blood-retinal-barrier and highly-regulated production and maintenance of aqueous and vitreous humor contribute towards immune-tolerance of eye-derived antigens (Caspi 2014).

The ocular tissues have characteristic differences compared to other tissues with regards to their immunologic make-up. The ocular milieu has lower expression of major histocompatibility factors, soluble cell-surface receptors, growth factors and pro-inflammatory cytokines. This results in a decreased T- and B-cell response in the eye and production of non-complement fixing antibodies (*anterior chamber-associated immune deviation - ACAID*). The biochemical microenvironment of the eye actively decreases the response of the immune system. Thus, ordinarily, systemic immune response to non-ocular antigens does not illicit damage to the eye (Taylor and Kaplan 2010).

However, various systemic autoimmune diseases are characterized by ocular manifestations that may precede systemic symptoms. These conditions are associated with breakdown of immune-privilege mechanisms of the eye, and frequently of the brain, as well. Various antigens that incite an inflammatory response are found both in the eye and the nervous system. Immune response against such antigens (e.g. Vogt-Koyanagi-Harada syndrome) results in disease manifestations that affect both organ systems.

36.3 Clinical Features of Select Autoimmune Diseases

36.3.1 Vogt-Koyanagi-Harada Disease

Vogt-Koyanagi-Harada (VKH) disease is characterized by diffuse, non-necrotizing granulomatous inflammation of the uveal tissue along with involvement of the central nervous system, auditory system and the integumentary system. VKH

consists of two entities: Harada's disease and Vogt-Koyanagi syndrome. Harada's disease (first reported by Einosuke Harada in 1926) is characterized by bilateral exudative retinal detachment along with cerebrospinal fluid (CSF) pleocytosis. Vogt-Koyanagi syndrome is associated with anterior segment inflammation (chronic anterior uveitis) along with alopecia, vitiligo and poliosis (described in 1906 by a Swiss resident in Ophthalmology—Alfred Vogt). These findings were brought together below one umbrella term, VKH, by Babel in 1932 (Herbert and Mochizuki 2007). Presently, the International Workshop on Vogt-Koyanagi-Harada Syndrome and Sympathetic Ophthalmia provides the guidelines for diagnosis, investigations, imaging interpretation and management of VKH.

36.3.1.1 Epidemiology

There is a wide geographic and ethnic variation in the incidence of VKH, with higher proportion of Japanese, Asian, Hispanic and Native Americans diagnosed with the disease. In the US, VKH disease accounts for less than 5% of all the uveitis clinic referrals. The incidence is higher in Japan, where VKH disease accounts for approximately 7% of all the uveitis referrals. Higher incidence of the disease among individuals with dark pigmented skin may be linked to the pathogenesis of the disease. Women may be more commonly affected. The most common age group for the occurrence of the disease is between the second to the fifth decade of life (Ohno et al. 1977).

36.3.1.2 Etiology and Pathogenesis

The etiology of VKH appears to be granulomatous inflammatory response to antigens located in the ocular, auditory and central nervous systems. Electron microscopic analysis of uveal tissues has revealed predominance of T-cell population in close association with melanocytes (Yamaki et al. 2000). In addition, histopathological and immunohistochemical findings suggest that VKH occurs as a result of altered T-cell response against yet undiscovered ligands of the melanocytic proteins (Sugita et al. 1996). Tyrosine-related proteins (Hayakawa et al. 2004), molecular mimicry (Sugita et al. 2007) and immunologic cross-reactivity with various external antigenic stimuli such as those caused by cytomegalovirus infection have also been shown to play a role in the etiology of VKH.

Derangement in cytokines and inflammatory mediators, such as increased levels of IL-17, IL-1 β (Li et al. 2010), IL-6 and tumor necrosis factor (TNF)- α and decreased levels IL-25 (Xu et al. 2014) has also been linked to the pathogenesis of VKH. Interleukin (IL) -21 may promote secretion of IL-17 resulting in pleiotropic effects on the immune system (Li et al. 2010). In addition, role of decreased IL-25 and increased release of IL-1 β , IL-6 and tumor necrosis factor (TNF)- α by peripheral blood mononuclear cells may play a role in the pathogenesis of the disease (Xu et al. 2014).

VKH disease has a strong immunologic basis with higher prevalence of the disease among individuals with HLA-DR1 and DR4 as compared to controls (Weisz et al. 1995). The predominant alleles associated with VKH include HLA-DRB1*0405 and HLA-DRB1*0410 (Shindo et al. 1994). The relative risk of developing VKH is higher with HLA-DR1 as compared to HLA-DR4 (4.11 versus 1.96) (Arellanes-Garcia et al. 1998; Tiercy et al. 2010). Thus, various population-based studies indicate that specific Human Leukocyte Antigen (HLA) genes may confer increased susceptibility for the development of VKH disease.

The demographic, immunologic and laboratory features of VKH disease closely resemble those of sympathetic ophthalmia (Rao 1997). This may explain the similarities in the morphological manifestations of both the diseases.

36.3.1.3 Clinical Features

Ocular features of VKH disease are divided into various stages including prodromal, acute, chronic and convalescent/recurrent stage. The disease may present initially with a viral-like illness. The patient may complain of malaise, headache, fever, orbital pain, meningisms and nausea. CSF analysis at this stage may indicate presence of pleocytosis in 80% patients. This stage is followed by the acute uveitic

stage characterized by *bilateral exudative panuveitis* that is usually symmetric (Fig. 36.1). There is anterior segment inflammation, vitritis, bilateral exudative retinal detachment (which may be multifocal and bullous), hyperemia and edema of the optic nerve head and retinochoroidal thickening detectable by ultrasound B-scan. Mutton-fat keratic precipitates and iris nodules may be visualized. Fundus fluorescein angiography reveals an impressive picture with multiple hypofluorescent dots initially followed by multiple, focal areas of hyperfluorescence (Fig. 36.1). Choroidal vessel leakage and hyperfluorescence can be detected using Indocyanine green angiography (ICGA) (Sakata et al. 2014; Cunningham et al. 2014).

This stage is followed by the stage of chronic uveitis heralded by integumentary signs such as vitiligo, poliosis and choroidal depigmentation. *Sugira's sign*, which is perilimbal vitiligo, may be observed at this stage. Depigmentation of the choroid gives an appearance of *sunset-glow fundus*. Retinal pigment epithelium (RPE) may show a moth-eaten appearance. Along with disc pallor, focal areas of yellow, well-circumscribed chorioretinal atrophy, especially in the inferior mid-periphery (earlier referred to as Dalen-Fuch's nodules), may be seen. Approximately two-third of the patients may have recurrent disease resulting in complications

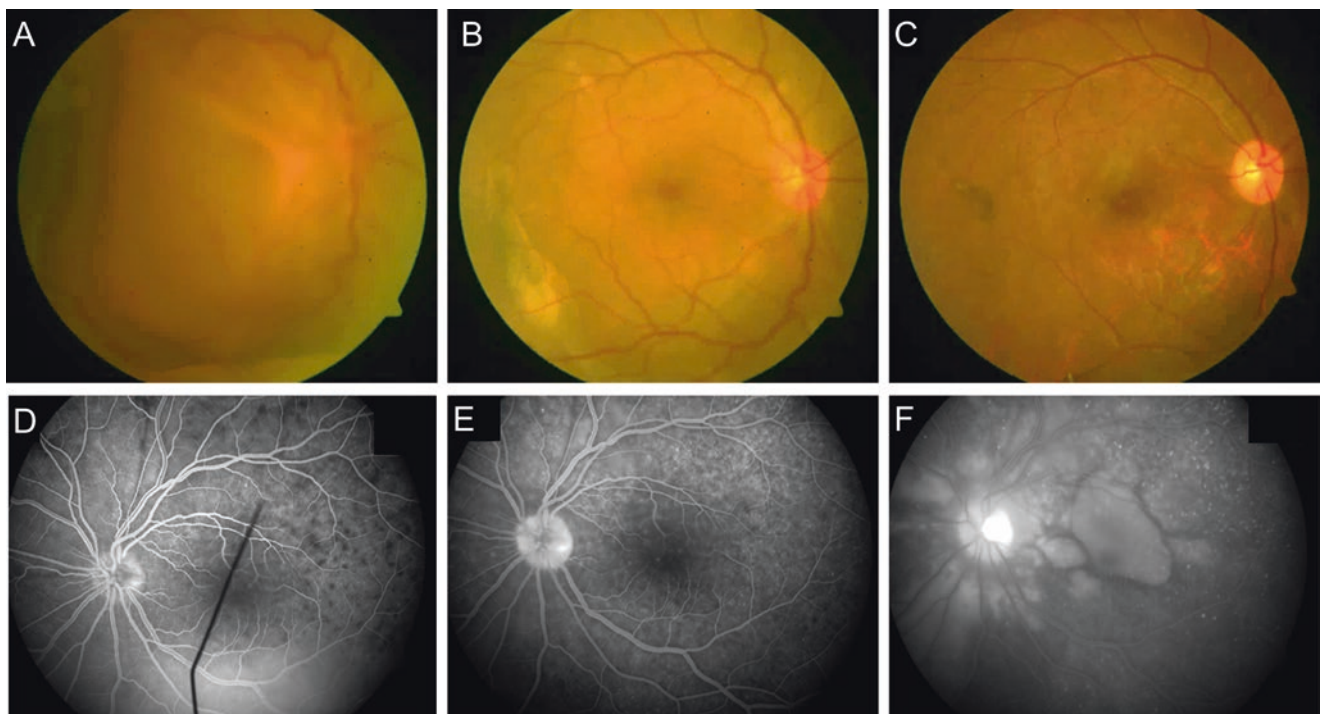


Fig. 36.1 Fundus photographs (right eye) of a 19-year-old girl diagnosed with Vogt-Koyanagi-Harada Syndrome. (a) shows the fundus photograph at presentation, with vitreous cells, optic nerve head edema and exudative retinal detachment. (b) shows improvement in the media clarity, decreased vitreous haze and exudation after 5 days of intravenous methylprednisolone (1 g/day). At 2 months (c), the media clarity has

much improved with resolution of the exudative retinal detachment. The patient received a course of oral prednisone and azathioprine. (d)–(f) show the fluorescein angiography at baseline (left eye) of the same patient. In the early phase (d), there are focal areas of hypofluorescence (temporal to fovea), which become progressively hyperfluorescent (e and f) along with pooling of the dye due to exudative retinal detachment

Table 36.1 Revised diagnostic criteria of Vogt-Koyanagi-Harada disease proposed by the first international workshop (Read et al. 2001)

I. Complete disease (criteria 1 to 5 must be present)	
	1. No history of penetrating ocular trauma or surgery preceding the initial diagnosis of uveitis
	2. No clinical or laboratory evidence suggestive of other ocular disease
	3. Bilateral ocular involvement (a or b criteria must be met, depending upon the stage of the disease at which the patient is examined)
	(a) Early manifestations of the disease
	– Diffuse choroiditis (focal areas of subretinal fluid; bullous serous retinal detachments), OR
	– Characteristic fluorescein angiography AND echography evidence of diffuse choroidal thickening
	(b) Late manifestations of the disease
	– History suggestive of prior uveitis with the above described characteristics AND ocular depigmentation (sunset glow fundus, Sugiura's sign)
	– AND other ocular signs (nummular chorioretinal depigmented scars, retinal pigment epithelium clumping and/or migration, or recurrent/chronic anterior uveitis)
	4. Neurological/auditory findings
	– Meningismus OR tinnitus, OR cerebrospinal fluid pleocytosis
	5. Integumentary findings (NOT preceding the onset of central nervous system or ocular disease)
	– Alopecia, OR poliosis, OR vitiligo
II. Incomplete disease (criteria 1 to 3 and either 4 or 5 must be present)	
III. Probable disease (isolated ocular disease; criteria 1 to 3 must be present)	

Table 36.2 Clinical findings predictive of Vogt-Koyanagi-Harada disease (Rao et al. 2010)

Acute stage	Chronic stage
Bullous retinal detachment ^a	Sunset glow fundus ^a
Choroidal thickening ^a	Sugiura's sign ^a
Subretinal fluid ^a	Vitiligo ^a
Alopecia ^a	Choroidal thickening
Hearing loss	Nummular choroidal scars
Vitiligo	Retinal pigment epithelium clumps/migration
	Hearing loss

The clinical findings with $\geq 50\%$ positive predictive value (PPV) and negative predictive value (NPV) have been listed in the table

^aIndicates clinical findings associated with $\geq 75\%$ PPV and NPV

such as subretinal fibrosis, neovascular glaucoma, cataract and painful blind eye (Rao et al. 2010).

The diagnosis of VKH may be challenging since patients may presented with a limited ocular disease without systemic manifestations. The international workshop on VKH has divided the disease into complete, incomplete and probable disease (Table 36.1).

Involvement of other melanin-containing organs presents with dermatological features (vitiligo over the head, trunk, eyelids; poliosis of the eyebrows; alopecia), neurological features (headache, meningo-encephalitis and/or focal neurological deficits) and auditory involvement (sensorineural hearing loss, tinnitus and vertigo). The frequency of distinguishing features and findings most predictive of VKH disease obtained from a cohort of 1147 patients has been summarized in Table 36.2 (Rao et al. 2010).

36.3.2 Adamantiades-Behçet's Disease

Adamantiades-Behçet's disease (ABD) is a multi-system inflammatory vasculitis characterized by autoimmune ulceration of mucous membranes, involvement of ocular, central nervous system, and articular and renal systems. Majority of the patients may present with isolated mucous membrane or ocular involvement, making the diagnosis challenging.

ABD is named after two eminent clinicians, a Greek ophthalmologist, Benedictos Adamantiades and a Turkish dermatologist, Hulusi Behçet. The disease is more prevalent in the Middle East and the Mediterranean region, or the silk route of Europe. The description of association of ocular signs and other systemic involvement dates back to the writings of Hippocrates (Zouboulis and Keitel 2003). However, due to the growing recognition of the potential visual-

blinding complications of this condition despite aggressive therapy, an International Workshop on Granulomatous Uveitis and Ocular Behçet's Disease has proposed criteria for the diagnosis and classification of ABD.

ABD is characterized by non-necrotizing uveitis along with necrotizing obliterative retinal vasculitis. It affects many organs and tends to recur every 1–2 months without therapy. Data from large population based studies from countries such as Japan, Turkey, Germany and USA suggest that the aggressive disease course may lead to legal blindness within 4 years in the absence of immunosuppression (Evereklioglu 2005).

36.3.2.1 Epidemiology

The highest prevalence of ABD has been reported from Turkey, with as many as 420 cases per 100,000 in Istanbul. The prevalence in US and Europe ranges from 0.12 to 7.5 cases per 100,000 population. The incidence of ABD is $\leq 20\%$ among all uveitis in Japan, Taiwan, Israel, Saudi Arabia, Australia and China. Thus, the disease may be associated with HLA alleles more common in certain ethnic groups. Dissemination of genetic information across gene pools may be responsible for spread of the disease to the Asian and Western population (Zouboulis and Keitel 2003).

The disease usually affects younger individuals between the second and third decades of life. Recently, ABD has been recognized as an important cause of pediatric uveitis with cases reported in patients as young as 2 months of age (Hatemi et al. 2014).

Population-based studies from the Middle East have shown that the male:female ratio of the disease is approximately 2.3:1 and it is believed to have worse prognosis in males as compared to females, who may present with less organ involvement. The rates of ocular involvement vary from 28 to 50% cases depending upon the sampled cohort.

Tables 36.3 and 36.4 provide the diagnostic criteria for ABD as per the International Team for the Revision of the International Criteria for Behçet's disease (ITR-ICBD) (2014) and the International Uveitis Study Group on Behçet's disease (1990).

36.3.2.2 Etiology and Pathogenesis

The exact etiology of ABD is unknown. The disease is multifactorial and it is accepted that ABD occurs in immunologically and genetically susceptible population, triggered by environmental agents or infections. Bacterial cross-reactivity with human antigens, cytokines and circulating immune-complexes are also implicated in the pathogenesis of this condition (Park et al. 2014).

Although classical Mendelian inheritance pattern has not been described for ABD, strong association with specific HLA genotypes has been shown. Subjects with HLA-B51/B5 have an increased risk of developing ABD. The prevalence of HLA-B51 allele among Japanese, Greek, Iranian, Italian and Saudi Arabian patients varies between 60 and 77%. The most common allele associated with ABD is HLA B*5101. Apart from HLA B51, there are several other genes located in close proximity that may be implicated in the pathogenesis of ABD (Wallace 2014).

A large number of infectious agents have been linked to the pathogenesis of ABD. Among these, the most common associations have been found with herpes simplex virus (HSV-1), *streptococcus* spp and *mycoplasma fermentas* based on oral, salivary and respiratory tract cultures. Streptococcal antigens may increase the T-lymphocyte cell-mediated IL-6 and interferon (IFN) γ release. Various antigens such as heat shock proteins, cytotoxins of *Helicobacter pylori* may play a role in the vascular damage typical of ABD (Hatemi and Yazici 2011).

Pathological immune dysfunction involving both the innate and adaptive immunity may occur in ABD. Low levels of mannose binding lectins, altered expression of toll-like receptors in monocytes and activation of adenosine deaminase may contribute towards increased T-cell expression in patients with ABD. Increased levels of TNF- α , IL-6, IL-8, IL-10 and IL-12 may increase leucocyte migration, activation of neutrophils, fibroblast proliferation and synthesis of prostaglandins. In ABD, both Th1 and Th2 cells seem to be involved in the pathogenesis. In addition to the T-cells, B cell population also plays an important role by presenting the antigens to the T-cell population (Park et al. 2014).

Table 36.3 International criteria for Behçet's disease (ICBD) by the international team for the revision of the international criteria for Behçet's disease (ITR-ICBD) (2014) (Davatchi et al. 2014)

Sign/symptom	Points
Ocular lesions	2
Genital aphthosis	2
Oral aphthosis	2
Skin lesions	1
Neurological manifestations	1
Vascular manifestations	1
Positive pathergy test ^a	1

Score of ≥ 4 indicates Behçet's disease

^aPathergy test is optional and primary scoring system does not include the test

Table 36.4 International study group for Behçet’s disease diagnostic criteria (1990) (IUSG 1990)

Mandatory: Recurrent oral ulceration (<i>minor aphthous, major aphthous, or herpetiform ulceration observed by a physician or patient, which recurred at least three times in 1–12 month period</i>)
Plus two of the following criteria:
Recurrent genital ulceration (<i>aphthous ulceration or scarring, observed by physician or patient</i>)
Eye lesions (<i>anterior uveitis, posterior uveitis, or cells in the vitreous, or retinal vasculitis observed by ophthalmologist</i>)
Skin lesions (<i>erythema nodosum observed by physician or patient, pseudofolliculitis, or papulopustular lesions, or acneiform nodules observed by physician in post-adolescent patients not on corticosteroid treatment</i>)
Positive pathergy test (<i>read by physician at 24–48 hours</i>)

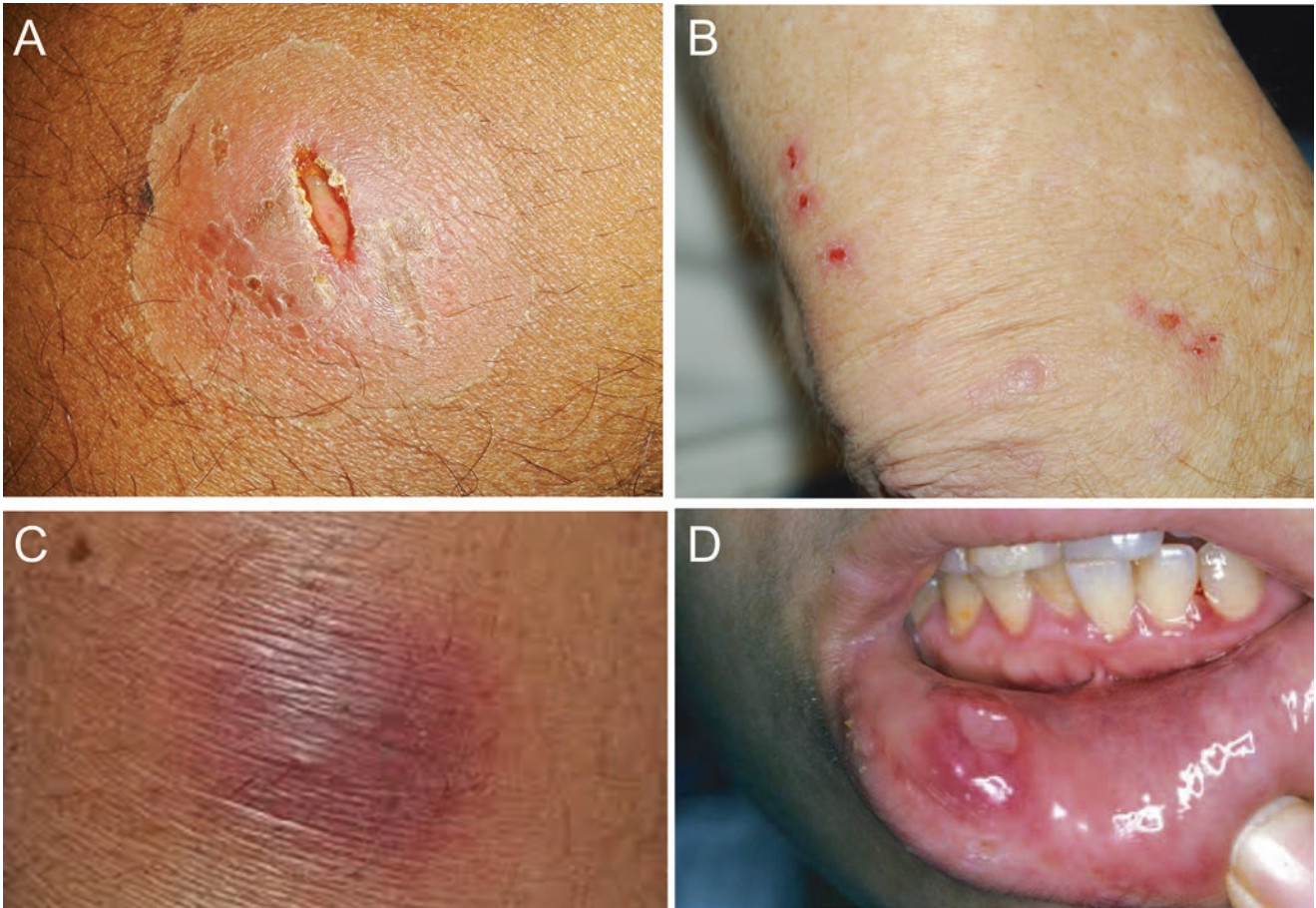


Fig.36.2 Various skin and mucous membrane lesions in Adamantiades- Behçet’s disease (ABD). (a) shows a large, pustulo-nodular lesion with central ulceration in the thigh of a young male patient. (b) shows multiple erythematous, acneiform lesions at the elbow in another Caucasian

patient with ABD. (c) shows a dark, plaque-like lesion during the active stage of the disease. (d) shows a large, oral aphthous ulcer on the buccal mucosa of a patient with ABD

Vascular thrombosis in ABD is associated with increased levels of nitric oxide, plasma metabolites, low concentration of Protein C, increased platelet activation and lower plasma levels of tissue plasminogen activator. These may occur as a result of endothelial dysfunction leading to a generalized state of hypercoagulability, especially in cases with neuro-Behçet’s disease. Plasma and cerebrospinal fluid concentrations of vascular endothelial growth factor have been shown to increase in patients with neurological complaints in ABD (Evereklioglu 2005; Hatemi et al. 2014).

36.3.2.3 Clinical Features

The most frequent, disease-defining clinical feature of ABD is painful oral *aphthae* and mucocutaneous ulcers. These are more common in males and appear round-bottomed, yellowish-white surrounded by an erythematous halo. Major ulcers (more than 1 cm in diameter) may leave behind residual scarring. Genital ulcers are less common and not mandatory for the diagnosis of ABD. They usually involve the scrotum or vulva. Epididymitis and salpingitis are rarer complications of the disease. Acneiform skin lesions, erythema

nodosum and pyoderma gangrenosum-like lesions are other dermatological manifestations of ABD (Fig. 36.2).

Vascular involvement may lead to aneurysms, cardiovascular events, hemoptysis, dyspnea and gastrointestinal manifestations due to Budd-Chiari syndrome. Aches and pains due to asymmetric, non-erosive, non-deforming arthritis are common in ABD (Mat et al. 2014).

Central nervous system involvement may occur due to thrombosis of dural venous sinuses. Neurological involvement usually occurs after 5 years of the diagnosis and affects 5% patients with ABD. There may be small vessel vasculitis (*vascular-Behçet*), focal neurological deficits, headache, pyramidal, cerebellar or cognitive defects, memory loss, aseptic meningitis, extrapyramidal symptoms, euphoria and disinhibition, or magnetic resonance imaging (MRI) abnormalities but absence of symptoms (*silent-Behçet*). Neuromuscular involvement may present with multiple mononeuritis or myositis (Siva and Saip 2009; Saip et al. 2014).

Ocular manifestations may occur in as many as 83–95% males and 67–73% females with ABD. Ocular symptoms usually follow mucocutaneous lesions and recurrence is common. All the structures of the eye may be involved in ABD, leading to severe debilitating visual loss. The classical finding in the anterior segment is presence of iridocyclitis with *mobile hypopyon*, which may be seen in less than one-third cases. Anterior chamber may show cells, flare, fibrin and endothelial dusting suggestive of non-granulomatous inflammation. There may be evidence of episcleritis or conjunctival ulcers. Posterior segment may show vitritis and retinal vasculitis involving both arteries and veins. Perivascular sheathing and hemorrhages may be observed. Vascular thrombosis may lead to branch or central retinal vein/artery occlusion. Papillitis and papilledema may occur that may progress to optic atrophy. Retinitis may be seen in as many as 52% patients with ocular involvement. Larger lesions of retinitis may lead to scarring resembling viral retinitis. Exudative retinal detachment may also occur (Khairallah et al. 2009; Bonfioli and Orefice 2005) (Fig. 36.3).

Fluorescein angiography may reveal vascular tortuosity, occlusion, staining of vessel walls and capillary leakage (Fig. 36.4). Shunt vessels and neovascularization may develop due to widespread ischemia. ICGA may reveal irregular filling of choriocapillaris and other choroidal perfusion defects (Durrani et al. 2007).

36.3.3 Sarcoidosis

Sarcoidosis is a multi-system chronic inflammatory disease characterized by presence of non-caseating granulomas most commonly affecting the respiratory system, lymph nodes, central nervous system, skin and the eyes. This condition was first recognized by Hutchinson in 1869 and the term '*sarkoid*' was coined by a Norwegian dermatologist, Caeser

Boeck in 1899 to describe the skin lesions. Association of sarcoidosis with uveitis was first described in early twentieth century as a combination of facial nerve palsy and uveoparotid fever (Heerfordt's syndrome). Acute systemic inflammation may present as Löfgren's syndrome (fever, polyarthritis and erythema nodosum). In the last few decades, there has been a great increase in knowledge regarding the pathogenesis and manifestations of the disease.

Ocular disease may occur in up to one-third patients with the disease at any time during the natural history of the condition. Uveitis is the most common ocular manifestation and is usually bilateral. Granulomas may involve the periorbital, lacrimal gland, extraocular muscles and adnexa (conjunctiva and lid). The spectrum of disease manifestations is very wide; neuro-ophthalmologic signs such as optic neuropathy or Horner's syndrome may also occur in sarcoidosis.

The diagnosis of sarcoidosis is often challenging due to variable manifestations, which may often appear to overlap with other clinical entities. Since most of the patients have a chronic progressive disease, early recognition and treatment can prevent ocular complications and systemic sequelae of sarcoidosis. The diagnostic criteria for ocular sarcoidosis by the first International Workshop on Sarcoidosis (IWOS) is provided in Table 36.5.

36.3.3.1 Epidemiology

Sarcoidosis accounts for nearly 5% of all the adult uveitis cases and 1% of the pediatric cases (Hoover et al. 1986). Ocular involvement in sarcoidosis may be in up to 40% cases. The incidence of sarcoidosis is higher among African Americans as compared to Caucasians, with an incidence of 35.5 per 100,000 individuals versus 10.9 per 100,000 individuals respectively. The African American population is more likely to have a chronic, progressive course with higher number of patients developing ocular manifestations. Thus, blinding complications are also more common in this ethnic group. The incidence of sarcoidosis peaks between the age groups of 20–39 years. The incidence of the disease is higher among women across all racial and ethnic groups. Overall, the highest annual incidence is seen in northern European countries (Iannuzzi et al. 2007).

36.3.3.2 Etiology and Pathogenesis

The etiology of Sarcoidosis remains unknown. Due to multi-system involvement, environmental causes and occupational influences such as irritants, inorganic particulate matter and metals in furnaces, have been evaluated as possible causative agents. Investigators have also reported mycobacterial DNA in the sarcoidal tissue. A number of ubiquitous environmental influences may play a role in the alteration of immune response in sarcoidosis.

The most common HLA associated with sarcoidosis is HLA B8. Genetic factors located in the HLA class II region,

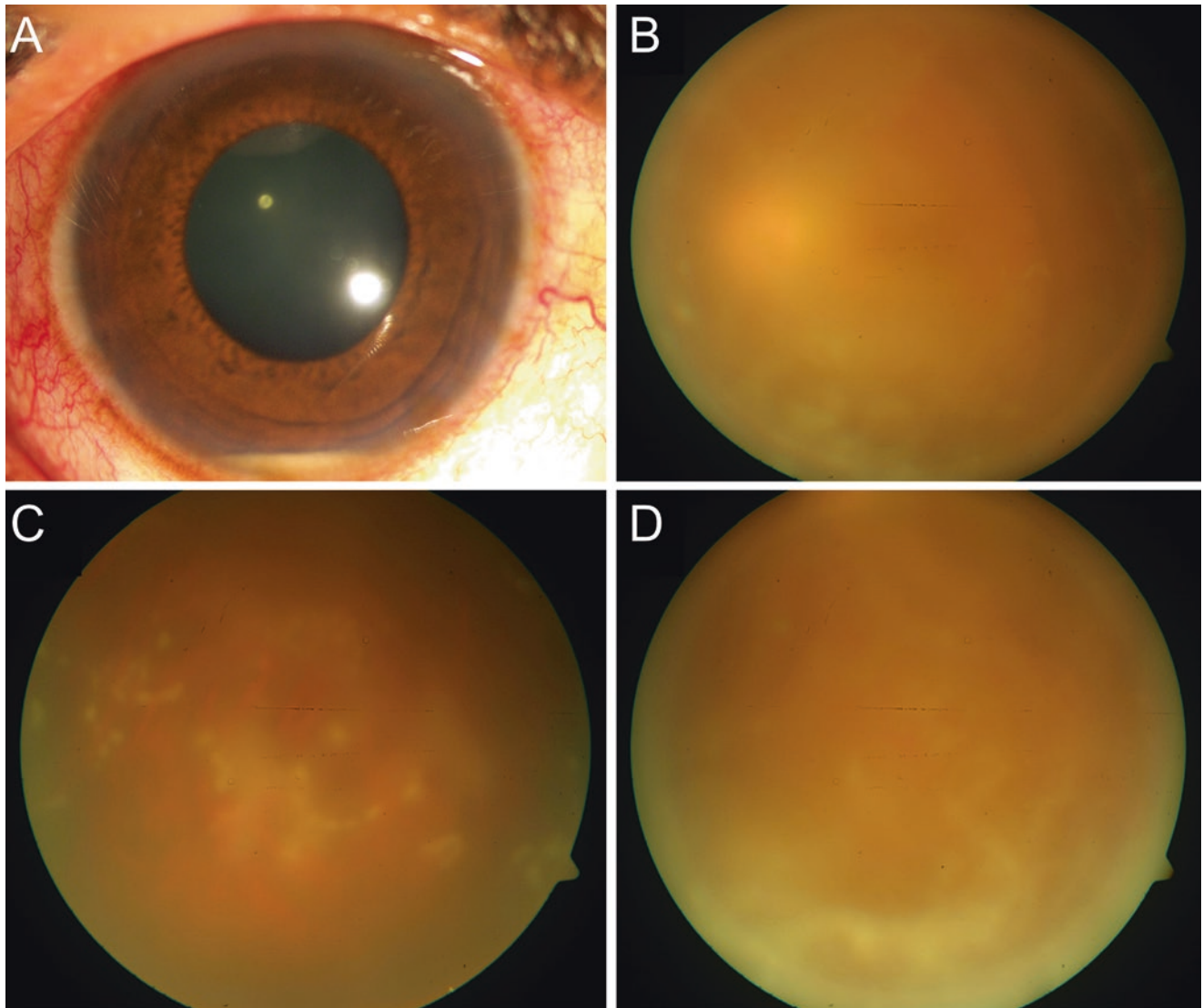


Fig. 36.3 Fundus photographs of an 18-year-old male diagnosed with Adamantiades-Behçet's disease. Slit-lamp photography of the anterior segment (a) shows ciliary congestion, cells in the anterior chamber and

a hypopyon. Posterior segment examination revealed dense vitritis (b), multiple exudates in the vitreous cavity (c), forming posterior hypopyon (d)



Fig. 36.4 Fluorescein angiography of the same patient as in Fig. 36.3 shows media haze in the early frame (a), diffuse hyperfluorescence in the mid-phase (b), optic disc leakage and vascular staining in the late

phase (c), suggestive of active vasculitis and optic neuritis in a case of Adamantiades Behçet's disease

Table 36.5 Diagnostic criteria^a for ocular sarcoidosis by the first international workshop on ocular sarcoidosis (IWOS) (2009) (Herbert et al. 2009)

Definite ocular ^b sarcoidosis	Biopsy-supported diagnosis with a compatible uveitis
Presumed ocular ^b sarcoidosis	Biopsy not done; presence of BHL with a compatible uveitis
Probable ocular ^b sarcoidosis	Biopsy not done; negative BHL; presence of three suggestive intraocular signs and two positive investigational tests
Possible ocular ^b sarcoidosis	Biopsy negative; four suggestive intraocular signs positive; two positive investigational tests

BHL bilateral hilar lymphadenopathy

^aAll other causes of uveitis, especially tuberculosis must be ruled out in all cases

^bThe term 'ocular' is used for both intraocular inflammatory lesions in patients with systemic disease and in patients with disease seemingly limited to the eye without any clinically detectable involvement of any systemic organ

i.e. DRB1 and DQB1 have been also implicated in the pathogenesis. HLA DQB1*0201 and DRB1*0301 have been associated with a better prognosis. In addition, familial pattern of disease occurrence has also been described. Sarcoidosis is also noted to occur after organ transplantation (Iannuzzi et al. 2007; Umur et al. 2012).

CD4 T helper cell population that interacts with antigen presenting B cells may play a central role in the pathology of sarcoidosis. Th1 cells produce IL-2, IFN- γ and TNF- α resulting in information of multisystem granulomas. Hyperglobinemia and hypercalcemia are other laboratory abnormalities in sarcoidosis (Boyd et al. 2001).

36.3.3.3 Clinical Features

Sarcoidosis most commonly presents with anterior uveitis. Posterior uveitis, retinal vasculitis, vitritis, choroidal granulomas and papillitis are other manifestations of the disease. The disease may involve the lacrimal gland resulting in enlargement, orbital inflammatory disease (*pseudotumor*), myositis, scleritis, keratitis and cranial nerve palsies. Uveitis can precede systemic involvement in 30% cases of sarcoidosis (*limited ocular sarcoid*).

Anterior uveitis may be non-granulomatous or chronic granulomatous (more common) with large mutton-fat keratic precipitates, iridocyclitis and attacks of glaucoma. Interstitial keratitis and conjunctival nodules may be noted.

Posterior segment disease may present with vitreous snowballs and debris. The classical perivascular sheathing in sarcoidosis is termed as '*candle wax drippings*' (*taches de bougie*). There may be multiple, round, punched-out choroidal lesions associated with macular edema (Fig. 36.5). Choroidal granulomas are observed as whitish elevated mass. Optic nerve leakage may be seen on fluorescein angiography. There may be papillitis, optociliary shunt vessels and macro-aneurysms. Clinical signs suggestive of ocular sarcoidosis are provided in Table 36.6.

Neurological manifestations associated with sarcoidosis (*neurosarcoid*) include encephalopathy, hypothalamic dysfunction, cranial nerve palsies, and pituitary disorders. Chiasmal syndromes and motility disorders may occur.

Direct infiltration of the sarcoid mass can affect the optic nerve, leading to optic atrophy (Fig. 36.5). Facial nerve palsy may be associated with significant corneal sequelae due to exposure keratopathy (Umur et al. 2012; Jamilloux et al. 2014).

36.3.4 Multiple Sclerosis

Multiple sclerosis (MS) is a chronic, relapsing demyelinating disease involving the central nervous system and the eye. Ocular and neuro-ophthalmic manifestations of the condition are diverse and the disease may affect virtually any anatomical structure including the components of the visual pathway. MS is one of the most common diseases of the central nervous system and its discovery dates back to the anatomical drawings from autopsies in the early nineteenth century. MS was recognized as a clinical entity in 1873 by Dr. Walter Moxon in England and Dr. Edward Seguin in 1878 in the US. Since then, efforts were made by scientists worldwide to establish the etiopathogenesis of the disease (Murray 2009).

Optic neuritis and uveitis are the two main clinical manifestations of MS in the eye. The association between MS and uveitis was first described in 1910. However, despite decades of intensive research and multicenter clinical trials, much needs to be learnt about the pathology of ocular disease in MS.

36.3.4.1 Epidemiology

MS affects individuals of all ages and race. The disease prevalence varies greatly and the incidence is higher in North America as compared to Asia. The typical onset of MS is between the second and the fifth decade of life. However, MS can occur in childhood as well as old age. Women are more commonly affected. Data from two large uveitis centers in the US and Germany showed that more than 90% patients presenting with uveitis associated with MS were females. The mean age of presentation in this cohort was 40.6 years (Toosy et al. 2014).

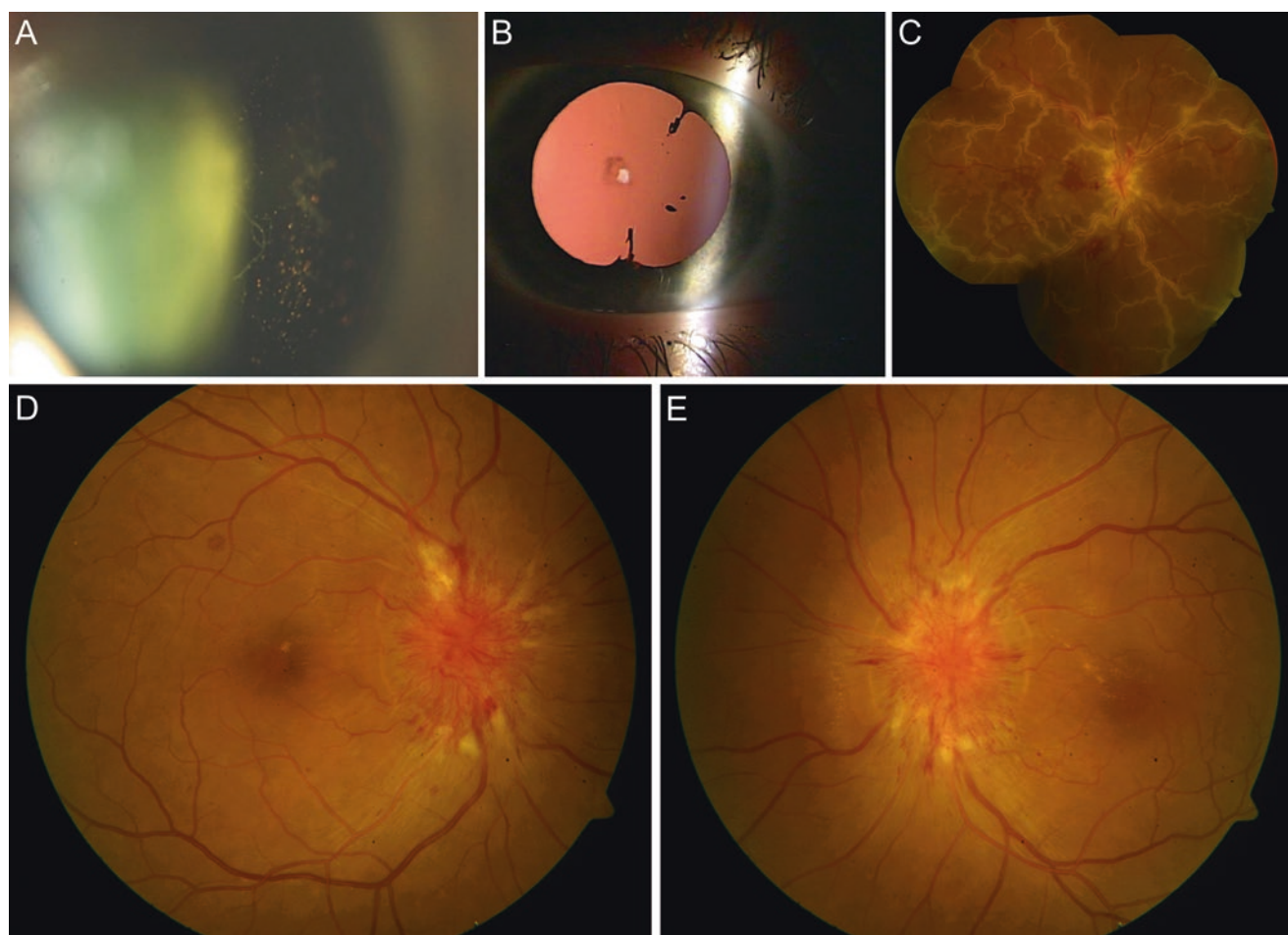


Fig. 36.5 Dense vitritis in a patient diagnosed with ocular sarcoidosis (a). (b) demonstrates tent-like posterior synechiae, observed in retroillumination anterior segment slit-lamp photograph of a patient diagnosed with sarcoidosis. (c) shows diffuse vasculitis (termed as frosted

branch angiitis) in another patient with sarcoidosis. (d) and (e) show fundus photographs of a patient diagnosed with neuro-sarcoidosis. The optic nerve heads in both eyes show direct infiltration with the sarcoid mass

Table 36.6 Ocular signs predictive of sarcoidosis as per the international workshop on ocular sarcoidosis (IWOS) (Herbort et al. 2009)

1.	Mutton-fat keratic precipitates (large/small) and/or iris nodules at pupillary margin (Koeppe) or in stroma (Bussacca).
2.	Trabecular meshwork nodules and/or tent-shaped peripheral anterior synechiae
3.	Snow-balls/string of pearls vitreous opacities
4.	Multiple chorioretinal peripheral lesions (active and atrophic)
5.	Nodular and/or segmental periphlebitis (\pm candle wax drippings), and/or macro-aneurysm in the inflamed eye
6.	Optic disc nodule(s)/granulomas(s) and/or solitary choroidal nodule
7.	Bilaterality (assessed by clinical examination or investigational tests showing subclinical inflammation)

36.3.4.2 Optic Neuritis and Multiple Sclerosis

Optic neuritis may be the presenting symptom of MS in 25 % of the cases (*MS-associated optic neuritis*). About 70 % patients with MS may develop optic neuritis during the disease course, usually in the relapsing-remitting stage. MRI studies have revealed disseminated white matter lesions suggestive of demyelinating disease in more than 50 % patients

with optic neuritis. As many as 72 % patients with MRI abnormalities convert to MS within 15 years (*clinically silent MRI lesions*). Bilateral simultaneous optic neuritis is rare, but sequential involvement of the fellow eye is common (Toosy et al. 2014).

Optic neuritis is usually preceded by periorbital or ocular pain in a majority of the patients. The pain may be

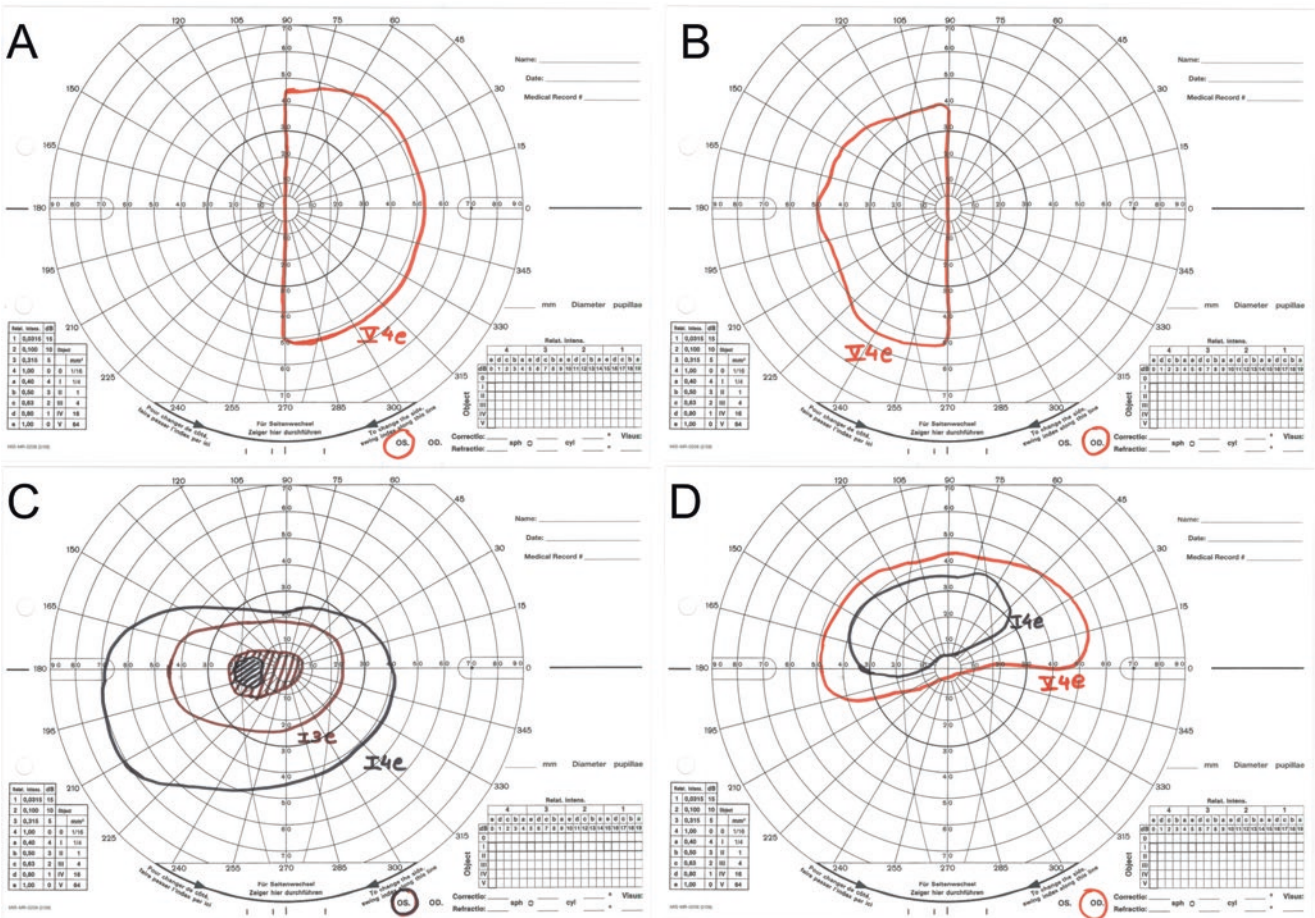


Fig. 36.6 Various visual field defects that can be observed in patients with optic neuritis secondary to multiple sclerosis using Goldman Visual Fields (GVF). (a) and (b) demonstrate bitemporal hemianopia. (c) shows a patient with cecentral scotoma. Panel (d) demonstrates altitudinal defect in a patient with optic neuritis

Table 36.7 Visual phenomena in optic neuritis associated with multiple sclerosis (Toosy et al. 2014)

Phosphenes	Bright fleeting, flashes of light that tend to be connected to eye movement
Uhthoff's phenomenon	Worsening of vision provoked by small increases in body temperature attributed to exercise, hot baths or showers, or hot weather conditions
Pulfrich's phenomenon	Anomalous stereoscopic perception of objects in motion due to asymmetrical conduction between the optic nerves

increased with extraocular movements and usually lasts several days. Optic neuritis usually presents with acute monocular vision loss, the severity of which may vary from mild visual field defects to no light perception. The peak usually reaches in about 2 weeks. Visual field defects include diffuse loss, central scotoma, arcuate and nasal-step scotomas, and altitudinal defects (Fig. 36.6). Visual field mapping with short wavelength automated perimetry may be more sensitive than conventional perimetry in detecting these losses. Color vision and contrast sensitivity may be impaired in more than 75 % patients. The disease course is characterized by foggy vision, dyschromatopsia (blue-yellow or red-green), phosphenes, *Uhthoff's phe-*

nomenon and *Pulfrich's effect* (Brodsky et al. 2008) (Table 36.7).

Clinical examination reveals relative afferent pupillary defect (Marcus Gunn pupil) in the affected eye. The appearance of the optic disc may be normal initially. However, later stages of the disease are characterized by swelling of the optic nerve head, peripapillary flame-shaped hemorrhages, and loss of spontaneous pulsations (Fig. 36.7). About two-third of patients may show normal appearance of the optic disc (*retrobulbar optic neuritis*). Retinal peripapillitis may occur but retinal exudates forming macular star, or vitreous cells should arouse a suspicion of an alternate diagnosis (Jacobs and Galetta 2004; Kaur and Bennett 2007).

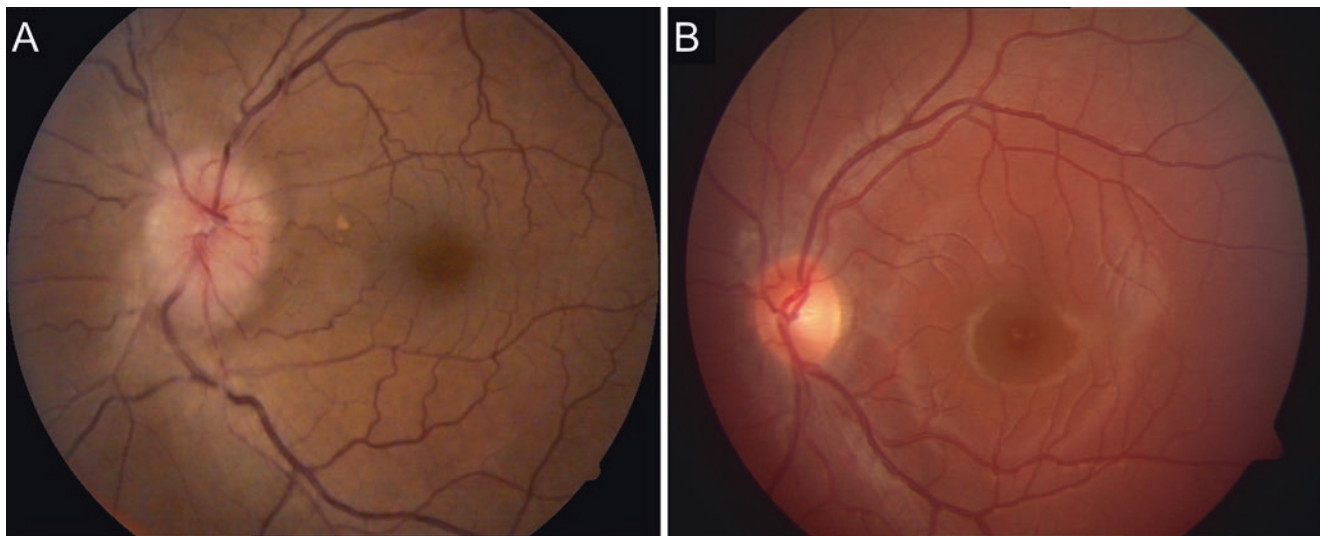


Fig. 36.7 Fundus photographs of two patients diagnosed with optic neuritis. (a) shows a patient diagnosed with papillitis with blurring of the disc margins, hyperemia of the optic nerve head and retinal nerve

fiber layer edema. (b) shows a normal appearing optic nerve head of a patient diagnosed with retrobulbar optic neuritis

Visual improvement in optic neuritis associated with MS correlates poorly with initial loss. Visual recovery is generally good with $\geq 90\%$ patients achieving visual acuity of 20/40 or better. However, the incidence of persistent defects such as color vision, contrast sensitivity, pupillary reactions and motion perception (indicating damage to the magnocellular pathway) are high (Beck et al. 1994).

36.3.4.3 Uveitis Associated with Multiple Sclerosis

The incidence of uveitis ranges from 0.4 to 26.9% among patients diagnosed with MS; however, it is infrequently recognized as an important association with the disease. Uveitis is 10 times more common in patients with MS as compared to the general population. Most cases are bilateral and present with intermediate uveitis that affects the vitreous, peripheral retina and the pars plana. Macular involvement with cystoid macular edema is the most important prognostic factor for visual outcome. The incidence of MS among patients with intermediate uveitis is 16% and is higher among individuals with HLA DR15 allele.

Intermediate uveitis is characterized by inflammation of the pars plana and the peripheral retina. There are vitreous cells, usually in the anterior vitreous. Pars plana exudates can result in formation of a snow-bank. The complications of this condition may lead to formation of cataract, glaucoma and epiretinal membrane.

Uveitis in MS may present as an isolated granulomatous anterior uveitis with mutton-fat keratic precipitates, similar to VKH or sarcoidosis. Since the clinical findings of anterior

uveitis are not pathognomic for any condition, a high level of suspicion is necessary to rule out MS (Kaya et al. 2014; Messenger et al. 2015).

36.3.4.4 Neuro-Ophthalmic Manifestations of Multiple Sclerosis

MS may be associated with a diverse range of ocular motor defects and nerve palsies. Isolated cranial nerve palsies most commonly affect the abducens nerve. Oculomotor and trochlear nerves are less commonly involved. This may lead to development of gaze palsies and deficits in pursuit, saccades and vestibular eye movement. Saccades, ocular flutter, opsoclonus and saccadic oscillations may also occur.

Lesions in the dorsal mid-brain may result in convergence retraction nystagmus, pupillary light-near dissociations. Nystagmus can be vertical, vestibular, pendular or periodic alternating. *Internuclear ophthalmoplegia* (INO) is the commonest form of nystagmus in patients with MS and occurs in up to 40% patients with the disease. INO is characterized by limitation of adduction of the ipsilateral eye and rapid nystagmus during abduction of the contralateral eye due to lesions in the medial longitudinal fasciculus. MS is associated with an increased risk of bilateral INO.

The other neuro-ophthalmic manifestations of MS include diplopia, skew deviation, oscillopsia and blurring of vision. These manifestations may greatly reduce the quality of vision among patients with MS (Kaur and Bennett 2007). The rarer manifestations of MS include Charles Bonnet Syndrome, characterized by complex visual hallucinations in a person with partial or complete blindness (Pula and Reder 2009).

36.3.5 Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease with protean ocular and neuro-ophthalmic manifestations. The presentations are diverse and the disease may have a life-threatening course. Formation of autoantibodies and presence of circulating immune complexes resulting in tissue damage are the hallmark of this condition. The altered immune response in SLE is associated with inflammatory and thrombotic effects. Anti-phospholipid syndrome is a component of SLE characterized by formation of autoantibodies against plasma proteins resulting in venous and arterial thrombosis (Tsokos 2011).

36.3.5.1 Epidemiology

Approximately 90% patients with SLE are females. The peak of the disease occurs between late teens to the fourth decade of life. The prevalence of the disease varies from 40/100,000 individuals in North Europe to more than 200/100,000 among African Americans (Tsokos 2011).

36.3.5.2 Etiology and Pathogenesis

Since the prevalence of the disease is higher among women, a role of female sex hormones has been described in the pathogenesis of SLE. Lupus can be drug-induced or may follow an antecedent viral-like illness. Other environmental factors linked with SLE include exposure to ultraviolet radiation (Mak and Tay 2014).

Genetic factors have also been implicated in the pathogenesis of SLE. The concordance rate is 25% among monozygotic twins and 2% among dizygotic twins. The pathogenesis of the disease has also been linked to HLA haplotypes such as HLA A1, B8 and DR3. The HLA subtype may be associated with enhanced response of the T cells to antigenic stimuli. In addition, genetic influences leading to deficiency of early complement factors such as C1q or C2 have also been linked to SLE.

Autoantibodies play a key role in the pathogenesis of SLE. Activation of T- and B lymphocytes occurs via various cellular pathways along with tissue injury due to formation of circulating immune complexes. Antibodies associated with SLE include anti-RO, anti-LA, anti-C1q, anti-Sm, anti-nucleosome and anti- α actinin, among several others (Tsokos 2011).

36.3.5.3 Clinical Features

Ocular manifestations may occur in more than one-third of patients diagnosed with SLE. SLE can occur at almost any anatomical location in the eye and the visual pathway. The most common finding of SLE is dry eye or keratoconjunctivitis sicca. SLE may be associated with secondary Sjögren's syndrome. Slit-lamp examination may reveal diffuse or localized corneal epitheliopathy and reduced tear production.

Severe corneal involvement may present with peripheral ulcerative keratitis, interstitial keratitis and corneal melt/ulceration. SLE may be associated with conjunctivitis, episcleritis and scleritis (Read 2004).

Anterior segment involvement can present with anterior uveitis. Lupus associated retinopathy may occur in 3–29% patients with the disease. SLE-associated retinopathy is secondary to microangiopathy caused by immune-complex mediated damage to retinal vascular endothelium. It is characterized by cotton wool spots, intraretinal hemorrhages, arteriolar narrowing, retinal edema and exudation. Severe vaso-occlusive disease is rare but can present with sight-threatening complications. Large vessel (central or branch retinal arteriolar/venular) occlusions may be observed. Patients with diffuse retinal vascular damage are at high risk for visual loss. Frosted branch angiitis and extensive macular exudation has also been reported in association with SLE (Palejwala et al. 2012). Although less known than retinopathy, lupus choroidopathy may be more common than what is generally appreciated. It usually serves as a sensitive indicator of lupus activity. The presence of SLE choroidopathy is generally indicative of coexistent (although sometimes occult) nephropathy, central nervous system (CNS) vasculitis, and other SLE visceral lesions (Nguyen et al. 2000).

Approximately 30–40% patients may present with involvement of the central nervous system (*neuropsychiatric lupus*). This condition is characterized by psychosis, neurological deficits resulting from thrombosis, limbic encephalitis, mononeuritis complex and polyneuropathy (Hanly 2014). Optic neuritis in association with SLE is rare (Lin et al. 2009).

36.3.6 Systemic Vasculitides Associated with Ophthalmic Manifestations

Systemic vasculitis is caused by an autoimmune process and is characterized by leucocytic infiltration of the vascular wall and necrosis. Rarely, systemic vasculitis may be associated with concomitant ophthalmic manifestations, including inflammation of the ocular vasculature. This is distinguished from retinal vasculitis, which is a term used to describe inflammatory involvement of retinal arterioles or venules. Retinal vasculitis usually occurs as an isolated, idiopathic disease.

Systemic vasculitis can have protean ophthalmic manifestations and these may precede systemic involvement, making the diagnosis challenging. Central nervous system involvement may result in neuro-ophthalmologic symptoms in addition to ocular inflammation. Among systemic vasculitis, granulomatosis with polyangiitis (GPA; previously known as Wegener's granulomatosis), polyarteritis nodosa (PAN) and giant cell arteritis (GCA) are common entities with ophthalmic manifestations (Abu El-Asrar et al. 2005).

36.3.6.1 Granulomatosis with Polyangiitis

GPA is a rare, systemic autoimmune disease associated with anti-neutrophil cytoplasmic antibody (ANCA)-mediated vasculitis and predilection to involve the respiratory tract and kidneys. Ocular involvement is known to occur in 28–59 % patients with GPA. On the other hand, neurological manifestations may occur in 10–45 % patients. The disease is most common in the Caucasian population and the peak incidence is observed in the fifth decade of life (Tarabishy et al. 2010).

The most common ocular manifestation seen in GPA is necrotizing scleritis, which may occur in more than 50 % patients with ocular GPA. Thinning of the sclera may result in perforation. GPA may also be associated with episcleritis and tarso-conjunctival disease resulting in formation of fibrosis, granulomas and eyelid abnormalities such as trichiasis and entropion. Orbital inflammatory disease (pseudotumor) may present with pain, epiphora, proptosis, lacrimation and movement disorders (Isse et al. 2013). Paranasal granulomas in GPA may result in *bony erosion* and secondarily involve the orbit and adnexa (Jiang et al. 2013). Optic nerve dysfunction may occur, either due to compression or direct invasion with granuloma formation. Ensuing fibrosis may result in orbital socket compression and enophthalmos (Tan et al. 2014).

Corneal involvement may present with peripheral ulcerative keratitis, interstitial keratitis or it may be associated with scleritis (*sclerokeratitis*) (Florine et al. 1993). Retinal disease results in severe visual morbidity. Retinal involvement may present with chorioretinitis, macular edema, exudative retinal detachment or retinal necrosis. Associated retinal vasculitis may result in central or branch retinal artery or vein occlusion.

36.3.6.2 Polyarteritis Nodosa

PAN is a multisystem necrotizing vasculitis affecting small and medium-sized arteries in the heart, kidneys, central nervous system and gastrointestinal system. Ocular involvement in PAN is seen in 10–20 % of the cases. The incidence of PAN is higher among males between the age-groups 40 and 60. The most common ophthalmic association is *choroidal ischemia*, which is usually recognized at autopsy (Vodopivec et al. 2014).

The ocular manifestations of PAN include retinal vascular diseases such as retinal vasculitis, hemorrhage, edema and central or branch retinal artery occlusion. Vascular supply of the optic nerve may be affected resulting in anterior or posterior ischemic optic neuropathy (Emad et al. 2007). Similar to GPA, PAN may be associated with scleritis, episcleritis, keratitis, non-granulomatous iritis and exudative retinal detachment.

Neurological disease may manifest with papillitis, Extraocular muscle palsies, amaurosis fugax, homonymous hemianopia and rarely, nystagmus. Mononeuropathy

multiplex is among the most common findings in patients with PAN (Paula De Carvalho Panzeri Carlotti et al. 2004).

36.3.6.3 Giant Cell Arteritis

GCA is the most common form of granulomatous medium-to-large vessel vasculitis. The most common vessels affected include aorta, extradural cranial vessels, ophthalmic artery, posterior ciliary arteries, superficial temporal artery and occasionally, the central retinal artery. The disease is most common above 50 years of age and the incidence increases with age. Individuals of Scandinavian or North European origin have the highest incidence of the disease. Familial clustering of the disease is noted along with HLA DRB1*04, DRB1*01 and DW6 associations (McAlinden et al. 2014).

Visual loss in GCA was first described by Jennings in 1938. Ophthalmic involvement in GCA presents as sudden-onset visual loss and is considered to be an *ophthalmic emergency*. The diagnosis of the condition is confirmed by biopsy of the temporal artery, which presents as nodular granulomatous infiltration of the vessel wall. However, the treatment with high-dose glucocorticosteroids is initiated prior to the biopsy results due to the high risk of blinding complications and fellow eye involvement. The involvement of the fellow eye can occur within 14 days in one-third of the untreated cases (Kale and Eggenberger 2010).

The most common ocular manifestation of GCA is arteritic anterior ischemic optic neuropathy (AAION), which can result in acute visual loss in 60 % patients. Up to 21 % patients may have no light perception. AAION manifests with a chalky white optic disc edema. Posterior ischemic optic neuropathy usually presents with a normal appearing optic nerve head. Central retinal artery occlusion can occur in up to 10 % patients with GCA (Kale and Eggenberger 2010).

Neuro-ophthalmic manifestations of GCA include extraocular muscle ischemia resulting in paresis/palsies, Horner's syndrome, anterior segment ischemia, hypotony and diplopia. Uveitis is an uncommon manifestation of GCA. Temporal or occipital headache, scalp tenderness, *jaw claudication* and constitutional symptoms such as fever, malaise, anorexia and myalgia are important clues to the diagnosis of this condition (Chew et al. 2009).

36.3.7 Systemic Arthritides Associated with Ophthalmic Manifestations

36.3.7.1 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is the most common systemic autoimmune condition affecting 1 % of the population. The principle target of the inflammatory damage in RA is the

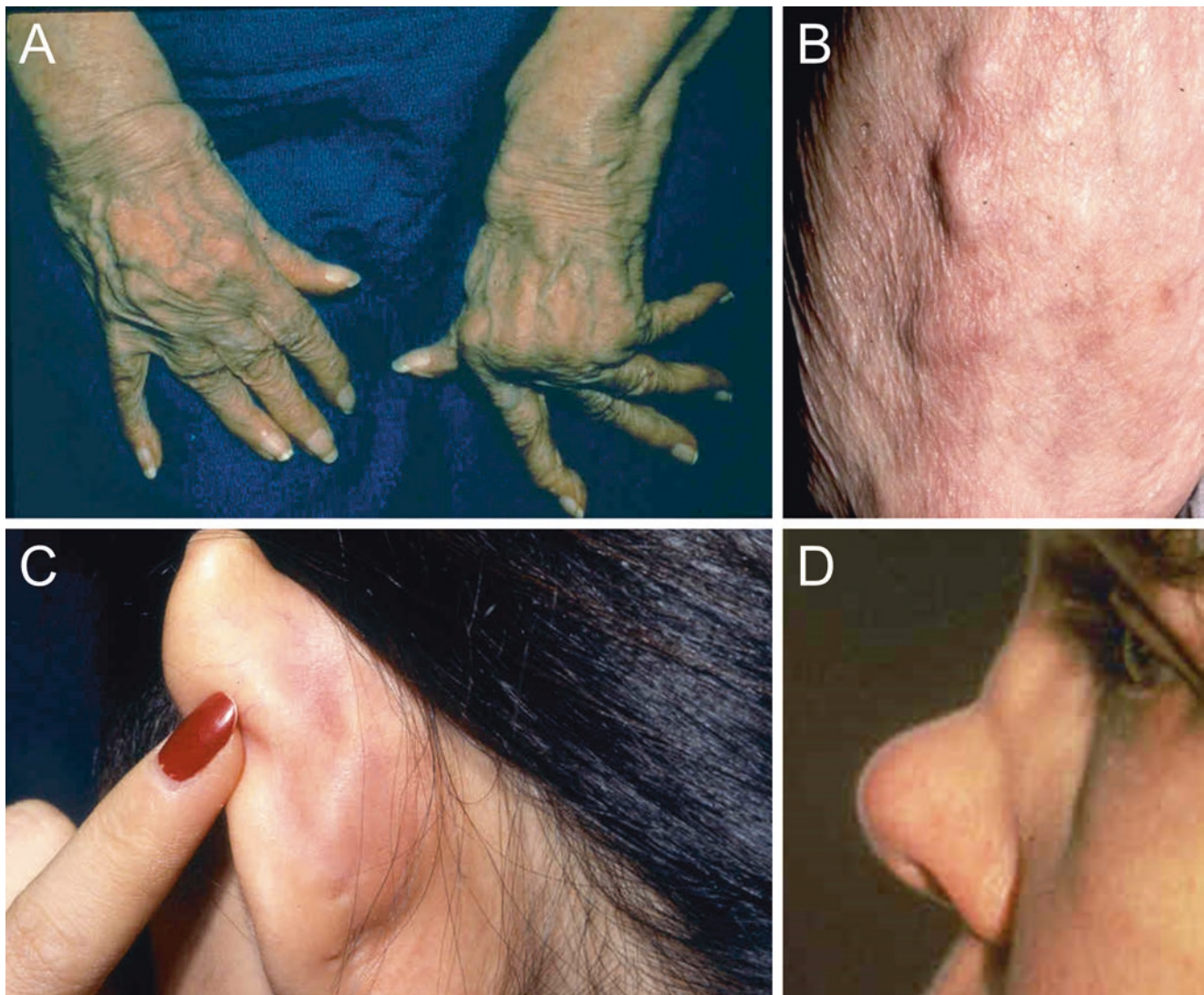


Fig. 36.8 Systemic deformities seen in patients with rheumatoid arthritis (RA) and relapsing polychondritis (RP). (a) shows characteristic ulnar drift, swelling of metacarpal joints and rheumatoid nodules in an elderly patient. (b) shows appearance of an inflamed vessel in a patient

with RA. (c) shows inflammation and destruction of the auricular cartilage in a patient with RP. (d) shows the characteristic saddle nose deformity due to cartilage destruction in a patient with RP. Figures A-D are courtesy of Professor C. Stephen Foster (Walham, MA, USA)

synovial tissue and cartilage (Fig. 36.8). However, RA may be associated with a number of extra-articular systemic manifestations including ophthalmic involvement. Autoimmune damage affecting other visceral organs in RA can be life-threatening.

The most frequent ophthalmic involvement in patients with RA is the damage to the ocular surface, resulting in dry eyes. The involvement of ocular surface in patients with RA has immunologic similarities to the joint disease, with evidence to suggest a heightened state of both, adaptive and innate immune system responsiveness. *Keratoconjunctivitis sicca* is characterized by increased number of antigen-presenting cells, and decreased anti-inflammatory cytokines such as IL-13 and decreased suppressor T cells (T_{reg}) (Tong et al. 2014). Dry eye may be complicated with chronic kera-

titis, ulceration and corneal melt due to poor tear-film. Frequency of dry eye syndrome may be as high as 45–71% in patients with RA.

Patients with RA may develop *peripheral ulcerative keratitis (PUK)*, an inflammatory thinning of the peripheral cornea that may lead to perforation. PUK occurs in approximately 3% patients with RA and is bilateral in 40% patients. Usually, PUK appears as a crescent-shaped peripheral corneal infiltration associated with scleritis. RA is the most common cause of scleritis and is associated with 20% of the cases. In 10% cases, the diagnosis of scleritis may precede the diagnosis of RA. Necrotizing scleritis is an extra-articular condition associated with an increased risk of death in patients with RA. Scleritis may be complicated by scleral perforation (*scleromalacia perforans*). Other ocular mani-

festations in patients with RA include conjunctivitis and episcleritis (Artifoni et al. 2014; Galor and Thorne 2007).

36.3.7.2 Sero-Negative Spondyloarthropathies

Sero-negative spondyloarthropathies (SSpAs) are a group of chronic inflammatory conditions affecting the axial skeleton, characterized by a negative serum rheumatoid factor. SSpAs are strongly associated with HLA B27 positivity. SSpAs include ankylosing spondylitis, reactive arthritis, psoriatic arthritis and arthritis associated with inflammatory bowel disease (Crohn's disease and ulcerative colitis), among other entities. Inflammatory eye disease is the most common extra-ocular manifestation of SSpAs with incidence of 25 % in patients with ankylosing spondylitis and 10 % with psoriatic arthritis (Zagora and McCluskey 2014; Zochling and Smith 2010).

The hallmark manifestation of SSpA is anterior uveitis, occurring in up to 40 % cases. Anterior uveitis is typically acute, unilateral or alternating between the two eyes. Epidemiological data suggests that there may be a mean of 5 attacks of uveitis during the course of the disease (Monnet et al. 2004). Ankylosing spondylitis may be associated with *hypopyon uveitis*. Episcleritis, scleritis and conjunctivitis may occur with SSpAs. Involvement of the posterior segment is less common. Infrequently, posterior or panuveitis may develop in patients with inflammatory bowel disease (Zagora and McCluskey 2014).

36.3.7.3 Relapsing Polychondritis

Relapsing polychondritis (RP), first coined by Pearson et al., is a multisystem chronic inflammatory disease primarily affecting the musculoskeletal system. The disease spectrum may vary from mild intermittent episodes of auricular and nasal cartilage pain to life-threatening airway disease. The peak age of onset of the disease is in the fifth decade of life and it is thought to be more common among women. The etiology of RP remains unknown thus far. Circulating antibodies against collagen II, IX and XI, as well as immune-complex deposition have been implicated in the pathogenesis of the condition (Sharma et al. 2013).

Most patients with RP present with auricular chondritis sparing the ear lobule. Progressive destruction and deformities may also involve the nasal cartilage in more than 80 % patients, resulting in *saddle nose deformity* (Fig. 36.8). Approximately 33 % patients may present with joint pain. The most common cause of death in these patients is due to laryngotracheal involvement leading to destruction, dislocation and stricture formation (Puechal et al. 2014; Sharma et al. 2013).

Ocular disease is seen in approximately 50 % patients with RP. Common ocular manifestations include scleritis, episcleritis and conjunctivitis (Rucker and Ferguson 1965). There may be associated corneal thinning. Uveitis is seen in

25 % patients and usually presents as sclerouveitis or iridocyclitis. Retinal involvement may present with exudates, hemorrhages and retinal vascular occlusion (Isaak et al. 1986). Orbital involvement manifests as proptosis, periorbital edema or extraocular muscle palsy (Yoo et al. 2011).

36.3.8 Myasthenia Gravis

Myasthenia gravis (MG) is a systemic autoimmune disease affecting the neuro-muscular junction and presenting with a wide variety of ophthalmic manifestations. Ocular MG is a localized form of the disease characterized by involvement of extraocular muscles, levator palpebrae superioris and orbicularis muscles with sparing of the pupillary muscles. This condition can easily masquerade cranial nerve palsies and INO due to nystagmus and limitation of movements. A history of weakness with diurnal variation and fatigue should raise a suspicion of ocular MG (Weinberg et al. 1994).

36.3.8.1 Epidemiology

Ocular MG may affect any age group and there is no racial or geographic predilection. The onset of the disease is rare in the first decade and after the seventh decade of life. Overall, women are affected more than men. However, the incidence of the disease is higher in men above the age of 50 years. Among individuals with pure ocular MG, the incidence is higher among men (Vaphiades et al. 2012).

36.3.8.2 Clinical Features

Among patients presenting with ocular disease, about 50–80 % progress to a generalized disease. Extraocular muscles are frequently affected in ocular MG as they are composed of fast-twitch muscle fibers and have a higher rate of synaptic firing. The tonic fibers, which are responsible for gaze, may be affected resulting in gaze paresis. Ptosis and diplopia are initial signs of the disease in over 50 % patients with MG. The ptosis worsens under stress such as prolonged upward gaze. Lifting of eyelid may result in enhancement of ptosis of the contralateral side. Ice-pack applied to the eyelid may result in improvement in the degree of ptosis. Ptosis can be unilateral or bilateral and is usually asymmetric (Elrod and Weinberg 2004).

Small amplitude upward movement of the eyelid (*hopping*) during lateral gaze is referred to as the *Cogan lid twitch sign*. Reverse Cogan lid twitch sign is the downward movement of the eyelid elicited by saccade to primary position from upgaze. Lid retraction and orbicularis weakness are other eyelid signs of MG. *Peek sign* is the increased visibility of the sclera due to drifting apart of the eyelid without forceful opening (Barton and Fouladvand 2000).

Extraocular muscle weakness in MG may mimic cranial nerve palsies or INO (*pseudo-INO*). However, gaze-evoked

vertical nystagmus, classical feature of INO, is absent in ocular MG. Medial rectus is the most commonly affected muscle. Ocular MG may thus, mimic incomitant strabismus. Saccadic movements and nystagmus is also common in MG (Vaphiades et al. 2012; Nair et al. 2014).

Various clinical tests such as the ice pack test, edrophonium (Tensilon) test or sleep test, and laboratory evaluations such as repetitive nerve stimulation or single-fiber electromyography may help in the diagnosis of this condition (Nair et al. 2014).

36.3.9 Graves' Disease

Graves' ophthalmopathy (GO), also known as thyroid eye disease, is an autoimmune thyroid disease with orbital manifestations. While more than 25 % patients with Graves' disease manifest with GO, subclinical disease can be found in over 70 % patients using imaging techniques such as orbital MRI. Unless treated appropriately, patients with severe GO may develop sight-threatening sequelae with a high risk of permanent blindness (Wiersinga and Bartalena 2002).

36.3.9.1 Epidemiology

The incidence of GO is approximately 16 per 100,000 in females and 2.9 per 100,000 in males in the United States. The estimated prevalence of the disease is 0.25 %, ranging from 0.1 to 0.3 % world over. The disease is more common in women with female to male ratio ranging from 9.3 to 1.4 depending upon the severity of the disease. Men are more commonly affected with severe forms of the disease with a female to male ratio of 1:4. The disease has bimodal age distribution with peaks in the 40s and late 60s. GO is rare in children with an incidence of <0.1 per 100,000 (before puberty). A number of racial, anatomical and environmental factors play a role in the epidemiology of GO. Cigarette smoking plays an important role in the development and occurrence of GO and adversely affects the severity of the disease. More than 80 % patients with GO have hyperthyroidism at the onset of the disease. Approximately 50 % patients with euthyroid Graves' disease develop hyperthyroidism within 2 years of the disease onset (Carter and Utiger 1992; McAlinden 2014).

36.3.9.2 Clinical Features

The most common presenting feature of GO are eye lid signs such as lid retraction and lid swelling. There may be widened palpebral fissure with decreased blink rate (*frightened face*). The patients may develop lid lag in downgaze (*von Graefe's sign*), and loss of eyebrows in the extreme third of the lid (*Hertoge's sign*). Hyperpigmentation of the superior eye lid folds is known as *Jellinek's sign*. Upper eye lid retraction resulting in excessive scleral show is known as *Stellwag's*

sign. Due to an increase in the orbital fat volume and infiltration by inflammatory cells, there is proptosis and enlargement of extraocular muscles (sparing the tendons) resulting in diplopia. If left untreated, the muscles may lose the ability to contract and become rigid, resulting in increased intraocular pressure in the up-gaze (*Sattler's sign*). Eventually, patients may develop paralytic strabismus and permanent lid lag. Lack of convergence of the eye balls is known as *Moebius's sign*. Patients may develop limitation of abduction and rotation of eyeball (*Jendrassik's sign*). Horizontal nystagmus or complete ophthalmoplegia may develop.

Complications such as exposure keratopathy may occur resulting in staining of the corneal surface. Neglected cases may develop corneal ulceration that may progress to perforation. Due to excessive mechanical stretch, the optic nerve may develop edema and atrophy (*dysthyroid optic neuropathy*), which may develop in approximately 3–7 % patients with GO. Corneal and optic nerve complications may lead to permanent visual loss among patients with GO (Saraci and Treta 2011; Yeatts 1995).

36.4 Miscellaneous Neuroimmune Diseases with Ophthalmic Involvement

36.4.1 Susac's Syndrome

Susac's syndrome (SS) is a rare, autoimmune microangiopathy characterized by infarcts in the brain, retina and cochlea. The classical triad of SS includes neurological dysfunction (*encephalopathy*), multiple branched retinal arteriolar occlusions (*BRAOs*) and sensorineural hearing loss. The disease typically occurs in young women and is often misdiagnosed as MS. Fluorescein angiography may detect presence of multiple BRAOs that may result in variable visual field loss. In addition to BRAOs, SS may be associated with retinal vasculitis (Greco et al. 2014). Unusual neurological manifestations, such as cauda equine syndrome, may occur in patients with SS (Allmendinger et al. 2014).

36.4.2 Cogan's Syndrome

Cogan's syndrome is characterized by non-syphilitic interstitial keratitis and bilateral audio-vestibular defects secondary to an autoimmune process. The autoantibodies may be directed against antigenic peptides expressed in epithelial cells in the inner ear. Corneal involvement presents with granular, irregular corneal infiltrate affecting the posterior stroma and endothelium. Ocular inflammation may manifest as scleritis, episcleritis, conjunctivitis, retinal vasculitis or papillitis. More than 90 % patients have associated systemic

symptoms. Neurological disease includes aseptic meningitis, hemiparesis or hemiplegia, pyramidal syndrome, vigilance disorders or trigeminal neuralgia. One-fourth patients may also present with cardiovascular, gastrointestinal or musculoskeletal disease (Kessel et al. 2014; Azami et al. 2014).

36.4.3 Reactive Arthritis

Reactive arthritis is a multi-system autoimmune seronegative disease that develops 1 to 3 weeks after gastrointestinal or genitourinary infection. Antigens from gram negative intracellular bacteria may trigger an inflammatory cascade with CD4 T Helper cell and CD8 response following mucosal invasion (Stavropoulos et al. 2015). While there are no diagnostic criteria established for reactive arthritis, the condition primarily affects the joints resulting in arthritis similar to psoriasis and is associated with urethritis and conjunctivitis/uveitis. Some forms of reactive arthritis developing the triad were previously referred to as 'Reiter's syndrome'. However, the term reactive arthritis encompasses a broader category of the disease (Selmi and Gershwin 2014). Ocular manifestations are often frequent in this condition, with more than 50% men experiencing conjunctivitis or acute anterior uveitis. Patients may also develop genital lesions and other visceral involvement, including cardiac valvular abnormalities and conduction defects.

36.4.4 Primary Sjögren's Syndrome

Sjögren's syndrome is a multisystem autoimmune condition with lymphocytic infiltration of the exocrine glands, such as the salivary and lacrimal glands. In addition, the disease can affect the nervous system in about 20% cases resulting in *sensory ganglionopathy* or transverse myelitis. Other visceral manifestations include interstitial lung or kidney disease, autoimmune hepatitis or pancreatitis. Ocular manifestations include dry eye, corneal haze/scarring, *sterile corneal melt* and papillary or cicatrizing conjunctivitis. Sjögren's may be associated with scleritis, episcleritis or uveitis (Akpek et al. 2015). Cases with optic neuritis and retinal vasculitis have been reported in literature (Tang and Wei 2013).

36.4.5 Dermatomyositis

Dermatomyositis is an inflammatory myopathy characterized by cutaneous manifestations that occur in the presence of minimal muscle involvement. Other features of the disease may include multiple systemic involvement and/or underlying malignancies. The typical rash of dermatomyositis is described as heliotrope rash (face), Gottron's papules (hands and elbows), and trunk (shawl and 'V' sign). The dis-

ease has two distinct forms—adult and juvenile (Luo and Mastaglia 2015). In a large series of 108 patients with juvenile dermatomyositis, eye lid manifestations (supraciliary heliotrope line with or without telangiectatic vessels, lid scarring, or pox-like lesions) were the most common manifestations. Other ocular manifestations include cataract (posterior subcapsular variety), retinal hemorrhages, and retinal vasculitis that may manifest as frosted branch angiitis (Lee et al. 2011).

36.5 Review Questions

- The following clinical features are seen during the acute stages of Vogt-Koyanagi-Harada disease except:
 - Exudative retinal detachment
 - Subretinal fluid
 - Choroidal thickening
 - Sun-set glow fundus*
- The pathogenesis and clinical features of Vogt-Koyanagi-Harada disease is most closely related to which of the following condition:
 - Serpiginous choroiditis
 - Sarcoidosis
 - Sympathetic Ophthalmia*
 - Graves' disease
- Choroidal manifestations in Vogt-Koyanagi-Harada disease include all of the following except:
 - Choroidal excavation*
 - Choroidal thickening
 - Choroiditis
 - Nummular choroidal scars
- Most common Human Leucocyte Antigen (HLA) associated with Adamantiades-Behçet's disease includes:
 - HLA B-27
 - HLA B51/5*
 - HLA A29
 - HLA DRB1
- Which of the following is the most common systemic manifestation of Adamantiades-Behçet's disease?
 - Central nervous system vasculitis
 - Genital ulcers
 - Oral ulcers*
 - Uveitis
- According to recent estimates, which of the following individuals are at an increased risk of developing sarcoidosis?
 - Caucasian males
 - Hispanic females
 - Caucasian females
 - African-American females*
- Which of the following cells play a central role in the pathogenesis of sarcoidosis:
 - CD4 -T Helper cells*
 - Cytotoxic CD8 cells

- c. Eosinophils
- d. Natural Killer cells
8. Worsening of vision provoked by small increases in body temperature attributed to exercise, hot baths or showers, or hot weather conditions among patients with Multiple Sclerosis is described as:
 - a. Sugiura's sign
 - b. Uhthoff's phenomenon
 - c. Pulfrich's phenomenon
 - d. Von Graefe's sign
9. Among patients with rheumatoid arthritis, the most common ocular involvement includes:
 - a. Dry eye syndrome
 - b. Optic neuritis
 - c. Choroiditis
 - d. Subretinal fluid
10. Susac's syndrome is characterized by presence of:
 - a. Central retinal artery occlusion
 - b. Branch retinal vein occlusion
 - c. Cilio-retinal artery occlusion
 - d. Branch retinal artery occlusion
11. Cogan Lid Twitch sign is described as:
 - a. Small amplitude upward movement of the eyelid during lateral gaze
 - b. Nystagmoid movements of the eyes
 - c. Presence of internuclear ophthalmoplegia
 - d. Ptosis

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Abstract

Neurogenesis is the complex process of generating new neurons in the central nervous system (CNS). Classically, neurogenesis was believed to only occur during embryonic development, never after the postnatal stage. However, the evolution of technologies in cellular and molecular biology, radiology, and so forth has resulted in the discovery of neurogenesis and neural stem cells (NSCs)/neural progenitor cells (NPCs) in the adult brain. While the discovery has led to the potential for the repair of the injured or diseased brain, there are a number of issues yet to be resolved. The complex-three dimensional structure and organization of the adult brain limits the differentiation and integration of implanted NSCs/NPCs and thereby for the repair. In this chapter, recent advances in the study of embryonic and adult neurogenesis will be described including the defining of mechanisms, molecular markers and regulatory factors operative during neurogenesis, as well as recent findings into the role of adult neurogenesis in brain disorders and brain repair.

Keywords

Doublecortin • Epidermal growth factor • Fibroblast growth factor • Granule cell layer • Hippocampus • Nestin • NeuN • Neural progenitor cells • Neural stem cells • Neurogenic niches • Neurospheres • Radial glia • Rostral migratory stream • Subventricular zone

37.1 Introduction

Neurogenesis is a complex process for generating new neurons in the brain and spinal cord. The process includes cell proliferation, production of migratory progeny, differentiation and functional integration into the neural circuitry in the central nervous system (CNS). The classical dogma of neurogenesis asserts that the process only occurs during embryonic development and prenatal stage, and new neurons are never generated after postnatal stage. Santiago Ramón y Cajal described that “in the adult centers the nerve paths are something fixed,

ended and immutable. Everything may die, nothing may be regenerated” (Ramón y Cajal 1913). This long-held theory was based on a concept that the brain is a complex-three dimensional structure with a very complex and hierarchically organized neural circuitry for the integration of new neurons as compared to other organs. However, the dogma was invalidated by Joseph Altman, who discovered new neuron generation in the brains of adult mammals, thus called adult neurogenesis, in the 1960s (Altman and Das 1965a, b; Altman 1969). Although the sensational discovery of the seminal phenomenon of adult neurogenesis was out of the neuroscience mainstream, it was re-recognized a few decades later in songbirds (Goldman and Nottebohm 1983) and then mammals including rodents, rabbits, monkeys and even in humans (Emsley et al. 2005; Ming and Song 2005; Martino et al. 2011). Moreover, the idea of adult neurogenesis was further affirmed by the discovery of neural stem cells (NSCs)/neural progenitor cells (NPCs) that can proliferate by growth

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factors: epidermal growth factor (EGF) and fibroblast growth factors (FGFs), and differentiate directly into neurons and glia throughout life in the mammalian brain (Reynolds and Weiss 1992; Lois and Alvarez-Buylla 1993). These observations have incited enormous and attractive interest among neurobiologists and clinicians to study adult neurogenesis in hopes of harnessing capability of brain repair.

Gliogenesis, the generation of new glial cells, occurs from late embryogenesis stage and throughout postnatal stages, as well as in the adult brain where both astrocytes and oligodendrocytes are produced (Rowitch and Kriegstein 2010; Gallo and Deneen 2014). In contrast, neurogenesis is initiated at an early developmental stage and continued throughout early postnatal stages. However, neurogenesis is inactive in the adult brain except in two main neurogenic niches: the subventricular zone (SVZ) of the lateral ventricle (LV) and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) (Gage 2000; Alvarez-Buylla and Lim 2004; Zhao et al. 2008; Ming and Song 2011). Over the last two decades researchers have studied adult neurogenesis in the two neurogenic niches as a model for understanding of which genetic factors and stimuli propel NSCs/NPCs toward a neuronal fate, differentiate them into neurons, guide their final destination and facilitate their integration into the neural circuitry. A thorough understanding of adult neurogenesis will ultimately contribute to exploiting these processes for brain repair to treat neurological disease. This chapter delineates recent advances in embryonic neurogenesis and in defining mechanisms, molecular markers and regulatory factors operative during adult neurogenesis. The descriptions also serve to introduce experimental outcomes for brain repair in animal models of neurological disease.

37.2 Neural Development

37.2.1 Early Embryonic Neurogenesis

In embryonic development, node cells intercalate and migrate toward rostral side of the embryo to generate the prechordal plate and notochord during gastrulation. Both prechordal plate and notochord are the “organizers” and signaling centers that induce formation of the monolayer neural plate including neuroepithelial cells (NECs) that serve as the primary neural precursors of the CNS. While the notochord defines the primitive axis that is ultimately replaced with the spinal cord, the prechordal plate plays a critical role in defining brain vesicles. The neural plate is shaped and then bended with V-shaped cross-section of the neural groove. In parallel, the neural plate is folded at its edges that are fused at midline of the embryo for neural plate closure, which is initiated at the hindbrain/cervical boundary and

extends simultaneously to what will become the brain and spinal cord. The closure results in formation of the early neural tube, constituted of pseudostratified epithelium with a central canal underneath ectoderm that completes at the anterior and posterior neuropores. This series of events, called primary neurulation, creates the primordial portions of the brain and most of the spinal cord (Colas and Schoenwolf 2001; Copp et al. 2003).

After neural tube formation, neurogenesis occurs in temporally and spatially defined configurations along the anterior-posterior (AP), ventral-dorsal (DV), and lateral-medial body axes (Taverna et al. 2014). Along these axes NECs are patterned by morphogen gradients that govern the pattern of guiding tissue and development and where and how various specialized neural cell types develop. Well-studied morphogens involved in this process include sonic hedgehog (SHH), bone morphogenetic proteins (BMPs), wntless/int (Wnt) proteins, and FGFs. For the DV axis, SHH is ventrally expressed in the floor plate and released toward the dorsal side. In contrast, BMPs and Wnts are dorsally expressed in the roof plate and contribute to the patterning of the dorsal-ventral axis of the neural tube. Importantly, SHH signaling antagonizes BMP signaling to generate a counteracting gradient that defines the spatially different expression patterns of transcription factors (Pax6, Pax7, Irx3, Dbx2, Olig2, Nkx2.2, Nkx6.1, among others) (Fig. 37.1a). Therefore, neural progenitor fates in each pattern are determined by the morphogens along the DV axis of the neural tube, which mostly becomes the spinal cord at the later stage (Ulloa and Briscoe 2007; Dessaud et al. 2008; Cohen et al. 2013). Regarding DV patterning in anterior parts (future brain region) of the neural tube, SHH plays as a ventral key center as seen in posterior parts (spinal cord). However, detailed mechanisms that establish the DV axis differ from those in posterior parts of the neuraxis. In particular, activity of Gli3 that acts as a repressor of its target genes is antagonized by SHH signaling to determine DV patterning in the development of the telencephalon (Rallu et al. 2002a). Deficiency of *Shh* or *Gli3* gene activity causes persistent expression of ventral telencephalic markers (Dlx2 and Gsh2), indicating that the balance of activities between SHH and Gli3 is critical for establishing DV patterning in the telencephalon (Rallu et al. 2002b).

The initial AP axis including specification of the forebrain is determined before and during gastrulation. At the gastrulation stage, non-neuronal tissues, anterior visceral endoderm (AVE) and the prechordal plate below the developing nervous system, are required for head induction and maintenance (Wilson and Houart 2004; Stern 2001). AVE and the prechordal plate play critical roles as signaling centers in specifying neural fate in the early forebrain development. The centers seem to produce morphogen antagonists to inhibit diffusible posteriorizing factors including Wnts,

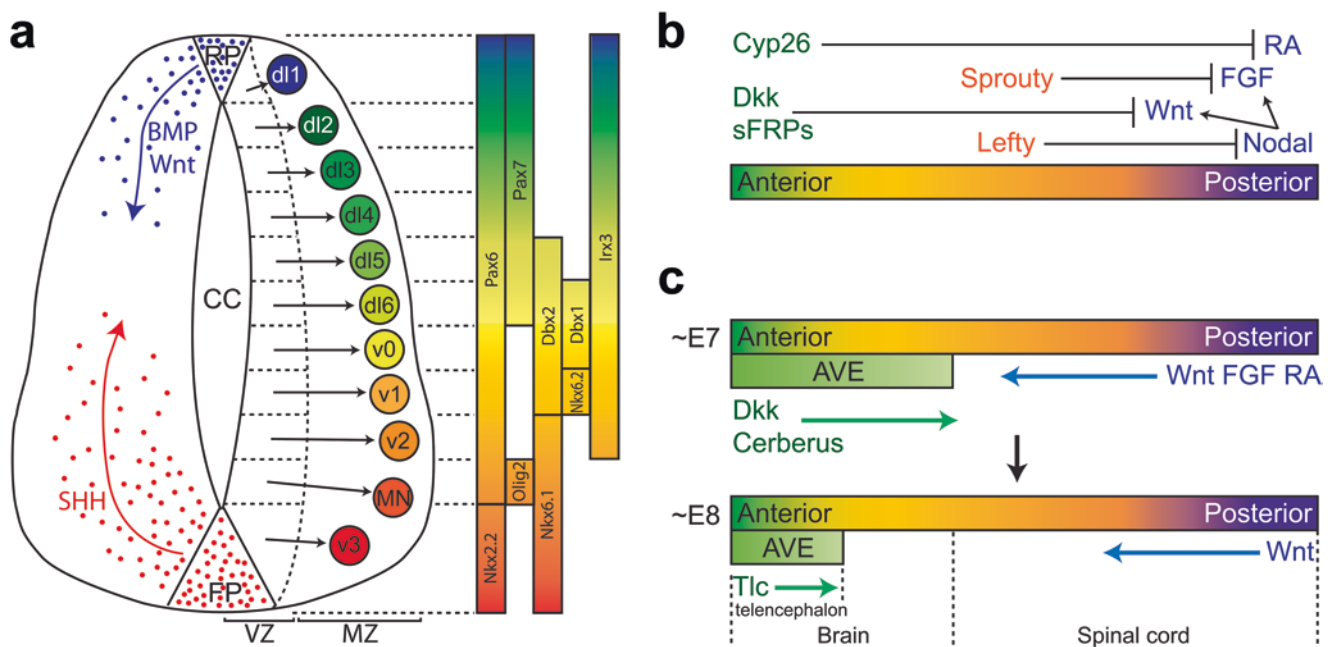


Fig. 37.1 (a) (Left) Diagram of a section of the spinal cord. The left half shows morphogen gradients of BMPs/Wnts from the roof plate (RP) and SHH from the floor plate (FP). The right half indicates the 11 neuronal progenitor domains located in the ventricular zone (VZ), medially adjacent to the central canal (CC). The morphogen gradients spatially control position identity of the progenitor domains where distinct neuronal subtypes are generated. Postmitotic differentiated neurons are divided into six types of dorsal domains (dl1–dl6), four types of ventral interneuron domains (v0–v3) and motor neurons (MN) in the mantle zone (MZ). (Right) The spatial pattern of morphogen gradients regulates transcription factor expression. Different combinations of transcription factors define individual progenitor domains. (b) Anterior-

posterior (AP) patterning during CNS development in fish and frogs. In neurulation, retinoic acid (RA) and Nodal, inducing Wnt and FGF, serve as diffusible posteriorizing factors. They are antagonized by Cyp26, Lefty, Frizzled-related proteins (Frzb: secreted FRPs) and dickkopf (Dkk), respectively, at the posterior part of embryos to specify the AP axis. (c) During mammalian development, Wnt, FGF and RA characterize the AP axis in the neural plate as in fish and frog embryos. Dkk and Cerberus are expressed in the anterior visceral endoderm (AVE) and antagonize Wnt signaling, shaping the anterior neural plate domain. Subsequently, Tlc, a member of FRPs, further antagonizes Wnt signaling to subdivide the domain, designating future telencephalon region. E, embryonic day

FGFs, Nodal and retinoic acid (RA) (Altmann and Brivanlou 2001; Schier 2001). Studies in fish and frogs have implicated that secreted Frizzled-related proteins (Frzb) and dickkopf (Dkk1–3 in mice) are expressed in the anterior endoderm (equivalent to the AVE) of frog embryos and antagonize Wnts. Nodal and FGF signaling are inhibited by Lefty and Sprouty, respectively, and Cyp26 proteins degrade RA into its polar metabolites to inhibit discrete levels of RA signaling at the posterior part of the embryo (Fig. 37.1b) (Tuazon and Mullins 2015). Although it is unclear how morphogen antagonists function in mammalian development, studies regarding removal or transplantation of the AVE, or mutations in genes such as *Otx2*, *Lim1* and *Hesx1* that are expressed in the AVE, support the concept that the AVE serves as a determinant to specify forebrain fate in unpatterned NECs (Rallu et al. 2002a). After anterior neural induction, Tlc, a member of Frzb, also serves as an antagonist of Wnt signaling, plays an important role in mediating establishment of telencephalon at the anterior neural ridge, and partitions the forebrain into its subdivisions of telencephalon, diencephalon and optic territories (Fig. 37.1c) (Houart et al. 2002).

37.2.2 Cerebral Cortex Development and the VZ/SVZ

The telencephalon gives rise to the cerebral cortex comprised of the neocortex and allocortex (also known as heterogenetic cortex). Fundamental telencephalon territory is determined at around mouse embryonic day (E) 9.5. NECs initially form symmetric cell divisions that provide lateral and radial expansion of their cell population for cerebral cortex development (Rakic 1995). The process is active at the anterior edge in particular, as well as in the area surrounding the lumen of the neuraxis. Meanwhile, NECs are polarized along the apical-basal axis with mitosis occurring at the apical-most germinal layer that is closest to the lumen and one of the germinal niches called the VZ. In the VZ, NECs form a belt of linkage called adherens junctions (AJ), connecting neighboring NECs to one another (Marthiens and French-Constant C 2009). NECs, now transformed into neural progenitor cells (NPCs), undergo interkinetic nuclear migration (INM), nuclear migration in the cytoplasm along the apical-basal axis of the neuroepithelium during S-phase in mitosis,

and form a pseudostratified epithelium (Sauer and Walker 1959; Taverna and Huttner 2010).

The earliest cortical neurons are generated from NPCs at around E10.5, and settle within the preplate (PP) (De Carlos and O'Leary 1992), which is split into the superficial marginal zone (MZ, Layer (L)1 of the postnatal cortex), the cortical plate (CP, the postnatal L2-L6) and the subplate (SP) underneath the CP. Starting at around E11-12, NPCs transform into radial glial cells (RGCs) with their somata locating at the VZ and integrating into AJ belts, their short processes reaching the ventricle (apical side), and their long processes reaching basal lamina. RGCs express astroglial markers such as glial fibrillary acidic protein (GFAP), glutamate/aspartate transporter (GLAST) and brain lipid binding protein (BLBP) (Campbell and Gotz 2002). Like NPCs, RGCs undergo INM in the VZ, and begin cell divisions both symmetrically and asymmetrically to produce daughter RGCs and intermediate progenitor cells (IPCs) or neurons (Gotz and Huttner 2005). Newly formed neurons migrate radially from the VZ toward the pial surface through the intermediate zone using RGC basal processes as guiding scaffolds and then reach their final destination (Rakic 1972, 1988). Cajal-Retzius (CR) cells have a major role in neuronal cell migration. The CR cells are generated as the earliest cortical neurons, and lie in the superficial MZ, and serve as “a director” to regulate the identity of radial glia and migration of neurons during cerebral cortex development. They are transient as most of the cells disappear by cell death at postnatal stages (Derer and Derer 1990; del Rio et al. 1995; Soriano et al. 1997). IPCs migrate from AJ belts, accumulate and line up above the VZ, and form a second germinal zone, called the SVZ. IPCs lose their processes and AB polarity, down-regulate astroglial markers, and undergo one round of symmetric neurogenic division to generate a pair of neurons that also radially migrate from the SVZ to their final destination using RGC basal processes (Gal et al. 2006). Newly generated neurons from both RGCs and IPCs migrate into the cerebral cortex and reach the most superficial side of the CP to form the mantle layer, overlaying leading neurons so that the CP is formed in order of L6–L2. At around E17, the end of embryonic neurogenesis, RGCs retract their basal processes and become gliogenic to generate astrocytes and ependymal cells (ECs) that line the ventricles (Fig. 37.2) (Kwan et al. 2012; Florio and Huttner 2014; Bjornsson et al. 2015).

37.2.3 Hippocampal Formation During Development

The allocortex is comprised of three subtypes: the periallocortex, paleocortex and archicortex. The hippocampal primordium is located within the archicortex. The hippocampus

originates from the caudomedial edge of neuroepithelium, a transient structure after the invagination of the roof in the dorsal telencephalon (Lee et al. 2000). Although the hippocampal DG originates from the VZ, the hippocampus formation including the DG is very unique. At around E 14.5, dentate NPCs migrate from the dentate neuroepithelium (also called the primary matrix), a part of the VZ in the archicortex and adjacent to the cortical hem (CH), toward the pial-midline side of the archicortex to form the secondary matrix. This migration is directed by orchestration of the CH-derived CR cells (Del Rio et al. 1997). Continuously, dentate NPCs migrate away from the secondary matrix towards the hippocampal fissure to form the tertiary matrix, which is a component of the future DG, using glial scaffolds that connect the CH (the future hippocampal fimbria) to the pial surface and the hippocampal fissure and generate granule cells (GCs) that form GC layer (GCL). In the VZ, RGCs undergo asymmetric cell divisions to give rise to daughter RGCs and hippocampal neurons, which migrate via RGC basal processes to the hippocampal cornu ammonis (CA) 1-3 to become pyramidal neurons. Dentate NPCs persist postnatally, but only in the tertiary matrix for a few weeks after birth; proliferation of NPCs is eventually restricted to the subgranular zone (SGZ) of the DG (Urban and Guillemot 2014; Yu et al. 2014).

37.3 Neurogenesis in the Adult Brain

While neurogenesis occurs in the entire brain during embryonic development, it becomes restricted to specific neurogenic niches that include the SVZ of the LV and the SGZ of the hippocampal DG in the adult brain. In both specialized niches, radial glia “-like” cells undergo asymmetrical cell division to produce progenitors, followed by generation of neuroblasts that migrate to their final destination, differentiation into neurons and integration into the neural network. Many of the features and the functions of progenitors and neuroblasts are similar in both niches, although the names of discrete progenitor cell types, markers that are expressed by each progenitor, and their final cell types at final destination differ in the SVZ and SGZ. While neuroblasts from the SVZ migrate rostrally to the olfactory bulb (OB) and differentiate mainly into GABAergic granule interneurons without axons and form dendro-dendritic synapses, dentate neuroblasts give rise to glutamatergic dentate granule cells and mature in the GCL of the DG. Indeed, their microenvironmental cues, including extracellular matrix molecules, growth factors and local neurotransmitters are essential for regulating their niche-specific proliferation and differentiation of progenitors (Basak and Taylor 2009; Conover and Notti 2008; Ming and Song 2011; Lepousez et al. 2015).

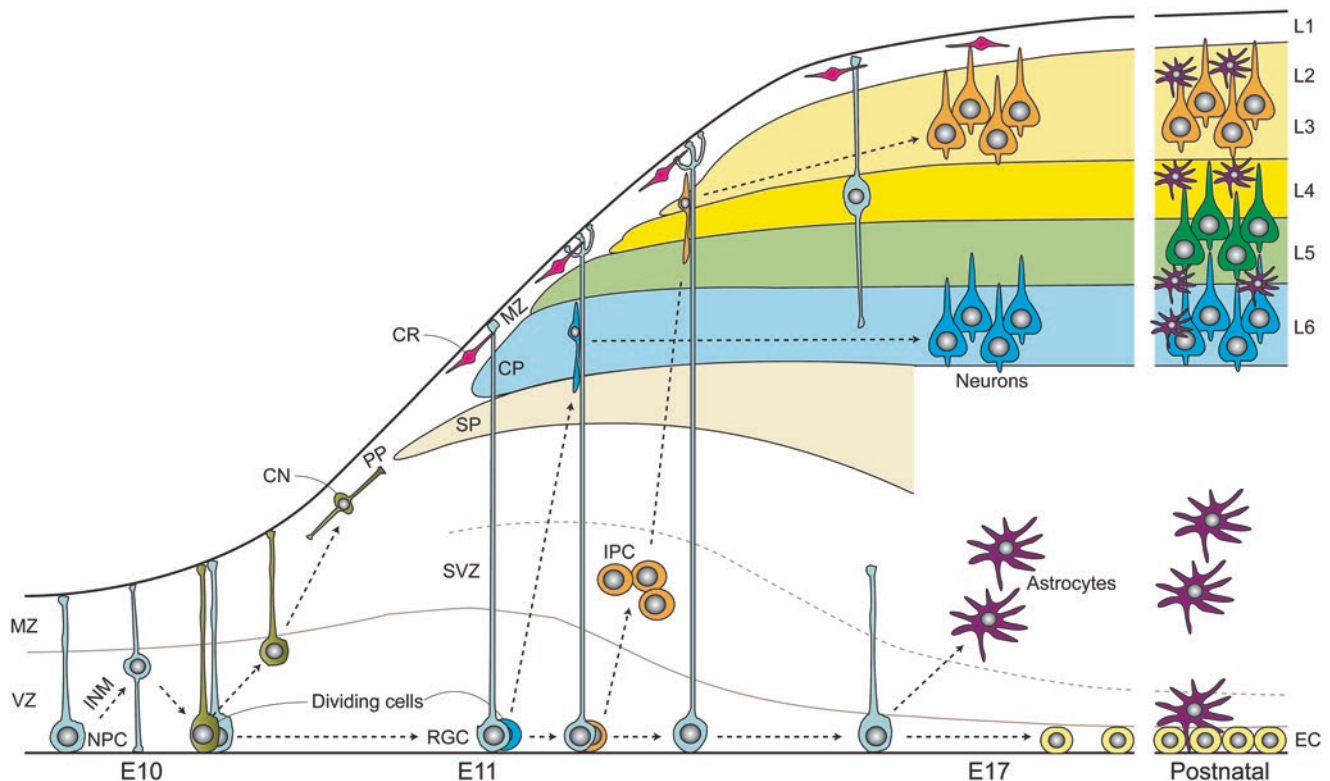


Fig. 37.2 Schematic of neuronal generation and migration in mouse neocortical development. The earliest cortical neurons (CNs) are generated from neural progenitor cells (NPCs) at around E10.5, and settle within the preplate (PP), which is split into the superficial marginal zone (MZ or Layer (L)1 of the postnatal cortex), the cortical plate (CP, the postnatal L2-L6) and the subplate (SP) at the later stages of development. At around E11-12, NPCs transform into radial glial cells (RGCs) with their somata positioned at the ventricular zone (VZ) and their long processes reaching basal lamina. RGCs divide both symmetrically and asymmetrically to produce daughter RGCs and intermediate progenitor cells (IPCs) or neurons. Newly formed neurons radially migrate from the VZ toward the pial surface through the intermediate zone using RGC basal processes as guiding scaffolds, and reach their

final destination. The Cajal-Retzius (CR) cells lie in the superficial MZ, and serve as “directors,” regulating the identity of radial glia and the migration of neurons. IPCs migrate above the VZ, form a second germinal zone called the subventricular zone (SVZ), and undergo one round of symmetric neurogenic division to generate pairs of neurons that also radially migrate from the SVZ to their final destination using RGC basal processes. Newly generated neurons from both RGCs and IPCs migrate into the cerebral cortex and reach the most superficial side of the CP to form the mantle layer, overlaying leading neurons to form the CP in order of L6 to L2. At around E17, RGCs retract their basal processes and become gliogenic, generating astrocytes and giving rise to ependymal cells (ECs) that line the ventricles

37.3.1 Adult Neurogenesis in the SVZ of the LV

Adult NSCs/NPCs are present in the SVZ, a shallow niche between the ependymal cell layer (ECL) lining the CSF-filled ventricles and the striatal walls (Fig. 37.3a) (Doetsch et al. 1999; Shen et al. 2008). The putative NSCs are quiescent but slowly proliferating radial glia-like cells called type B cells, which exhibit hybrid characteristics of astrocytes and immature progenitors and express nestin, vimentin and GFAP. Cell bodies of the type B cells line the ECL, and project their processes into the ventricles through the ECL to contact the CSF, and onto a planar SVZ vascular plexus where they contact blood vessels (Mirzadeh et al. 2008; Tavazoie et al. 2008). The type B cells receive their microenvironmental cues from both the vascular plexus and CSF

compartments, as well as from ECs, to regulate their proliferation and differentiation. The type B cell in the adult SVZ niche seem to have similar functional aspects to, but differ from RGCs in the embryonic VZ/SVZ, due to the state of maturation and location surrounding the cells, which results in distinct cue signatures. *First*, ECs are immature during embryonic development but postnatally mature. *Second*, ECs produce cue molecules to regulate neurogenesis in the adult SVZ. *Third*, cue molecules produced from other sources such as microglia, pericytes, blood vessels and the CSF including choroid plexus in the adult SVZ are distinct from cue molecules in the embryonic SVZ (Bjornsson et al. 2015).

Upon activation, type B cells divide and develop into transient-amplifying progenitor cells called type C cells, which express *Ascl1* (achaete-scute complex homolog 1)

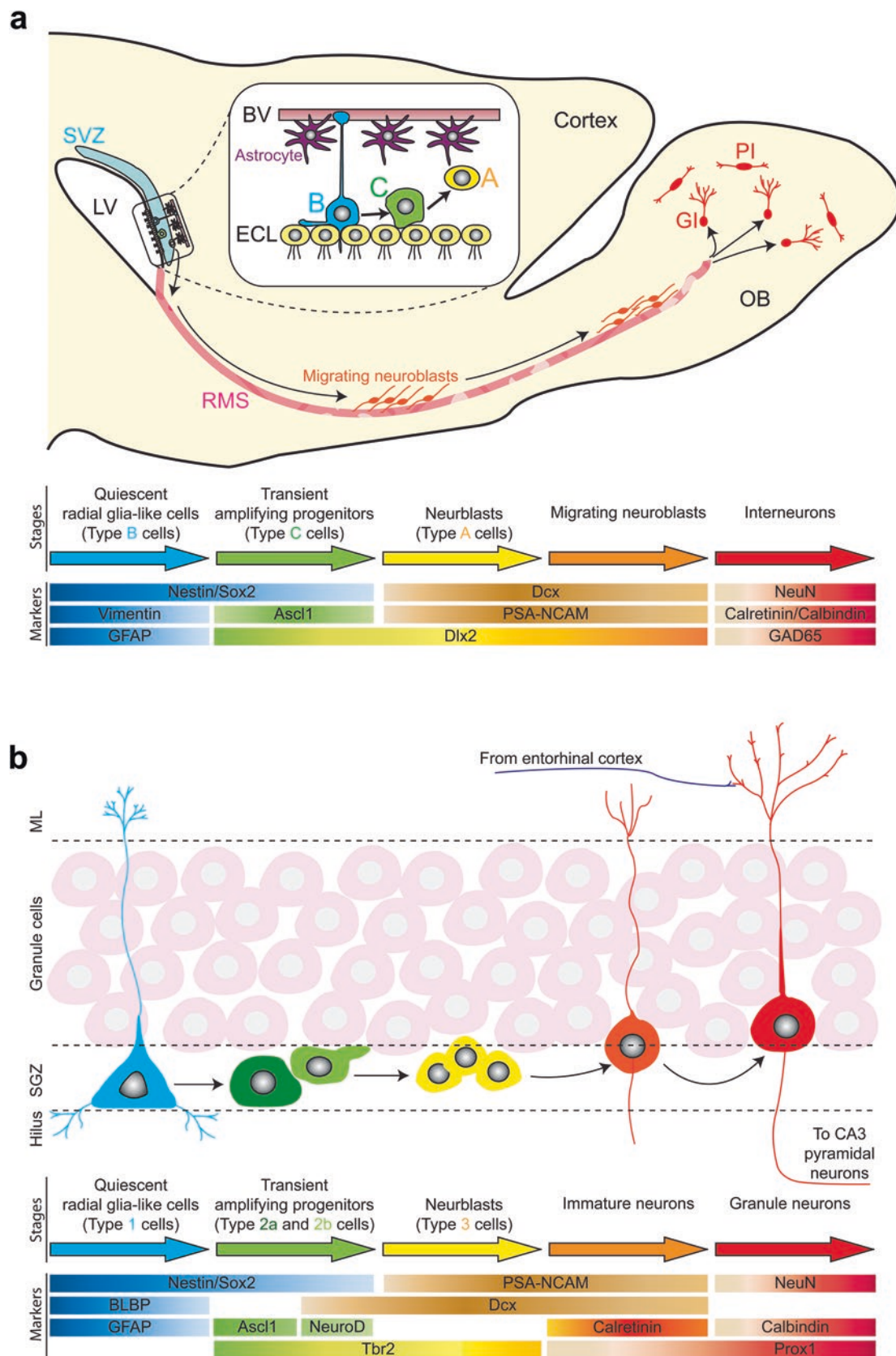


Fig. 37.3 Adult neurogenesis in the mouse brain. **(a)** Adult neural stem cells (NSCs)/neural progenitor cells (NPCs) are present in the subventricular zone (SVZ), a shallow niche between the ependymal cell

layer (ECL) lining the CSF-filled lateral ventricle (LV) and the striatal walls. The putative NSCs are quiescent but slowly proliferating radial glia-like cells called type B cells. Cell bodies of the type B cells line the

and *Dlx2*, and amplify their daughter cells to expand the population of the cells. The proliferating type C cells rapidly divide and give rise to type A cells, immature neuronal marker doublecortin (*Dcx*)-expressing neuroblasts or glial cells (oligodendrocytes or astrocytes). The type A neuroblasts exit the SVZ to enter chains along the rostral migratory stream (RMS), a dense channel of olfactory ensheathing glia, and migrate towards the OB, where SVZ-derived neuroblasts differentiate mostly into GABAergic granule interneurons in the GCL of the OB, but also into a variety of phenotypes including GABAergic or dopaminergic periglomerular interneurons and glutamatergic juxtglomerular neurons in the glomerular layer, and then integrate into the existing neuronal circuitry. The neuroblasts also differentiate into oligodendrocytes (Carleton et al. 2003; Brill et al. 2009; Kriegstein and Alvarez-Buylla 2009; Kosaka et al. 1995; Tatti et al. 2014).

37.3.2 Adult Neurogenesis in the SGZ of the DG

NSCs are believed to be quiescent radial glia-like cells called type 1 cells residing in the SGZ of the hippocampal DG. The cells are self-renewing and multipotent. They generate granule neurons through several steps (Fig. 37.3b), where a finely tuned balance regulated by both intrinsic and extrinsic stimuli, including signaling of BMP, IGF (insulin-like growth factor), Notch, Wnt, transcriptional factors, lipid metabolic processes and excitatory GABAergic inputs determines whether the cells keep their quiescent status or undergo proliferation and differentiation (Lledo et al. 2006; Braun and Jessberger 2014; Urban and Guillemot 2014). The type 1 cells express stem cell markers *Sox2* and *nestin*, but also have astrocytic features as shown their molecular markers such as GFAP and BLBP. The type 1 cells extend their radial processes through the entire GCL and ramify in the inner molecular layer (ML) of the DG. Upon activation, they undergo asymmetric divisions to generate intermediate

NPCs called type 2 cells. They are transient amplifying cells that continue to express *Sox2* and *nestin*, but lose GFAP/BLBP expression and their radial processes. The type 2 cells are categorized into two subtypes (type 2a and type 2b) based on differences in molecular markers between the subtypes. While the type 2a is *Ascl1*-positive during early stage of type 2 cells, the type 2b is *NeuroD* and *Dcx*-positive during late stage. Subsequently the type 2b cells give rise to *Dcx*-positive neuroblasts called type 3 cells, and expand the neuroblasts pool. The type 1, 2 and 3 cells are in mitotic cycles and have the ability of self-renewal. Once exiting the cell cycle, the type 3 neuroblasts enter the post-mitotic phase, start to branch out their processes, and undergo neuronal differentiation through a tightly regulated process. The neuroblasts differentiate into *Dcx* and calretinin-positive immature neurons around 2 weeks after the first division of the type 1 cells. Subsequently, the immature neurons migrate up into the GCL, undergo neuronal maturation regulated by *Cdk5* and *Disc1*, and then eventually become neuronal nuclei (*NeuN*)/calbindin/*Prox1*-positive glutamatergic dentate granule cells. Over a period of 3–6 weeks, newly developed neurons in the GCL extend their large dendritic arbor into the ML, and project axons into the hilus and CA3 regions for integration into existing hippocampal circuitry, and become fully functional. The integration is dependent on glutamate-mediated NMDA receptor responses (Kempermann et al. 2004; Mu et al. 2010; Bonaguidi et al. 2011; Imayoshi and Kageyama 2011; Ming and Song 2011; Urban and Guillemot 2014).

37.4 Methods to Detect Newly Generated Cells

37.4.1 Cell Proliferation Markers

Altman and colleagues first used a radiolabelled nucleoside, ^3H -thymidine ($[^3\text{H}]\text{dT}$), to show existence of newly generated neurons in the OB and DG of the hippocampus in adult

Fig. 37.3 (continued) ECL, and project their processes into the ventricles through the ECL to contact the CSF, and onto a planar SVZ vascular plexus where they contact to blood vessels (BV). Upon activation, the type B cells divide and amplify their daughter cells to expand the population of the cells as well as give rise to transient-amplifying progenitor cells called type C cells. The proliferating type C cells rapidly divide and produce type A neuroblasts, which exit the SVZ to enter chains along the rostral migratory stream (RMS), a dense channel of olfactory ensheathing glia, and migrate towards the olfactory bulb (OB). Here, SVZ-derived neuroblasts differentiate into GABAergic granule interneurons (GI), among a variety of other phenotypes including GABAergic or dopaminergic periglomerular interneurons (PI), and integrate into the existing neuronal circuitry. At each of these stages, cells express stage-specific markers. (b) The type of NSCs/NPCs in the SGZ of the hippocampal dentate gyrus (DG). NSCs are quiescent but self-renewing and multipotent radial glia-like cells (type 1 cells). The type 1 cells have radial processes that extend through the entire granule cell layer (GCL) and ramify in the inner molecular layer (ML) of the DG. Upon activation, they undergo asymmetric divisions to generate type 2 cells, which are intermediate, transient amplifying cells, and lose their radial processes. The type 2 cells are further categorized into two subtypes (type 2a and type 2b) based on differences in a set of molecular markers between the subtypes. The type 2b cells give rise to type 3 neuroblasts, and expand the neuroblast pool. The type 1, 2 and 3 cells are in mitotic cycles and are able to self-renew. Once exiting the cell cycle, the type 3 neuroblasts enter the post-mitotic phase, in which they start to branch out their processes, and undergo neuronal differentiation through a tightly regulated process. The neuroblasts differentiate into immature neurons. Subsequently, the immature neurons migrate up into the GCL, undergo neuronal maturation and become mature glutamatergic dentate granule cells. Newly developed neurons extend their large dendritic arbor into the ML, and axons into the hilus and area CA3 for integration into existing hippocampal circuitry, and become fully functional.

rats (Altman and Das 1965a; Altman 1969). Exogenous [^3H] dT is incorporated into newly synthesized DNA in cells at the S-phase during cell cycles, inherited into cell progeny, and can be visualized with autoradiography. This was based on a new stoichiometric technique to label dividing cells with [^3H]dT by intraperitoneal injection developed in late 1950s, and to allow quantitative analysis of proliferation, differentiation, and survival of new-born cells (Sidman et al. 1959; Kempermann et al. 1997; Miller and Nowakowski 1988). However, this methodology had disadvantages including limited detection depth from surface of sections due to the small half-distance of the beta-particle emitted by tritium atom decay ($\sim 1\text{ }\mu\text{m}$), long-term processes of developing autoradiographs (3–12 weeks), the logistics of handling radioactive materials and cost (Bisconte 1979; Duque and Rakic 2011).

The introduction of bromodeoxyuridine (BrdU), another synthetic thymidine analog, has brought a marked, rapid progress in the field of the adult neurogenesis. BrdU is also intraperitoneally administrated into animals and incorporated into newly synthesized DNA as an S-phase marker during cell cycles like [^3H]dT (Gratzner 1982). BrdU can be detected with immunohistochemistry and immunofluorescence with significant penetration of antibodies to BrdU that labels all proliferating cells throughout the section. This methodology can be used to perform stereological quantification and phenotypic analysis of proliferation, differentiation, and survival of newly generated cells that can be confirmed with various phenotypic markers (Kempermann et al. 1997; Miller and Nowakowski 1988). Thus, this is currently the most common technique to detect newly generated cells, especially for studies of the neurogenic niches of the adult brain. BrdU was also used to show the first evidence of adult neurogenesis in humans. BrdU staining in human postmortem brain tissues from cancer patients, who received BrdU administration for diagnostic purposes, revealed existence of dividing cells in both the SGZ and the SVZ of the human brain (Eriksson et al. 1998). Recently, a small molecule 5-ethynyl-2'-deoxyuridine (EdU) has been developed as another alternative thymidine analog to label newly synthesized DNA in embryos and adult rodents (Salic and Mitchison 2008). EdU can be detected using a sensitive fluorescent reagent containing an azide, that effectively permeates alive and fixed tissues and accesses EdU in genomic DNA, by “click” chemistry reaction, a copper-catalyzed covalent reaction between an azide and an alkyne in the EdU nucleoside without DNA denaturation.

These thymidine analogs are incorporated not only into synthesized DNA but also into nicked and damaged DNA undergoing active DNA repair even with a low frequency (Selden et al. 1993). It is also possible to mark cell cycle re-entry in postmitotic neurons before apoptosis using thymidine analogs (Katchanov et al. 2001; Kuan et al. 2004).

Therefore, care must be taken upon data conclusion for incorporation of both ^3H -thymidine and BrdU. To properly examine neurogenesis, careful analyses with confocal microscopy using multiple cell-type-specific markers along with the thymidine analogs, mainly with BrdU, are necessary (Rakic 2002). Moreover, detectable levels of BrdU incorporation with a single administration are limited and reduced after several rounds of cell division (Hayes and Nowakowski 2002). Results of BrdU uptake for proliferative, repairing or dying cells are dependent on each experimental paradigm, thus dose and duration of BrdU administration must be appropriately planned to avoid misunderstanding the data.

As an alternative to thymidine analogs, Ki-67 can be utilized to detect proliferating cells with immunohistochemistry. Ki-67 is a nuclear protein, expressed in dividing cells during the entire mitotic process in mammals including rodents and humans, and detectable with Ki-67-specific antibodies (Endl and Gerdes 2000; Scholzen and Gerdes 2000). Since Ki-67 is an endogenous protein, it has no adverse effect on living cells unlike [^3H]dT or BrdU, which are known to be toxic by intraperitoneal administration, causing stress affecting neurogenesis (Ehmann et al. 1975; Kolb et al. 1999; Breunig et al. 2007; Taupin 2007). Despite the lack of adverse effects, the short half-life of Ki-67 renders it less suitable in the analysis of differentiation and survival of newly formed cells.

37.4.2 NSC/NPC Markers

Nestin is an intermediate filament protein that is expressed by NECs and NSCs/NPCs during CNS development (Alvarez-Buylla et al. 2001). In the adult brain, it is observed in the neurogenic niches of both the SVZ and the SGZ where nestin expression is found in radial glia-like cells (type B and type 1), continues through transient progenitor stages in both niches (type C and type 2a/2b), and disappears in neuroblasts (type A and type 3) (Fig. 37.3) (Kempermann et al. 2004; Ming and Song 2011). Using a transgenic mouse expressing green fluorescent protein (GFP) under the control of the regulatory regions of the nestin gene (Yamaguchi et al. 2000), NSCs/NPCs can be labeled and visualized with GFP that allows for analyses of morphology and identification of newly formed cell types using other molecular markers in the adult brain (Fig. 37.4a) (Kempermann et al. 2004).

Retroviruses have a single-stranded RNA viral genome that integrates into the host genome for viral replication with the reverse transcription process and transgene expression in the host cell. Retroviruses without nuclear import mechanisms carry out viral integration only during mitosis when the nuclear membrane disappears (Lewis and Emerman 1994), they can be used to label and track only proliferating NSCs/NPCs by a live reporter such as GFP, allowing for

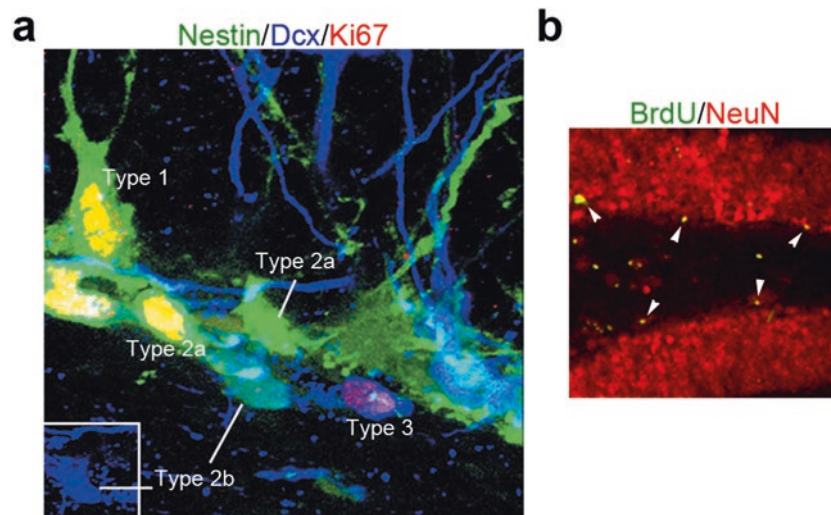


Fig. 37.4 (a) Identification of cell types in hippocampal adult neurogenesis using a transgenic mouse expressing green fluorescent protein under the control of the regulatory regions of the nestin gene. Confocal image shows multi-stained cells of Nestin (green)/Ki67 (red)-positive type 1 cell, Nestin/Ki67-positive proliferating type 2a cell, Nestin/Ki67-negative type 2a cell, Nestin/Dcx-positive type 2b cell, and

Nestin-negative and Dcx/Ki67-positive proliferating type 3 neuroblast. Cited from (Kempermann et al. 2004) (b) BrdU (green)/NeuN (red) staining in the subgranular zone of the hippocampal dentate gyrus 3 weeks after BrdU administration shows BrdU incorporation into matured newly formed neurons and neuronal survival (yellow, arrowheads)

visualization and analysis of living newly developed cells (van Praag et al. 2002). Since GFP is distributed throughout infected neurons, the entire morphology of the newly formed neurons including their dendritic trees and axonal arborization can be observed, enabling electrophysiological analysis of the labeled cells. This methodology, however, has several disadvantages. *First*, invasive stereotaxic injection is required to introduce the virus to areas of interest. *Second*, since retroviral infection is sporadic and unpredictable, labeling efficiency is not constant between individuals, thus hindering accurate quantification. *Third*, spurious GFP expression caused by fusion with retrovirus-infected microglia is found in mature neurons (Ackman et al. 2006), resulting in immune-mediated side effects and data misinterpretation. With these limitations, this methodology may not be ideal for studies of neurogenesis in the neurogenic niches (Gould 2007).

37.4.3 Neuronal Markers

The *Dcx* gene was originally identified as a causative gene of human cortical disorders. Mutant alleles of the gene affect NPC migration during cortical development, which leads to X-linked lissencephaly, a neuronal migration disorder associated with epilepsy and mental retardation in humans (des Portes V et al. 1998; Couillard-Despres et al. 2001). *Dcx* is a microtubule-associated protein expressed at high levels during neuronal migration in mouse CNS development, and retained within the two neurogenic niches in the adult brain (Francis et al. 1999; Nacher et al. 2001). It is expressed

throughout neurons including in the cytoplasm, nuclei, leading processes and growth cones of neurites (Brown et al. 2003; Schaar et al. 2004). In the adult brain neurogenic niches, *Dcx* expression is transient, seen mainly in type A migrating neuroblasts in the SVZ, and type 2b, 3 and postmitotic immature neurons in the SGZ (Brown et al. 2003; Ming and Song 2011).

Polysialylated neural cell adhesion molecule (PSA-NCAM) is a cell surface glycoprotein in the CNS. It is a polysialylated form of NCAM, which is post-translationally modified and is considered to facilitate NPC migration, neural plasticity, axon pathfinding and synaptogenesis. The level and distribution of PSA-NCAM are correlated to learning and memory (Becker et al. 1996; Fox et al. 2000). It is expressed widely in the brain during embryonic development, postnatally in immature neurons in the SGZ and the SVZ-RMS-OB system of the adult brain, and overlaps with *Dcx* in migrating neuroblasts (Bonfanti and Theodosis 1994; Ming and Song 2005). PSA-NCAM is also found in mature neurons in paleocortex layer II, the septum and the amygdala (Kiss and Rougon 1997; Rutishauser 2008; Nacher et al. 2013).

NeuN is a neuron-specific nuclear protein in the vertebrate CNS. It was originally identified by immunoreactivity with a monoclonal antibody (clone A60) that was generated by immunization of BALB/c mice using cell nuclei isolated from the brains of a strain, *Mus caroli*. It is expressed in postmitotic neurons with some exceptions including cerebellar Purkinje cells and Golgi cells, mitral cells in the OB, and photoreceptor cells in retina (Mullen et al. 1992). In the neurogenic niches in the adult brain, it is hard to distinguish newly

developed neurons from existing neurons by NeuN expression, since Dcx expression is down-regulated before NeuN expression is initiated in postmitotic neurons. A number of studies employ BrdU incorporation and fluorescent doublestaining with antibodies to BrdU and NeuN to evaluate newly formed and matured neurons and their survival (Fig. 37.4b) (Butovsky et al. 2006; Becker et al. 2007; Choi et al. 2008; Kiyota et al. 2011; Kiyota et al. 2015).

37.5 Factors Regulating Adult Neurogenesis

37.5.1 Aging

Aging is a major negative factor in adult neurogenesis. While embryonic neurogenesis occurs in the entire brain during development, postnatal adult neurogenesis is tightly restricted only in the two neurogenic niches aforementioned. Adult neurogenesis is a dynamic process that produces many thousands of neuroblasts, followed by neuronal differentiation, everyday throughout life in both neurogenic niches in rodents, primates and humans (Cameron and McKay 2001; Kempermann and Gage 2002; Knott et al. 2010; Curtis et al. 2007; Sanai et al. 2007; Spalding et al. 2013). Although a significant number of new cells are produced, only a small fraction of neuroblasts and immature neurons survive and are able to become functionally integrated into pre-existing neural circuitry, and most of them are eliminated by apoptosis and cell death (Carleton et al. 2003; Dayer et al. 2003). Therefore, aging causes rapid decline in adult neurogenesis (Kuhn et al. 1997; Heine et al. 2004b; Manganas et al. 2007). Recent studies report a significant decline in SVZ neurogenesis after infancy in the human SVZ/OB system (Sanai et al. 2011; Bergmann et al. 2012). In the DG, granule cells generated during embryonic development are replaced by postnatally generated granule cells with aging-dependent reduction in the hippocampal neurogenesis (Spalding et al. 2013). Aging-associated decline of neurogenesis can be attributed to changes in sizes of quiescent and active NSC pools (Lugert et al. 2012; Mira et al. 2012), or to termination of differentiating ability of NSCs into neurons in the hippocampus (Encinas et al. 2011).

37.5.2 Life Habit

Various environmental stimuli dynamically regulate the process of adult neurogenesis. Factors include exercise, enriched environment, dietary modulation, and so forth (Zhao et al. 2008; Lucassen et al. 2010). Generally, exercise is beneficial to our quality of life. In laboratory rodents, certain voluntary running exercises enhance the number of cell proliferation

and newly developed cell survival, and accelerates the formation of mushroom spines in the SGZ (van Praag et al. 1999; Zhao et al. 2006). This has also been observed in aged animals, and associated with improvement in learning and memory functions in the Morris water maze (MWM) (van Praag et al. 2005). Voluntary running-dependent neurogenesis is likely mediated by trophic growth factors: brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF), and is associated with long-term potentiation (LTP) induction in the DG (Olson et al. 2006). Although exercise can be beneficial, an excess amount of running negatively affects the hippocampal progenitor proliferation. Long-term running for 24 days results in a significant reduction of progenitor proliferation rate, because of a gradual activation of the hypothalamic–pituitary–adrenocortical (HPA) axis. During the activation, corticotrophin-releasing factor (CRF) is released from neurons of the paraventricular nucleus in the hypothalamus, and promotes adrenocorticotrophic hormone release from the pituitary gland. The adrenocorticotrophic hormone stimulates the adrenal cortex to synthesize and release glucocorticoid hormones (GCs; corticosterone in rodents; cortisol in humans). Elevated GCs affect the hippocampal neurons to reduce dendritic branching causing atrophy (Sapolsky 2000). While it is suggested that the GCs regulate the hippocampal neurogenesis through the serotonin system, the mechanism behind this process is not exactly determined yet (Bruehl et al. 2007; Alenina and Klempin 2015). The HPA axis can be attenuated by adjusting the daily running distance to an appropriate level (Ulrich-Lai and Herman 2009; Lucassen et al. 2010).

Classically, an enriched environment—equipped with nesting materials, a rearrangeable set of plastic tubes, tunnels and running wheels in a cage—has been known to induce experience-dependent neuroanatomical plasticity, which includes morphological changes in the size of hippocampus, augmented dendritic arborization and an increased number of glial cells. Survival of newly formed cells is increased in the hippocampal DG of animals exposed to an enriched environment (Kempermann et al. 1997). Although many newly developed cells die within 4 weeks after birth, environmental enrichment promotes cell survival of immature neurons, especially within the 1–3 weeks after birth, associated with VEGF expression and subsequent improvement of learning and memory function in the MWM task (Kee et al. 2007; Tashiro et al. 2007; Zhao et al. 2008).

Dietary modulation influences adult neurogenesis and hippocampal function. Simple sugars and saturated fats are major components causing excessive energy intake that facilitates insulin resistance, obesity, diabetes and cardiovascular disease (Gross et al. 2004; Everitt et al. 2006). In the rodent brain, high-calorie diets affect learning and memory associated with deficits in hippocampal neurogenesis and synaptic plasticity, as well as reduced BDNF expression in

the hippocampus (Molteni et al. 2002; Lindqvist et al. 2006; Farr et al. 2008; Stranahan et al. 2008). In contrast, dietary restriction (DR) not only extends life span but also has a beneficial role in enhancing hippocampal neurogenesis (Park and Lee 2011). DR mimetics such as 2-deoxy-D-glucose, metformin and resveratrol protect hippocampal neurons in models of neurodegenerative diseases, and facilitate survival of newly generated neurons (Lee et al. 1999; El-Mir et al. 2008; Rocha-Gonzalez et al. 2008). Similarly, several antioxidants have been shown to boost and protect adult neurogenesis. For instance, curcumin, an yellow substance from the root of the plant *Curcuma longa*, has its neuroprotective effect associated with prevention from cognitive impairment (Frautschy et al. 2001). Its neuroprotective effect is attributed to an increase in the number of hippocampal proliferating cells rather than enhancing cell survival (Xu et al. 2007). These findings are associated with elevated levels in hippocampal BDNF expression and other neurotrophic factors such as neurotrophin-3 (NT-3) and IGF-1 (Park and Lee 2011).

37.5.3 Stress

Stress is one of the most potent environmental factors to negatively impact adult neurogenesis (Mirescu and Gould 2006). An ever-present part of life, the stress response can be induced by a number of physical and psychological stimuli from social pressures, job demands, illness, sleep disruption, inappropriate diet, excess exercise, exposure to an unfamiliar environment, and so on. They are mostly correlated to perturbations of a biological nature associated with metabolic changes, blood loss, and/or inflammation, and eventually bring physical and emotional symptoms of depression (Lucassen et al. 2010). Both acute and chronic stress exposures negatively affect cell proliferation, neuronal renewal and survival (Mirescu and Gould 2006; Oomen et al. 2007). Acute stress reduces cell proliferation and increases the number of apoptotic cells. Chronic stress seems to reduce apoptosis but it also decreases cell proliferation through activation of cell cycle inhibitor p27Kip1 (Heine et al. 2004a; Heine et al. 2004c). A variety of stress paradigms causes elevated levels of adrenal GCs through the activated HPA axis, thus reducing cell proliferation in the SGZ. Stress-mediated suppression of the proliferation can be prevented by adrenalectomy (Mirescu and Gould 2006).

Stress impacts levels of neurotransmitters including serotonin, norepinephrine or noradrenalin, GABA, and dopamine, resulting in reduced SGZ neurogenesis. Administration of antidepressants can lessen these effects (Warner-Schmidt and Duman 2006). Stress has been also linked to changes in levels of growth and neurotrophic factors (BDNF, IGF-1, nerve growth

factor (NGF), EGF, and VEGF), altering the regulation of adult neurogenesis. An enriched environment can mimics antidepressant effects by reducing expression of these factors, stimulating SGZ cell proliferation, thereby increasing neurogenesis (Zhao et al. 2008; Lucassen et al. 2010).

37.6 Adult Neurogenesis in Brain Disorders and Brain Repair

Finding that neurogenesis continues to occur in the neurogenic niches throughout life led to further investigation into the role of neurogenesis in various neurodegenerative diseases using animal models of human diseases. In fact, adult neurogenesis is strikingly altered in the diseased/damaged brains in two main ways. *First*, overall reduction of NSC/NPC activity and/or cell survival causes a decline in neurogenesis. *Second*, aberrant maturation of newly developed neurons results in an increased number of cells and abnormal integration into neural circuitry. Neurogenesis is particularly up-regulated in the neurogenic niches in certain diseases such as stroke, seizures, and traumatic brain injury (TBI) (Quadrato et al. 2014). Although up-regulated neurogenesis in disease conditions indicates a compensatory mechanism for self-repair in the damaged brain, it cannot completely repair the brain insults where survival and integration of newly formed neurons into the neural circuitry is abnormal. Therefore, understanding the exact mechanisms that modulate endogenous stem cells in the brain under various diseased conditions, and developing strategies to increase the number of functional NPCs and their survival may allow us to develop therapeutics.

Furthermore, the transplantation of somatic or pluripotent stem cells isolated from exogenous sources into the damaged brain also has therapeutic potential as another cellular-based strategy to repair neurological insults. During the last few decades, drastic advancements in the field of regenerative medicine have been made toward repairing injured organs including the brain. Well-known stem cells include embryonic stem cells (ESCs), mesenchymal stem cells (MSCs), immortalized human NSCs/NPCs, primary isolated NPCs, and induced pluripotent stem cells (iPSCs) (Liu et al. 2009; Martino et al. 2011). The goal of stem cell transplantation is to replace damaged and dysfunctional cells with new functional ones, and it has been effective in disease and injury models (Pluchino et al. 2003; Rafii and Lyden 2003; Cummings et al. 2005; Le Blanc et al. 2008). Although there are many challenging issues to be resolved regarding therapeutic use of grafted stem cells, some of the cells have the features of resident stem cells allowing for appropriate integration into the neural circuitry. In the following the relevance and therapeutic potential of adult neurogenesis in several brain disorders will be discussed.

37.6.1 Ischemia

Cerebral ischemic injury triggered by vascular occlusion in the brain is the most common form of stroke causing adult-acquired behavioral and cognitive disabilities. Focal (stroke) or global (e.g. cardiac arrest) cerebral ischemia arises from temporary or permanent blockage of blood supply, causing oxygen and glucose deprivation to the brain. Several events such as edema, deafferentation, and inflammation occur acutely around the infarct after ischemic injury, consequently triggering a secondary complex chain of events: oxidative stress, excitotoxic neurotransmitter release, disruption of the blood-brain barrier, and eventually cell necrosis affecting neurons, glia and blood vessels (Broughton et al. 2009). Early functional recovery can resolve the injurious events to a limited extent through incompletely understood mechanisms. Accumulating evidences suggest that a number of endogenous regenerative responses, including immunomodulation, altered gene expression, enhanced angiogenesis, neuroplasticity and neurogenesis, may be involved in the longer-term recovery of function following cerebral ischemia (Ergul et al. 2012; Hermann and Chopp 2012). Notably a number of studies demonstrate that cerebral ischemia potently stimulates neurogenesis in both the SVZ and the hippocampal DG (Liu et al. 2009). For example, it enhances neuroblast production in the rat SVZ, as well as in the cortex, outside of the neurogenic niches, in both rat stroke models and biopsy specimens of human brains (Shimada et al. 2010; Jin et al. 2006). Such reactive responses are considered to be attempts of self-repair in the damaged brain after injury. However, they are insufficient in generating adequate amounts of new neurons that can appropriately survive, integrate into the neural circuits, and recover functionality. Hence it is important to understand the cellular and molecular mechanisms of the post-ischemic responses in order to establish therapeutic strategies for treating stroke patients.

Recent therapeutics targeting stem cells promise a means of augmenting brain repair. Therapeutic targets include promotion of endogenous neurogenesis reinforced by stimulators and transplantation of various kinds of exogenous stem cells. Stroke is non-progressive and involves a focal loss of all cell types around the infarct, not targeting specific cells as in other neurodegenerative disorders. As described in the former section, adult neurogenesis is also increased in response to ischemia in both within and outside of the neurogenic niches. Thus stem cell therapy is not restricted to a paradigm of replacing only specific cell types, instead it may be able to dynamically act on multiple mechanisms in a temporally and spatially regulated microenvironment as stromal cells. Enhancing therapeutic effects, the use of extrinsic molecules such as hepatocyte growth factor, statins and fluoxetine have also been shown to boost neuroblast production, associated with angiogenesis, synaptogenesis and increased VEGF expression (Shang et al. 2011; Chen et al. 2003; Sun et al. 2015).

The therapeutic effects of a variety of stem cells have been explored in animal models of stroke. While the use of ESCs yet face ethical, religious and moral objections, *in vitro* fertilization programs currently utilize ESCs for medical purposes (Kalladka and Muir 2014). Studies using ESCs in animal models of middle cerebral arterial occlusion (MCAO) inducing stroke show that transplanted ESC-derived NSCs within the infarct core can survive and differentiate into mature glial cells and electrophysiologically functional neurons with high yield. Transplantation exhibits no chromosome abnormalities, no tumor formation and improved sensory recovery (Buhnenmann et al. 2006; Daadi et al. 2008; Drury-Stewart et al. 2013). Intracerebral implantation of human NSCs shows cell migration to ischemic regions, behavioral improvements, more than 50% cell survival beyond 2 months, and some differentiated neurons with forming synapses in rats after MCAO (Darsalia et al. 2011; Zhang et al. 2009; Song et al. 2011; Daadi et al. 2009). Invention of iPSCs enables resolution of the religious, legal, moral and ethical issues of ESCs. iPSCs produce patient-specific autologous cells, although there are still several clinical hurdles for this approach such as limited yields, long-term processes, and potential risks of tumorigenesis from viral transfection and oncogenic transcription factors (Malik and Rao 2013). Intracerebral implantation of undifferentiated iPSCs results in the production of neuroblasts and differentiated mature neurons, but also teratoma formation in MCAO rat models (Kawai et al. 2010). However, transplanted human iPSC-derived long-term self-renewing neuroepithelial-like SCs can survive without forming tumors, differentiate into mature neurons that exhibit proper electrophysiological properties, and receive synaptic input from host neurons, suggesting that predifferentiation of SCs may be essential to minimizing the risk of tumorigenesis (Oki et al. 2012). Alternatively, bone marrow-derived MSCs and many other cell types with similar properties to the MSCs have been used almost exclusively as therapeutics over the last decade. Numerous studies with animal stroke models have demonstrated beneficial roles of implanted MSCs, which produce trophic/growth factors (BDNF, glial-derived neurotrophic factor (GDNF), NT-3, VEGF, FGFs and thrombospondins) at the ischemic boundary in response to the local microenvironment, associated with stimulation of neurogenesis, angiogenesis and immunomodulation. Several Phase I and II clinical trials using MSCs are ongoing (for more detail see a review: (Liu et al. 2009; Kalladka and Muir 2014)).

37.6.2 Epilepsy

Epilepsy is a debilitating and multifarious disease, frequently associated with other mood disorders such as anxiety and depression. It is clinically defined by at least two or more

unprovoked seizures, which originate from different regions of the brain. While epileptic seizures induce the proliferation of NPCs including neuroblasts in both neurogenic niches of the adult brains, many studies exclusively focus on investigating adult neurogenesis in the hippocampal DG, since chronic epilepsy is strongly associated with cognitive problems and memory disturbances (Hermann and Seidenberg 2007). To generate animal models of seizures, chemoconvulsant drugs such as pilocarpine or kainic acid are used to induce status epilepticus. The drugs cause precipitating injuries and acute cell loss, followed by spontaneous seizures during phases lasting between days to weeks, becoming more frequent later (Williams et al. 2009). In parallel, dentate neurogenesis is dramatically increased in rodent models of temporal lobe epilepsy. However, a majority of newly formed cells after the epileptogenic injury integrate aberrantly (Murphy et al. 2011). During normal adult neurogenesis in the dentate SGZ, matured granule cells correctly migrate into the GCL and project their apical dendrites into the dentate molecular layer. In the neurogenesis in the epileptic brain, the seizure has several unique effects. *First*, it leads to mossy fiber sprouting of pre-existing neurons, which forms recurrent excitatory synaptic connections with neighboring granule cells and is hypothesized to promote hyperexcitability in the hippocampus. *Second*, it induces ectopic migration of new granule cells into the dentate hilus. *Third*, it promotes the granule cells to project their basal dendrites into the dentate hilus and receive excess recurrent input. Seizure induces abnormal functional integration of new neurons into the neural circuitry. Therefore, enhanced neurogenesis seems to be not a compensatory mechanism in response to neuronal death, and eventually declines to basal or even lower levels. However, declined neurogenesis can be recovered by antidepressant fluoxetine administration at the later stage. Conversely, valproic acid, an antiepileptic drug, blocks seizure-induced neurogenesis in rats with status epilepticus, associated with improved learning and memory (Barkas et al. 2012; Jessberger et al. 2007). The level of the neurogenesis has no correlation with epileptic susceptibility, thus the role of seizure-induced aberrant neurogenesis is yet under investigation (Zhao et al. 2008; Danzer 2012).

37.6.3 TBI

TBI is mostly caused by physical impact shocks including direct collision (e.g.: in traffic accidents and sport related injuries) and blast shock waves from explosion in battlefields. Patients suffering from TBI have deficits in sensory motor function, cognition, and become comatose in the worst cases. Within a few seconds such shocks lead mechanical tissue damage as the primary insult. The secondary insult consists of many events including ischemia, metabolic dysfunction, release of purinergic signaling molecules and excitatory

neurotransmitters, which affect resident astro/microglial function causing production of pro-inflammatory mediators, and eventually induce hippocampal neuronal cell death (Davalos et al. 2005; Laird et al. 2014; Sun 2014). TBI increases cell proliferation in both the SVZ and the hippocampal DG in mouse and rat models of TBI including fluid percussive injury, controlled cortical impact and diffuse weight drop injury (Dash et al. 2001; Chirumamilla et al. 2002; Gao et al. 2009), newly formed neurons that integrate into the neural circuitry in the hippocampus (Emery et al. 2005; Sun et al. 2007), suggesting innate attempts of regenerative response to repair brain tissues after TBI. Administration of growth factors and drugs including EGF, FGF-2, VEGF, erythropoietin, progesterone, statins, and imipramine has shown to enhance neurogenesis in both the SVZ and the DG (Sun 2014). Recent stem cell therapies using NSCs, NPCs, MSCs, iPSCs and human ESCs in animal models of TBI, or autologous bone marrow MSCs in patients with TBI have shown improved behavior and neuropathology (Harting et al. 2009; Bakhtiyari et al. 2011; Galindo et al. 2011; Heile and Brinker 2011; Dunkerson et al. 2014; Chang et al. 2015).

37.6.4 Alzheimer's Disease

Alzheimer's disease (AD) is the most common form of cognitive impairment affecting the elderly. Massive neuronal loss and brain atrophy are major diagnostic signs and neuropathogenic features. AD is characterized by the presence of extracellular senile plaques and intraneuronal neurofibrillary tangles (NFTs) in affected brain tissues. While NFTs consist of abnormally hyperphosphorylated microtubule associated protein tau, senile plaques are the aggregation of amyloid- β peptides (A β), which induce neuroinflammation, consequent synaptic and neuritic injuries, tau hyperphosphorylation and ultimately neuronal death (Hardy and Selkoe 2002). How adult neurogenesis is altered in human AD pathology is incompletely understood, since there seems to be conflicting observations about whether hippocampal neurogenesis is a positive or negative effect. Although one report shows that hippocampal TUC-4 (TOAD/Ulip/CRMP family protein 4) and Dcx-positive cells are increased in human AD brains (Jin et al. 2004b), others show decreases in Sox2 and Dcx-positive cells, associated with increased BMP6 levels as a negative regulator of neurogenesis, and failure in maturation of newly developed neurons (Li et al. 2008; Crews et al. 2010). While the vast majority of AD cases are late-onset sporadic form, transgenic AD animal models with familial AD (FAD) mutations in transgenes including A β precursor protein (APP) and presenilin, causing an early-onset form of AD, show altered adult neurogenesis. In such animal models, however, the positive or negative effects on neurogenesis regulation depend on types of transgenes, their expression,

promoters, age of animals and onset of the disease. Most AD animal models with FAD mutants lead to impaired neurogenesis in the hippocampal DG (see reviews for the detail: (Lazarov and Marr 2010; Mu and Gage 2011)). Conversely, some strains such as J20 transgenic mice expressing human APP (hAPP) with Swedish and Indiana mutations driven by a PDGF promoter, and APP23 mice expressing hAPP with Swedish mutation driven by a Thy-1 promoter show increased NPC proliferation and differentiation. Facilitation of neurogenesis may be a compensatory response representing self-repair mechanisms within the diseased brain, providing a potential therapeutic strategy to treat AD (Jin et al. 2004a; Lopez-Toledano and Shelanski 2007; Ermini et al. 2008). Based on those findings, a number of compounds, drugs and molecules have been tested to investigate whether they could modulate endogenous stem cells resident in the brain of AD animal models. These treatments include antidepressants, anti-inflammatory drugs, antioxidants, cholinesterase inhibitors, dietary modifications, immunomodulators and lithium, and all able to improve impaired neurogenesis (Butovsky et al. 2006; Kotani et al. 2008; Kim et al. 2013; Crupi et al. 2013; Maruszak et al. 2014; Fiorentini et al. 2010). Infusion of neurotrophic/growth factors such as NGF, BDNF, GDNF and FGF2 has a beneficial effect by enhancing neurogenesis or neuroprotection, associated with improved synaptic plasticity and performance in learning and memory (Nagahara et al. 2009; Revilla et al. 2014; Schaeffer et al. 2009; Kiyota et al. 2011). Some studies report that AD symptoms are ameliorated by cell transplantation-based therapies in AD animal models. For example, engrafted NSCs, isolated from postnatal day 1 GFP transgenic mice, differentiate into neurons, astrocytes, and oligodendrocytes in the recipient hippocampus. Transplantation of the NSCs results in improved synaptic plasticity, learning and memory, and increased BDNF levels in AD mouse models (Blurton-Jones et al. 2009; Zhang et al. 2014). An shRNA-mediated down-regulation of BDNF levels abolishes the improvements, suggesting that BDNF is critical for NSC therapy to treat AD (Blurton-Jones et al. 2009). Transplantation of NSCs stably overexpressing NGF transduced by adeno-associated virus promotes survival and differentiation of these cells, and improves cognitive function in AD rats (Wu et al. 2008). It is unclear whether such improvements are due to maturation, differentiation or integration of transplanted cells into the preexisting neural circuitry. However, embryonic MGE-derived progenitor cells of interneurons transplanted into the hippocampal hilus of aged AD mice differentiate into mature interneurons and integrate into the hippocampal circuitry, showing improvements in learning and memory. These findings indicate a significant therapeutic potential of utilization of human MGE-like cells, which might survive well and function in the human diseased brains (Tong et al. 2014). Additional

studies using human ESCs and iPSCs show that GABAergic interneurons are immature with limited integration or without fast spiking interneurons (Nicholas et al. 2013; Maroof et al. 2013), suggesting incomplete therapeutic potential for methodologies employing these cell types.

37.6.5 Neuroinflammation and Microglia

Neuroinflammation is not strictly a CNS disorder as is beneficial, neurophysiological responses to maintain brain homeostasis and plasticity. However, it is mostly associated with neurodegenerative disorders and brain injuries, influencing adult neurogenesis within both detrimental and beneficial consequences, depending on how resident microglia, infiltrated macrophages, and astrocytes are activated and how long the inflammation continues in affected areas of the brain (Russo et al. 2011; Hu et al. 2015). Notably microglia is the first immunological barrier against pathogens and environmental insults as they generally clear apoptotic cellular debris by phagocytosis (Hanisch and Kettenmann 2007; Sierra et al. 2010). Activated microglia produce proinflammatory cytokines (e.g. interleukin (IL)-6, IL-1 β , tumor necrosis factor- α : classic activation), and affect neurogenesis with detrimental consequences for neuronal viability as well as proliferation, differentiation and survival. These factors are also secreted by reactive astrocytes, meaning reactive astrogliosis has a negative impact on adult neurogenesis and contributes to the neuropathogenesis in the diseased brain. NPCs also regulate microglial function with NPC-derived secreted factors and expression of several neuroimmunoregulatory factors, thereby maintaining the balance between adult neurogenesis and microglial activation in the neurogenic niches (Biber et al. 2007; Mosher et al. 2012). Conversely, alternative activation of microglia under certain pathological conditions may positively regulate neurogenesis, and orchestrate neuronal restorative processes in the adult brain (Butovsky et al. 2006; Kiyota et al. 2012; Mathieu et al. 2010). Gene delivery of anti-inflammatory cytokines, IL-4 and IL-10 into the hippocampus protects hippocampal neurogenesis and restores impaired spatial learning in animal AD models (Kiyota et al. 2010; Kiyota et al. 2012). In particular, IL-4 directly induces CD200 expression in cultured neurons (Varnum et al. 2015). CD200 is a type I transmembrane glycoprotein with an immunoglobulin superfamily domain, and has anti-inflammatory effects through interaction of CD200-CD200 receptor expressed by microglia (Clark et al. 1985; Lue et al. 2010). Thereby overexpression of CD200 restores impairment in both proliferation and differentiation of hippocampal neurons in an AD mouse model (Varnum et al. 2015). Thus, adult neurogenesis can be both down-regulated by neurotoxin-induced inflammation and restored by anti-inflammatory treatments (Fuster-Matanzo

et al. 2013; Hu et al. 2015). Additionally, recent studies demonstrate that immunological processes such as the complement system and Toll-like receptor-mediated innate immunity may play a role in adult neurogenesis regulation with different molecular mechanisms from general immune responses (Zhao et al. 2008).

37.7 Closing Remarks

Since the first discovery of adult neurogenesis about half a century ago, a vast amount of information has been gathered. It includes the establishment of *in vitro* cultures of SVZ and SGZ-derived NSCs as neurospheres that are able to self-renew and differentiate into neurons and glia (Reynolds and Weiss 1992; Kilpatrick and Bartlett 1993; Gage et al. 1995), as well as methods to detect proliferating/differentiating NSCs/NPCs in the adult brain, along with comprehensive cellular and molecular biological and stem cell technologies. These breakthroughs have provided crucial platforms to further investigate the developmental potentialities of NSCs/NPCs, the mechanisms of proliferation and differentiation, and the applications to brain repair. Despite such progresses, however, the clinical applications of utilizing drugs that promote endogenous neurogenesis or implanting NSCs/NPCs have not yet materialized. This is due to challenging hurdles yet to overcome. Notably the brain is a very complex three-dimensional structure consisting of the extensive heterogeneity of neuronal subtypes alongside other cells such as glia and endothelial cells. During the development of the brain, different types of neurons are produced and organized temporally and spatially in tightly regulated manner driven by molecular signaling. Thus, complication of structure and organization of the adult brain limits the differentiation of implanted cells into neuronal subtypes and their integration into specific areas for repair. Even though endogenous or exogenous cells can successfully migrate and functionally integrate into the preexisting neural circuitry, the number of the cells is very small and the vast majority of them fail. One possible strategy is to develop methods to directly differentiate cells into the specific subtypes and then transplant. Further investigation into these methodologies will open a door to overcome the limitations of repairing the diseased brain.

37.8 Review Questions

1. What are the distinctions between neural stem cells and neural progenitor cells?
2. As currently identified, which growth factors contribute to maintaining neural progenitor cells?
3. Briefly summarize the production, migration, and maturation of new neurons into the dentate granule cell layer.

4. What primary region is supplied with new neurons from the SVZ in the adult brain? What type of neurons? Placement of neurons? Pathway for migration?
5. Explain the flow that new neurons are derived from GFAP-positive radial glia-like cells.
6. What stimuli are required to maintain a neurogenic environment?
7. What is important to develop therapeutics targeting successful adult neurogenesis following injury?
8. What types of stimuli have been shown to enhance basal levels of adult neurogenesis?
9. How can the microglia and astrocyte responses occurring with brain injury influence neurogenesis?

37.9 Answers

1. Stem cells can replicate indefinitely, whereas progenitor cells can divide only a limited number of times.
2. EGF and FGF.
3. Quiescent but self-renewing and multipotent radial glia-like cells called type 1 cells in the subgranular zone are activated and undergo asymmetric divisions to generate daughter radial glia-like cells and intermediate, transient amplifying type 2 cells, which give rise to type 3 neuroblasts, and expand the neuroblast pool. The neuroblasts differentiate into immature neurons. Subsequently, the immature neurons migrate up into the granule cell layer, undergo neuronal maturation and become mature glutamatergic dentate granule cells.
4. SVZ-derived neuroblasts migrate through chains along the rostral migratory stream, and differentiate mostly into GABAergic granule interneurons in the olfactory bulb.
5. GFAP-positive radial glia-like cells co-express stem cell markers nestin and Sox2. They undergo asymmetric divisions to generate daughter radial glia-like cells but also intermediate progenitor cells (IPCs), which lose GFAP expression. The IPCs become Dcx-positive and eventually NeuN-positive matured neurons.
6. Factors include exercise, enriched environment, dietary modulation, and so forth.
7. Although up-regulated neurogenesis in disease conditions indicates a compensatory mechanism for self-repair in the damaged brain, it cannot completely repair the brain insults where survival and integration of newly formed neurons into the neural circuitry is abnormal. Therefore, understanding the exact mechanisms that modulate endogenous stem cells in the brain under various diseased conditions, and developing strategies to increase the number of functional NPCs and their survival may allow us to develop therapeutics.
8. Administration of growth factors and drugs including EGF, FGF-2, VEGF, erythropoietin, progesterone, statins,

and imipramine has shown to enhance neurogenesis in both the SVZ and the DG.

9. Notably microglia is the first immunological barrier against pathogens and environmental insults as they generally clear apoptotic cellular debris by phagocytosis. Activated microglia produce proinflammatory cytokines (e.g. interleukin (IL)-6, IL-1 β , tumor necrosis factor- α : classic activation), and affect neurogenesis with detrimental consequences for neuronal viability as well as proliferation, differentiation and survival. These factors are also secreted by reactive astrocytes, meaning reactive astrogliosis has a negative impact on adult neurogenesis and contributes to the neuropathogenesis in the diseased brain.

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Abstract

Chronic traumatic encephalopathy (CTE) is a progressive neurodegenerative disorder that can only be diagnosed post-mortem and has been retrospectively linked to a history of neurotrauma involving repeated concussive and subconcussive head injuries. Such a pattern of neurotrauma is commonly found among military veterans and athletes participating in contact sports. A post-mortem diagnosis depends on the detection of abnormal hyperphosphorylated-tau (p-tau) neuropathology that is characterized by an irregular, focal, perivascular distribution largely localized to cortical sulci. Although p-tau is not unique to CTE, this distribution pattern helps distinguish it from other degenerative tauopathies. High molecular weight p-tau proteins tend to accumulate in neurons and glia as pretangles, neurofibrillary tangles, extracellular tangles and neuropil threads. Supportive non-p-tau pathologies include TDP-43 and β -amyloid in neuronal cytoplasmic inclusions and dot-like structures in the tangles. Progressive tauopathies can share a broad range of neuropsychiatric sequelae, which for CTE are identified through next-of-kin interviews or verbal autopsies. A better understanding of how CTE relates to other neuropsychiatric and neurodegenerative disorders will require carefully controlled prospective studies involving both in vitro and in vivo model systems as well as parallel efforts to develop and validate fluid biomarkers and tau-specific neuroimaging technologies. Such an approach will improve the odds of advancing multiple, targeted therapies from preclinical studies to clinical trials. This chapter covers etiology, pathophysiology, experimental model systems, biomarkers and potential therapeutic strategies pertaining to CTE.

Keywords

Alzheimer's disease • Biomarkers • Chronic traumatic encephalopathy • Neurofibrillary tangles • Traumatic brain injury

38.1 Introduction

Chronic traumatic encephalopathy (CTE) refers to an incurable brain pathology induced by direct physical head injury that can trigger a slowly progressive pathophysiological cascade producing abnormal neuronal protein aggregates (hyperphosphorylated tau pathology) that lead to brain inflammation and irreversible neurodegeneration (Iqbal et al. 2016). The primary risk factor for CTE is a long-term history of repetitive traumatic brain injuries (TBI, e.g., concussive

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and subconcussive impacts). Not only is this neurodegenerative disorder incurable, it's further complicated by the facts that symptoms often aren't manifested for years or decades after the last traumatic event and a definitive diagnosis can only be made post-mortem. Because CTE can assume a progressive neurodegenerative process, its symptoms run the gamut of cognitive, affective/mood, and motor dysfunction, many of which are shared with other neurodegenerative diseases, such as Alzheimer's and Parkinson's. Although the tau pathology generally characteristic of CTE might not develop until long after a series of typically sports-related, repetitive concussive and subconcussive impacts, it can also arise from single severe traumatic events (Johnson et al. 2012, 2013) that are more isolated yet involve tremendous forces, such as vehicle collisions, falls or bomb blasts. During head trauma, the brain may be subjected to the compressive and shearing forces of rapid acceleration and deceleration. Goldstein et al. (2012) hypothesized that the rapid acceleration-deceleration forces of concussive impact trauma (e.g., the "bobblehead effect") were key to CTE pathogenesis in both sports- and blast-induced TBI. These authors confirmed their hypothesis in a mouse model wherein mice were exposed to a blast wind inside a shock tube. Only mice whose heads were not restrained developed CTE neuropathologies. In the case of blast-induced head trauma, even without direct cranial impact, tissue damage can also result from a blood surge throughout the cerebrovasculature and implosive cavitation of dissolved gases induced by the massive overpressure shock wave. Thus, depending on the type of head trauma, their frequency and cumulative impact, the resulting physical brain injuries can range from mild to severe and be either localized contusions in specific brain regions (e.g., coup-contrecoup injuries) or extensive and diffuse, which may influence the suite of symptoms that are manifested based on the brain regions affected. Although head trauma is the proximate cause of CTE, whether or not an individual will develop CTE may depend on other risk factors, including genetics (Jordan et al. 1997; Gandy and DeKosky 2012) that may predispose them to the underlying pathophysiological processes.

Martland (1928), publishing his opinion in the medical literature regarding the punch drunk syndrome, was the first physician to posit a link between multiple concussions, the development of progressive degenerative lesions, and symptoms characteristic of the Parkinsonian syndrome. As accounts of the neurological effects of boxing grew, a new term was coined, dementia pugilistica (Millspaugh 1937). With the attention still on boxing, Critchley (1957) appears to be the first to apply the term "chronic traumatic encephalopathy". However, aside from the neurological studies aimed at boxing throughout much of the twentieth century, other high-impact or contact sports garnered much less attention toward the potential lingering or chronic effects of repeated concussive or subconcussive blows to the head. Beginning in the late 1980s, research was being published on

the potential for brain injury in soccer players, with particular focus on the practice of heading the ball. For example, Tysvaer and Løchen (1991) reported that, out of 37 retired soccer players tested, 81 % exhibited cognitive deficits. But it was not until after the early 2000s that CTE became a hot topic with both news and social media outlets. This is when medical professionals began publishing case studies of retired National Football League (NFL) players with a history of both multiple concussions and the psychological and neurological correlates of traumatic brain injury (Guskiewicz et al. 2005; Omalu et al. 2005, 2006; Cantu 2007). In the U.S. alone, the number of contact sports- and recreation-related concussions occurring each year has been estimated to range between 1.6 and 3.8 million (Langlois et al. 2006). More recently, an examination of the brains of former NFL football players revealed that 87 of 91 tested CTE-positive (Concussion Legacy 2015). Furthermore, just as the scope of sports-related CTE was encompassing the NFL, the U.S. was engaging in two wars in Afghanistan and Iraq. Due to significant improvements in armored transport, body armor, and battlefield medicine, the ratio of wounded to deceased soldiers is at an all-time high (IOM 2014) and the 'signature' wounds now are post-traumatic stress (PTSD) and traumatic brain injury (TBI). In the war on terror, roughly 20 % of veterans are estimated to have experienced TBIs (McKee and Robinson 2014). Post-mortem studies of the neuropathology of war veterans published by Omalu et al. (2011a, b) and Goldstein et al. (2012) indicated that blast-induced TBI could likely contribute to the development of progressive tau protein-linked neurodegeneration, which is the hallmark of CTE seen years after sports-related concussions.

Thus, whether acquired while engaging in contact sports, or during active military service, or even in an injury accident, a history of TBIs is now recognized as a risk factor for CTE. Functional deficits due to both primary and secondary brain injuries contribute to TBI outcomes (Table 38.1). Primary injury caused by an immediate mechanical insult occurs followed by secondary injury that results from the pathophysiological changes, including a persistent heightened neuroimmune response (Blaylock and Maroon 2011; Bramlett and Dietrich 2014), occurring over a period of days to months to years leading to chronic progressive neurodegeneration. Single moderate-to-severe TBI has been associated with accelerated neurodegeneration and increased risk of AD, PD and ALS, whereas repetitive mild-to-severe TBI is associated with persistent, long-term debilitating effects, progressive neurodegeneration and CTE (McKee et al. 2009, 2010, 2013; Goldstein et al. 2012; Hall 2016). Not surprisingly, the socioeconomic costs of TBI/CTE are staggering. The Centers for Disease Control and Prevention (2015) estimates the direct and indirect medical costs in 2010 at \$76.5 billion. Other aspects of the costs associated with neurotrauma were reviewed by Humphreys et al. (2013). The development of effective therapies for CTE will

Table 38.1 Primary injuries and secondary damage in TBI

Mild TBI	Moderate TBI	Severe TBI
Primary Injury: Mild to Severe CNS Trauma		
Predominantly non-penetrating blast	Frequently mixed, non-penetrating blast	Complex, penetrating blast
Loss of consciousness: <30 min	Loss of consciousness: >30 min <24 h	Loss of consciousness: >24 h
Amnesia: <24 h	Amnesia: >24 h	Amnesia: >7 days
GCS: 13–15	GCS: 9–12	GCS: <9
Imaging: negative	Imaging: transient changes	Imaging: positive, lasting abnormalities
Secondary damage: Mild to severe diffuse injury, impaired axonal transport, neuronal circuit damage and cell death		
<u>Neuropathological deficits</u>		
Contusion, mild edema, uncertain short-term neuropathology, abnormal protein aggregates, neuro-fibrillary tangles (tau pathology)	Significant neuropathological deficits, abnormal protein aggregates, neurofibrillary tangles (tau pathology), β -amyloid pathology	Severe, chronic neurological deficits, chronically impaired neuronal homeostasis, abnormal protein aggregates, amyloid- β pathology
<u>Neuropsychiatric deficits</u>		
Short-term abnormalities, long-term abnormalities after repeated TBI	Significant neuropsychiatric abnormalities	Severe and variable, chronic physical and neuropsychiatric disabilities
<u>Comorbidities</u>		
CTE, PTSD, Dementia pugilistica, pugilistic Parkinsonism	PTSD, AD and PD	Polytrauma, such as multiple-organ injuries, AD

require concerted efforts at unraveling the cellular-molecular mechanisms underlying its pathogenesis. The pre-clinical research towards this end relies on animal models that recapitulate the chronic cognitive and neuropathological symptoms documented in human patients. It is imperative that we move beyond the current understanding of CTE, which rests on retrospective clinical-pathological correlations, to establishing causal links between TBIs and the clinical and neuropathological manifestations labeled as CTE. This will require both animal and human studies of CTE based on randomized before-after control-impact designs employing protein-based assays, biofluid-based biomarkers and neuroimaging.

38.2 Clinical Signs and Symptoms of CTE

CTE represents a chronic disease process (Masel and DeWitt 2010) initiated by mechanical force-induced head trauma that can progressively worsen in response to repeated head trauma. Individual head injury events can range from mild to severe, and as the severity increases so does the robustness of associated acute clinical symptoms (McCrory et al. 2013) within the first hours and days after exposing the head to a biomechanically forceful impact. However, these acute symptoms typically dissipate within days or weeks. Although mild, moderate and severe TBIs are recognized clinical entities, operational definitions of what constitutes these different forms can get complicated (McCrory et al. 2013; Reith et al. 2015). Since neuropathological evidence is required to confirm CTE, our understanding of how CTE might manifest itself as motor, cognitive, or affective symptoms has emerged retrospectively

from ‘verbal autopsies’, involving interviews of the families of those neuropathologically diagnosed with CTE post-mortem. Stern et al. (2013) conducted next-of-kin phone interviews for 36 deceased men with a diverse history of contact sports, ranging from high school only on up to collegiate and professional sports. The brains from these men, selected from 81 brains neuropathologically confirmed with CTE at the Boston University Center for the Study of Traumatic Encephalopathy brain bank, were free of comorbid brain disorders and the interviews were performed blind to their respective neuropathological results. In addition to documenting changes in cognitive, behavioral, psychological, and motor function, the interviewers also obtained complete histories on demographics, military service, occupation, athletic activity, physical and mental health treatments, daily social habits, and cause of death. Although three of the subjects were asymptomatic, the authors fit the remaining 33 subjects into one of only two CTE variants based on their initial symptoms—behavior/mood or cognition—thereby excluding a motor variant since none of the subjects exhibited motor impairment as the initial symptom. Subjects ascribed to the behavior/mood variant exhibited their initial symptoms at a significantly younger age than those with the cognition variant, but they also died significantly younger. Less than one-third of the subjects ever exhibited motor impairment or Parkinsonism, whereas 94% eventually showed signs of dementia in the form of progressive memory impairment; signs are features that can be verified by an independent observer. Dementia was a more common outcome in the cognition variant. Interestingly, 40.9% of the behavior/mood group had been diagnosed as early-stage CTE (Stages I-II out of IV, (McKee et al. 2013)), in contrast to only 9.1% of the cognition group,

which was primarily late-stage CTE. Behavioral features were manifested as explosivity, verbal and physical violence and impulsivity. Prominent mood changes included primarily depression, hopelessness, suicidal ideation and anxiety. Cognitive dysfunction included memory loss, executive dysfunction, loss of concentration or attention, impaired language and visuospatial difficulties. They added that the behavior/mood variant comprised signs and symptoms that were more stable in contrast to the more progressive features of the cognition variant.

Recognizing the limited external validity inherent in such a small non-random sample from a CTE brain bank comprised of athletes' brains donated by families who may have felt compelled to donate based on the symptoms they had witnessed, the same investigators refined their definitions of CTE variants by expanding the number of neuropathology cases from 36 to 202 athletes (Montenigro et al. 2014). They accomplished this by conducting a systematic review of the published literature of case series that had reported enough information allowing each case to be classified according to Jordan's (2013) clinical criteria of definite, probable or possible CTE neuropathology. Given the vast array of signs and symptoms associated with neuropathologically defined CTE, Montenigro and colleagues resorted to research diagnostic subtyping to clinically define what they called traumatic encephalopathy syndrome (TES). They arrived at four subtypes—a behavioral/mood variant, a cognitive variant, a mixed variant, and TES dementia—requiring that five general criteria be met, at least one of three core clinical features from the cognition, behavioral or mood domains and at least two of nine supportive features. Besides a history of repeated TBI ranging from subconcussive to severe loss of consciousness, other key general criteria include a minimum of 12 months of clinical signs or symptoms and the absence of neurological co-morbidities that could otherwise account for those features. Supportive features included impulsivity, anxiety, apathy, paranoia, suicidality, headaches, motor signs, progressive decline and a delayed onset of at least 2 years post-TBI. Modifiers were applied to identify the clinical course as progressive, stable or unknown/inconsistent, and whether motor signs were manifested. Because they delved back to the earliest CTE-related literature (1928–2010), the proportion of boxers jumped from 8 to 70% of cases, which prompted them to address the perceived divide between 'classic' CTE and the post-2005 phenomenon of 'modern' CTE. A designation of classic CTE was reserved for cases having prominent signs of motor impairment, such as ataxia, dysarthria, Parkinsonism, gait anomalies and tremor, with the clinical domains of cognition, mood, and behavior featuring much less prominently. They attributed the perceived clinical divide as an artefact borne out of the preponderance of boxers in earlier cases. To support this claim, they took a closer look at the 80 brains from athletes

examined by McKee et al. (2013) and determined that 71% of professional boxers presented motor signs compared to only 13% of professional football players. Furthermore, of the CTE Stage IV cases, 83% of the boxers had exhibited cerebellar pathology compared to only 57% of footballers. The biomechanical differences in repetitive head trauma experienced by boxers and football players might explain the patterns observed (Stern et al. 2013; Montenigro et al. 2014). Punches exert powerful torsional forces through the jaw to the brainstem and cerebellum, whereas the accelerative and decelerative forces exerted on the brain during collisions in football are largely linear or transverse. Besides the type of brain injury, clinical researchers also need data on other risk factors such as the number, frequency and severity of TBIs, the age range of exposure, lifestyle choices, and genetic susceptibility (e.g., Apolipoprotein E4). At this time, however, Montenigro and colleagues emphasized that in a clinical setting their research diagnostic scheme sacrifices specificity (avoiding false positives) for high sensitivity (avoiding false negatives). In the future, improving our ability to use a cluster of signs and symptoms as the clinical manifestations that diagnose or predict the underlying neuropathology of CTE will also require a fuller understanding of the risk factors that help transform brain trauma events into a progressive neurodegenerative disease.

38.3 Neuropathology of CTE

38.3.1 Historical Overview

While numerous clinical and neuropathological descriptions of CTE have been reported during the past decades, it remains unclear whether the pathological changes causally lead to the clinical observations given the current lack of epidemiological, longitudinal, cross-sectional and prospective data. Most of our understanding of CTE is based on the retrospective examination of patients at autopsy stages.

The neuropathological features of CTE were first described in a case report of a 51-year-old ex-boxer who developed post-traumatic dementia in 1954 by Brandenburg and Hallervorden (1954). The authors described Alzheimer's disease-like neuropathological changes, including the presence of senile plaques with neurofibrillary tangles (NFTs) in the cortex, basal nuclei, and to a lesser extent, in the cerebellum. This initial neuropathological report was followed by a number of case reports or small cases of boxers with varying degrees of pathologic findings associated with the disorder (Constantinidis and Tissot 1967; Courville 1962; Grahmann and Ule 1957; Mc 1959; Neuburger et al. 1959). Many of these reports described shared pathological abnormalities, such as cerebral atrophy, particularly of the frontal lobes. In 1973, Corsellis and colleagues (1973) performed a detailed

histopathological analysis on the brains of 15 ex-boxers ranging in age from 57 to 91 years. This seminal research outlined the major gross and microscopic neuropathological changes of CTE in boxers, demonstrating a pathologically widened and fenestrated cavum septum pellucidum, enlarged lateral and third ventricles, thinning of the hypothalamic floor, cerebellar and cerebral scarring, marked loss of pigment in the substantia nigra and uniquely distributed NFTs. In 1990, Roberts et al. (1990a), in a well-controlled study re-examining brains from the Corsellis series plus six additional retired boxers using a refined immunohistological staining technique, discovered the presence of β -amyloid ($A\beta$) plaques in nearly all cases. In a separate study (Roberts et al. 1990b), these same authors also examined the neuropathological features of CTE in the first non-boxer case, an elderly woman with a history of domestic violence and repeated blows to the head, revealing neuropathology identical to that seen in boxers. An autopsy study by Hof et al. (1991) of a 24-year-old autistic woman who had a long history of head banging showed the formation of NFTs in layer II and III of the cerebral cortex, especially in the temporal region, and amygdala in the absence of neuritic plaques or $A\beta$ deposits. Geddes et al. (1999), investigating the early neuropathological changes of CTE in the brains of five young men, ranging in age from 23 to 28 years, who had suffered from repetitive head injuries, reported no detectable gross changes to the brain, but demonstrated marked histological changes in all five cases involving formation of phosphorylated tau-positive, neocortical NFTs and neuropil threads (NTs) in striking clusters around intracortical blood vessels. $A\beta$ deposits were not found in any of the cases. The authors suggested that neocortical NFT formation and cytoskeletal abnormalities through damage of blood vessels or perivascular elements may contribute to the pathogenesis of this disease.

Omalu et al. (2005) reported the first case of CTE in a retired NFL player in 2005. Autopsy revealed mild pallor with mild dropout of pigmented neurons in the substantia nigra and mild neuronal dropout in the frontal, parietal, and temporal neocortex despite the apparent absence of cortical atrophy. Microscopic examination showed sparse NFTs and tau-positive NTs with diffuse amyloid plaques in the neocortex. The following year, Omalu et al. (2006) reported a second case of CTE in former NFL players, demonstrating tau-positive NFTs and NTs in all regions of the brain without detectable amyloid plaques and cerebral amyloid angiopathy. The third case of CTE in a former NFL player, characterized by diffuse cerebral tauopathy (NFTs and NTs) without any neuritic amyloidopathy, was reported in 2010 by the same research team (Omalu et al. 2010b). Omalu and colleagues also published neuropathological evidence of CTE in a 40-year-old professional wrestler (Omalu et al. 2010a) and in an Iraqi war veteran (Omalu et al. 2011a, b). Both

cases exhibited wide-spread tau-positive NFTs and NTs without atrophy. In 2009, McKee et al. (2009) carefully reviewed the clinical and neuropathological characteristics of 47 cases of neuropathologically-confirmed CTE in the world literature and also described the clinical and histological findings of CTE from an additional three retired athletes ranging in age from 45 to 80 years, including a former professional football player. The authors summarized the neuropathological features of CTE, including atrophy, ventricular dilation, fenestration of the cavum septum pellucidum, tau-positive NFTs, NTs and astrocytic tangles, and they documented $A\beta$ deposition as an inconsistent pathologic feature of CTE. Later, McKee et al. (2010) identified a widespread TAR-DNA binding protein 43 (TDP-43) proteinopathy in the frontal and temporal cortices, medial temporal lobe, basal ganglia, diencephalon, and brainstem in 10 of 12 examined CTE cases. The largest case study to date is that of McKee et al. (2013), who comprehensively investigated postmortem brains from a cohort of 85 head trauma patients between the ages of 17–98 years and showed neuropathological evidence of CTE in 68 cases. The subjects of this study involve 64 athletes, 21 military veterans and one individual with head-banging behavior. In this study, McKee et al. also defined four stages of CTE based on stereotyped patterns of structural change and p-tau pathology (Fig. 38.1). Furthermore, McKee et al. introduced neuropathological criteria for diagnosing CTE: “(i) perivascular foci of p-tau immunoreactive astrocytic tangles and neurofibrillary tangles; (ii) irregular cortical distribution of p-tau immunoreactive neurofibrillary tangles and astrocytic tangles with a predilection for the depth of cerebral sulci; (iii) clusters of subpial and periventricular astrocytic tangles in the cerebral cortex, diencephalon, basal ganglia and brainstem; and (iv) neurofibrillary tangles in the cerebral cortex located preferentially in the superficial layers”.

38.3.2 Gross Pathological Features

The neuropathological features of modern CTE have been evolving over the last decade (Randolph 2014). Grossly, there are usually no identifiable pathological abnormalities in early-stage CTE, but sometimes a cavum septum pellucidum and mild enlargement of the frontal and temporal horns of the lateral ventricles may be observed. Intermediate and advanced stages of CTE are normally accompanied by generalized brain atrophy with reduced brain weight. Specifically, a marked cerebral atrophy, particularly in the frontal and temporal regions, was commonly reported with advanced CTE (Payne 1968; Corsellis et al. 1973; McKee et al. 2009, 2010, 2013; Mawdsley and Ferguson 1963; Brandenburg and Hallervorden 1954; Neuburger et al. 1959; Grahmann and Ule 1957; Roberts et al. 1990a; Nowak et al. 2009). In addi-

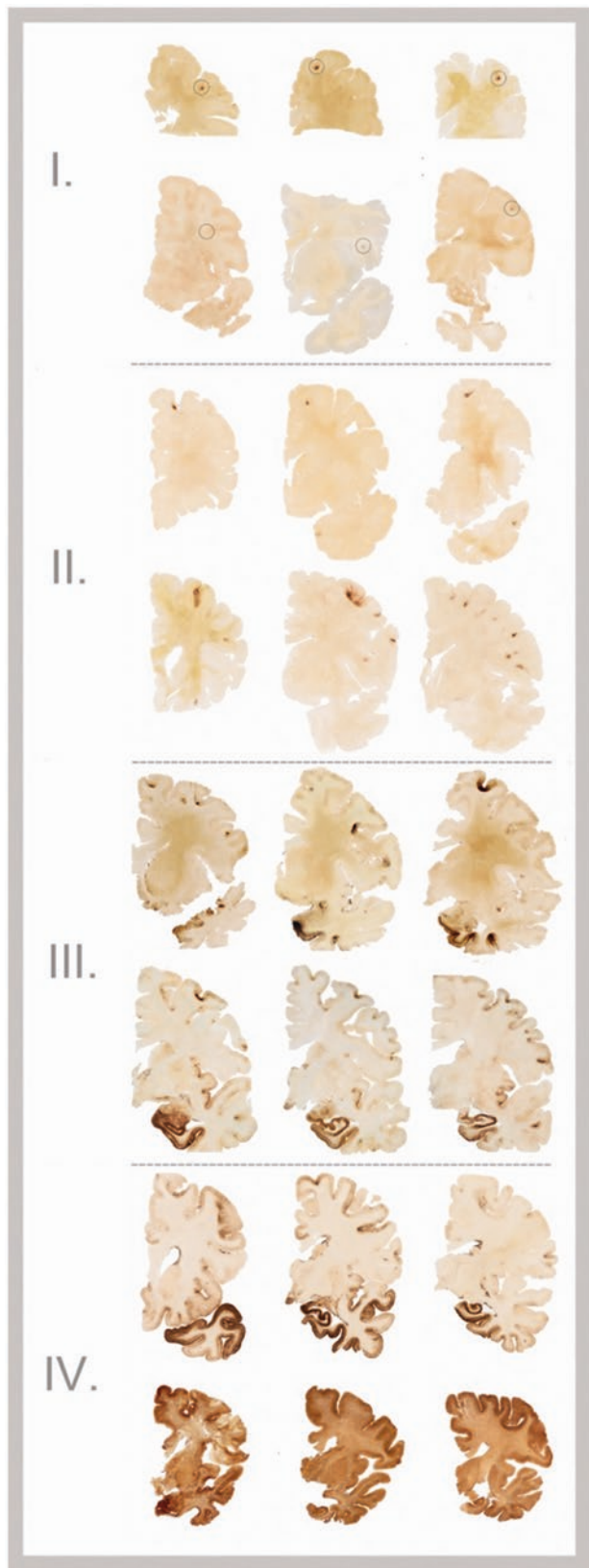


Fig. 38.1 The four stages of CTE. In stage I CTE, p-tau pathology is restricted to discrete foci in the cerebral cortex, most commonly in the superior, dorsolateral or lateral frontal cortices, and typically around small vessels at the depths of sulci (black circles). In stage II CTE, there

tion, atrophy of several other regions of the brain, such as parietal and occipital lobes, cerebellum, hippocampus, thalamus, mammillary bodies, entorhinal cortex and amygdala, has also been reported (Williams and Tannenberg 1996; Grahmann and Ule 1957; McKee et al. 2013). However, brain atrophy is not a significant gross feature in several cases of non-boxing athletes with CTE (Omalu et al. 2005, 2006, 2010a, b, 2011a, b). A second common gross pathological feature, especially in those with a history of boxing, is cavum septum pellucidum, which has been noted in 69% of CTE cases and was accompanied by a fenestrated septum in 49% of cases (McKee et al. 2009). Other gross neuropathology described in recent cases of CTE includes ventricular enlargement, thinning of the corpus callosum, dilation of perivascular spaces and pallor of the substantia nigra and locus coeruleus (McKee et al. 2009, 2013).

38.3.3 Microscopic Pathological Features

Histologically, CTE is characterized by extensive regional occurrence of p-tau-positive NFTs and NTs. In addition, the presence of p-tau-positive astrocytic tangles has been described in the McKee case series (McKee et al. 2009, 2013). The tau-immunoreactive neurofibrillary pathology is commonly found in the frontal, temporal and insular cortices, basal ganglia, brainstem, spinal cord, and diencephalon, and with distinct accumulations around the depths of cortical sulci and perivascular areas. Currently, the significance of this abnormal accumulation of tau around blood vessels is not yet clear, but some researchers hypothesize that damage to blood vessels during neurotrauma causes brain atrophy and the formation of NFTs (Saulle and Greenwald 2012). Although examinations of the brains of AD patients and two boxers with CTE revealed a similar tau isoform ratio and phosphorylation state between two distinct diseases (Schmidt et al. 2001), the abnormal distribution and localized nature of the tau pathology are unique to CTE and distinguish it from other degenerative tauopathies (McKee et al. 2009, 2013). The widespread presence of TDP-43-positive inclusions and

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Fig. 38.1 (continued) are multiple epicenters at the depths of the cerebral sulci and localized spread of neurofibrillary pathology from these epicenters to the superficial layers of adjacent cortex. The medial temporal lobe is spared neurofibrillary p-tau pathology in stage II CTE. In stage III, p-tau pathology is widespread; the frontal, insular, temporal and parietal cortices show neurofibrillary degeneration with greatest severity in the frontal and temporal lobe, concentrated at the depths of the sulci. Also in stage III CTE, the amygdala, hippocampus and entorhinal cortex show neurofibrillary pathology. In stage IV CTE, there is severe p-tau pathology affecting most regions of the cerebral cortex and the medial temporal lobe, sparing calcarine cortex in all but the most severe cases. All images, CP-13 immunostained 50- μ m tissue sections. [Reprinted in its entirety from McKee et al. (2013) with permission from the Oxford University Press and from the primary author.]

Table 38.2 Proposed supportive neuropathological criteria for CTE

1.	Macroscopic abnormalities of the septum pellucidum, disproportionate dilation of the third ventricles, mammillary body atrophy, or signs of previous brain injury
2.	Abnormal tau-positive neurofibrillary pathology in subcortical nuclei, including the mammillary bodies and other hypothalamic nuclei, amygdala, nucleus accumbens, thalamus, substantia nigra and midbrain tegmentum
3.	Abnormal tau-positive neurofibrillary pathology in superficial layers II and III
4.	Abnormal tau-positive neurofibrillary pathology in the hippocampus, particularly in CA2 and CA4 regions
5.	Tau-immunoreactive thorny astrocytes in the subpial and periventricular regions
6.	TDP-43-positive neuronal inclusions in the hippocampus, anteromedial temporal cortex and amygdala

Table 38.3 Proposed neuropathological criteria to exclude the primary diagnosis of CTE

1.	Prominent cell loss in the cerebellar dentate nuclei, coiled bodies in oligodendroglia and tufted astrocytes as seen in progressive supranuclear palsy (PSP)
2.	Predominant neurofibrillary degeneration of the hippocampal CA1 region with the presence of A β plaques as seen in AD
3.	Prominent lesions in the striatum and pallidum in association with extensive glial plaques in both cortical and subcortical areas as seen in corticobasal degeneration (CBD)
4.	Globular astrocytic inclusions as seen in globular glial tauopathy (GGT)

neurites is another common pathological finding reported in most cases of CTE. TDP-43 immunoreactivity is prominent in the frontal and medial temporal cortices, brainstem, insula, hippocampus, caudate, subcortical white matter, amygdala, putamen and hypothalamus and is occasionally co-localized with p-tau. Recent examinations of the brains of three athletes with CTE even revealed extensive TDP-43-positive inclusions in the anterior horns of the spinal cord (McKee et al. 2010). Deposition of A β -containing plaques, which are found in 40–50 % of individuals with CTE, is not a consistent feature of CTE. Furthermore, unlike AD, A β plaques observed in CTE are less dense and predominantly diffuse (McKee et al. 2009).

Very recently, the National Institutes of Health (NIH) held a consensus meeting to evaluate neuropathological criteria for the diagnosis of CTE. The consensus group agreed that abnormal p-tau pathology in neurons, glia and cell processes, when detected in an irregular, focal, perivascular distribution and at the depths of cortical sulci is sufficient for diagnosing CTE (McKee et al. 2016). Furthermore, they defined a group of supportive neuropathological features (Table 38.2) for diagnosis of CTE. Also, in the presence of co-morbid pathology, the group defined the neuropathological features for ruling out the sole diagnosis of CTE (Table 38.3).

38.4 Relevance to Neuropsychiatric Disorders

Even after a history of repetitive head trauma has been documented, establishing a link between this history and the development of neuropsychiatric sequelae can be problematic. Confounding factors include pre-existing mental traits, substance abuse, education and family dynamics. For instance, people who participate in collision or contact sports may have higher premorbid rates of impulsivity and aggression than the general population, and post-injury changes in personality could reflect exaggerations of pre-morbid traits (Nicholl and LaFrance 2009). CTE, or the syndrome as clinically defined by Montenigro et al. (2014), presents with symptoms and signs in one or more of the four clinical domains: mood, behavior, cognition and motor. Personality changes are not uncommon following traumatic brain injuries, however, the list of psychological and cognitive changes attributed to CTE may be too long to manifest unique clinical-pathological correlations (Randolph 2014). In a retrospective analysis of 60 Finnish patients 30 years post-TBI, Koponen et al. (2002) diagnosed the long-term psychiatric consequences of TBI according to DSM-IV and DSM-III-R criteria. During that 30-year period, they reported that 62 % had developed Axis I disorders, including major depression, alcohol abuse, panic disorder, specific phobias and psychotic disorders, which according to the authors, contrasted with 33 % for the U.S. Epidemiologic Catchment Area (ECA) survey. Furthermore, an Axis II personality disorder had been diagnosed in over 23 % of patients. The prevalence of current disorders among the TBI patients was 40 % compared to <16 % for the ECA. However, it should be noted that their study lacked age-matched controls and their sample was drawn entirely from a clinical psychiatric population. According to McKee et al. (2013), heightened suicidality is associated with mid- to late-stage CTE in former athletes. However, Iverson (2014) emphasized that such a claim is premature, finding little evidence linking suicidality with proteinopathy. Indeed, both Randolph (2014) and Iverson (2014) cite evidence that the risk of suicide for retired NFL players is much less than for same-aged men in the general population. CTE induced by repetitive head trauma has also been linked to neurocognitive deficits. One study of 41 NFL retirees found that they performed more poorly than 41 healthy controls on a battery of neurocognitive tests, however, their pattern of mild cognitive impairment (MCI) was indistinguishable from the scores of 81 amnesic MCI patients with no history of TBI (Randolph et al. 2013). Thus, it remains entirely feasible that the MCI patients and the retired NFL players with MCI shared a similar neuropathological process not dependent on a history of head trauma. Hart et al. (2013) reached a similar conclusion in their study of MCI and depression in retired NFL players, declaring that

none of them met the reported clinical profile for CTE. Hart and colleagues subjected 34 retired NFL players to a battery of psychological and cognitive tests as well as neuroimaging ($n=26$). All but two had a history of concussions, ranging from 1 to 13 episodes, and the group was contrasted with age-matched healthy controls. Also, players diagnosed with cognitive deficits and/or depression were compared to those without. The prevalence of depression and MCI in players was higher than expected in the general population, while the prevalence of dementia was less than expected. When comparing the cognitively impaired and unimpaired and when evaluating neurobehavioral outcomes, the authors failed to detect significant links with either concussion history or the number of years in the NFL (an indirect measure of subconcussive history). Importantly, retired NFL players displayed significantly greater white matter lesion volume than did healthy controls, whereas the lesion volume of symptomatic and asymptomatic players did not differ significantly, which perhaps hints at the influence of subconcussive impacts. A similar pattern emerged from changes in cerebral blood flow and in the distribution of low fractional anisotropy in athletes, indicative of white matter injury, in the temporal, parietal and frontal regions associated with neuropsychological outcomes.

38.5 Relevance to Neurodegenerative Disorders

It remains debatable whether the aggregations formed by hyperphosphorylated proteins are the root cause of neurodegenerative disease or simply a consequence of the disease process. The p-tau now associated with CTE can also be induced by hypoglycemia (Chu-Wan et al. 2013), hypoxia (Gao et al. 2013), cerebral ischemia or stroke (Wen et al. 2004), and heavy metal exposure (Cai et al. 2011; Xiong et al. 2012). Randolph (2014) listed 26 neurodegenerative conditions whose neuropathology includes elevated levels of cerebral tau aggregations. Oddly enough, Davis et al. (2015) produced a different table of 26 tauopathies. Taken together, the combined list of conditions, ranging from Alzheimer's disease to Parkinson's disease to white matter tauopathy with globular glial inclusions, also includes normal aging, thereby complicating any effort to prescribe a unique neuropathological signature to CTE. Indeed, Randolph (2014) postulated that since the overall death rate for retired NFL players was much lower than for men in the general population, then we might see a higher incidence of age-related neurodegenerative disease in these former athletes. Randolph's main argument, which is also championed in more detail by Davis et al. (2015), was that more carefully controlled studies were needed before the presence of p-tau could be causally linked to a specific neurodegenerative disease process or to cogni-

tive or neurobehavioral dysfunction. Puvanna et al. (2016) provide evidence that the presence of p-tau is not an etiologic mechanism induced by repetitive head trauma or other sources of axonal injury, but instead is a marker of chronic neurological dysfunction. They compared CTE-confirmed brains to both severe epileptic brains and healthy control brains. Their findings regarding the distribution pattern of insoluble p-tau in CTE brains corroborated those of others, however, they found the same macroscopic distribution pattern in epileptic brains without a history of neurotrauma. Microscopically, the only difference they discerned between CTE and epileptic brains was that CTE was characterized by the presence of high molecular weight tau tangles, whereas the tau in epileptic brains was mostly low molecular weight, diffuse tau. The authors cautioned that they could not rule out a history of comorbid epileptic seizures in their sample of CTE brains.

Since CTE's clinical presentation can be indistinguishable from Alzheimer's disease (AD), and because brain trauma is known to elevate A β levels in addition to abnormal tau, Stein et al. (2015) compared the patterns of A β deposits in the brains of 114 CTE-confirmed subjects. They also compared these brains with confirmed AD brains and with a large cohort of non-specific brains representing the general population-at-large. Diffuse or neuritic plaque deposits of A β were present in 52 % of CTE brains. However, compared to the nonspecific cohort, A β deposits appeared in CTE brains at a younger age and accumulated at an accelerated rate. Also, CTE brains with A β plaques exhibited more severe tau and Lewy body pathology independent of age. In CTE-AD comorbid subjects, the A β deposits were more common in the sulci than in gyri. Parkinsonian symptoms are also not uncommon in CTE patients displaying pallor substantia nigra (Smith et al. 2013). Gardner et al. (2015) demonstrated in a controlled epidemiological analysis that hospital patients aged 55 and older were at a 44 % increased risk of developing PD in the next 5–7 years if they had been hospitalized due to TBI as opposed to a non-TBI injury; fully 66 % of injuries in both groups resulted from falls. Increasing the potential relevance of their findings to CTE were the facts that greater TBI severity and multiple TBIs further increased the risk of PD. Acosta et al. (2015) investigated the chronic effects of TBI on PD-related pathology 60 days post-TBI in a rat model of controlled cortical impact (CCI). Their immunostaining studies of key markers of PD pathology revealed that the ipsilateral SNpc had been infiltrated by pro-inflammatory activated microglia resulting in an abundant overexpression of alpha-synuclein in the SN culminating in a significant decrease in the number of tyrosine hydroxylase-positive nigral neurons, which are hallmark features of PD. Examinations of some CTE-confirmed brains have also detected widespread TDP-43 proteinopathy, which is more often associated with amyotrophic lateral sclerosis, motor

neuron disease and frontotemporal lobar degeneration. McKee et al. (2010) examined CTE brains of contact sport athletes and found TDP-43 proteinopathy afflicting the frontal and temporal lobes, basal ganglia, diencephalon, brainstem and spinal cord. Presently, since both single and repeated TBI exposures have been linked to multiple neurodegenerative diseases with the abnormal accumulation of multiple proteins, a newly emerging view has been described at length by Washington et al. (2016), who are advancing the notion of polypathology, and recommending that we describe the spectrum of chronic disorders that surface after single or repeated TBI as traumatic encephalopathy or trauma-induced neurodegeneration.

38.6 Model Systems

CTE-related complications are many, ranging from trauma-induced dementia and psychosis to the development of Alzheimer's and Parkinson's diseases. With a greater prevalence of war and contact-sport related injuries, CTE will become more common. While it has been known at least since the 1898 case of Phineas Gage (Damasio et al. 1994; Van Horn et al. 2012; Koponen et al. 2002) that head trauma can lead to the development of neurological and psychological disorders, the pathobiological changes that influence the development of neurological complications are not well understood. Hence in vitro and in vivo models of CTE were developed to better comprehend these changes. Deciphering these pathobiological changes will help us to develop effective medicines to treat or even prevent the occurrence of CTE-related complications.

38.6.1 In Vitro Models of CTE

The advantages of using in vitro models of CTE are the ability to control the experimental conditions to obtain reproducible results without confounding systemic factors. With this, it is possible to understand the development and progression of trauma-induced inflammation and cell death. Various in vitro models of CTE have been developed to study the effects of various modes of brain injury (Morrison et al. 1998b). Among these, the most commonly used models are stretch-injury, liquid shear-stress-induced injury and barotrauma.

Transection: Following a penetrating brain injury, brain tissue at the wound site is often mutilated with microtubule loss and misalignment, contributing to neurodegeneration often seen in patients healing from such injuries (Brizuela et al. 2015; Povlishock et al. 1999). To study such axotomy in vitro, neurons and/or glia are plated on tissue culture plates. Using a blade (Mukhin et al. 1998), plastic stylet

(Tecoma et al. 1989) or rotating scribe (Mukhin et al. 1997), multiple cells are mechanically damaged or removed via transection. Glial activation, cell viability, and other cell-biological assays can then be measured (Tecoma et al. 1989). A micro-transection model was also developed for study of single-cell trauma (Gross et al. 1983). One of the main disadvantages of this model is the lack of standardized protocols regarding the amount of strain and force required to reproduce this model from one lab to another. Clinically speaking, secondary axotomy is a major consequence of CTE rather than primary axotomy. Hence, currently the focus has shifted to other models of CTE. Nevertheless, this technique is still used to test the efficacy of novel pharmacological agents that primarily increase axon growth via microtubule sprouting (Brizuela et al. 2015).

Barotrauma: Along with the transection model, barotrauma is one of the earliest in vitro models of CTE (Lewelt et al. 1982). While the transection model simulates a penetrating head injury, the barotrauma model recreates blast-induced and shockwave-generated neuronal injury. This is achieved by creating a pressurized chamber (≤ 15 atm) where the neuronal cells are kept for a certain duration of time (Murphy and Horrocks 1993). The severity of the injury depends on the overpressure and exposure time. However, because brain tissue is incompressible, the pressures required to cause cell compression are much higher and therefore not clinically relevant. A modification of this model is the weight-drop model where a free-falling weight drops from a specified height onto the cultured cells or brain slice. The force of impact determines if this injury is focal or diffuse. Another modified version of barotrauma is the fluid percussion model. A rapid pulse is delivered by dropping a weight onto a fluid-filled chamber containing neuronal or glial cells. This millisecond change in fluid pressure can cause axotomy of the cells present in the chamber. While this modified version of the barotrauma model provided a more realistic view of pulse pressure-induced injury, the force of pressure required to induce neuronal damage was still much higher than the relevant pressures that induce brain trauma.

Acceleration/deceleration injury (ADI): Being one of the major contributing factors to brain trauma, acceleration/deceleration injury is also one of the most widely studied brain injury models. This injury occurs due to inertial loading. An example of acceleration trauma is a blow to the head, after which the accelerating cranium impacts the stationary brain. Likewise, during an automobile accident, the accelerating brain collides with the stationary skull again leading to brain trauma. This trauma can be focal or diffuse and can lead to shear strain, axotomy and neuroinflammation (Davis 2000; Daneshvar et al. 2015). Various models of ADI have been developed, each recapitulating some aspect of this

complex injury. One of the earliest models of acceleration-induced neuronal injury required a flask of cultured cells to be subjected to a tangential force of 220 x g using an impacting pendulum (Lucas and Wolf 1991). After three consecutive accelerations, neuronal apoptosis occurred. At that time, the magnitude of force generated following ADI was not well known. Around 1990, physical model simulations of head injury were developed along with animal models of CTE (Margulies et al. 1990). It was found that shear strain of 0.10–0.50 produced at a strain rate of 10–50 per sec generated regional brain deformations linked to clinically relevant regional brain injuries.

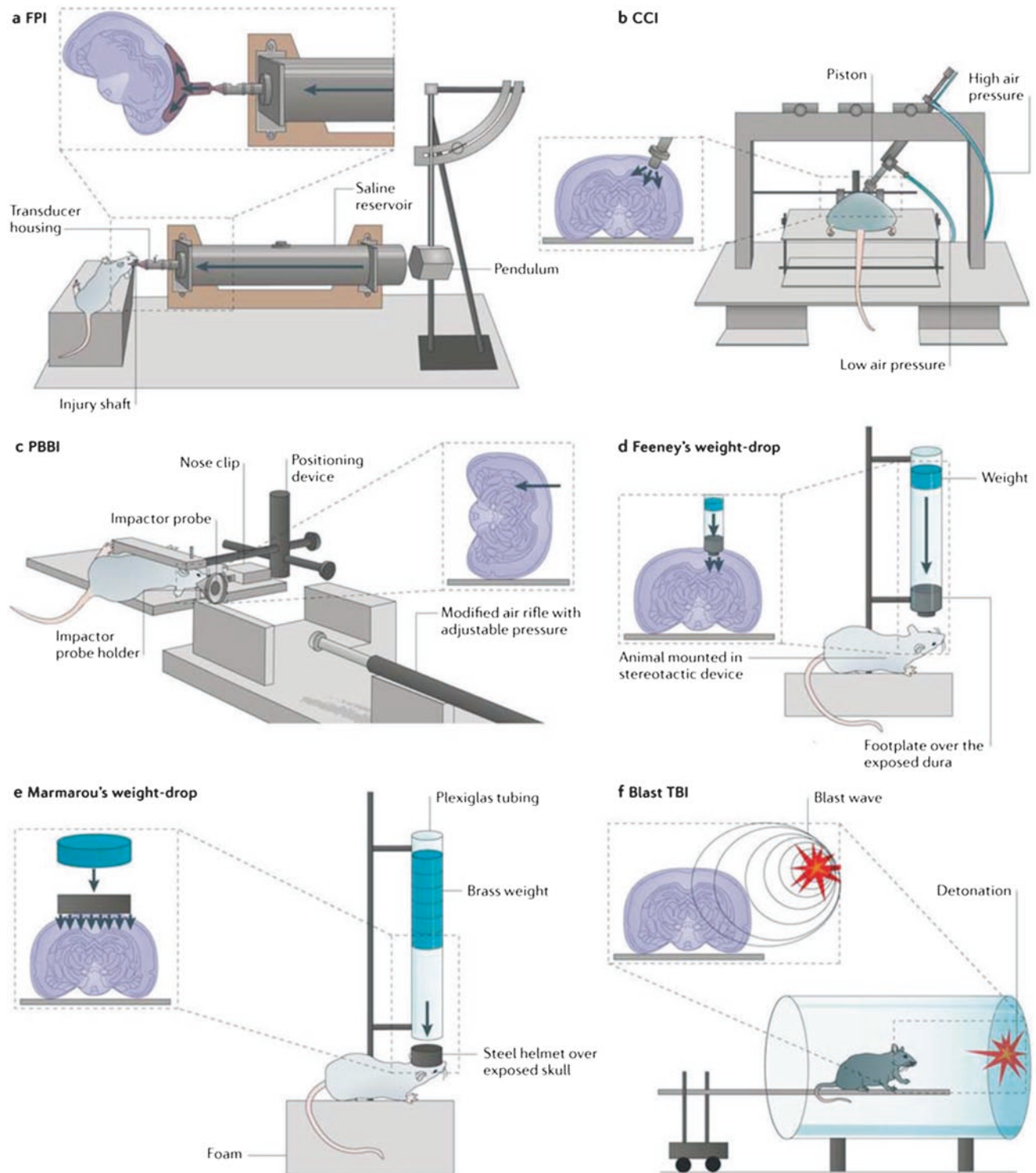
Stretch injury: One of the major disadvantages of the ADI model was its inability to account for stretch-induced mechanical strain on the axons, usually seen in ADI. During ADI-induced brain trauma, the brain collides with the cranium causing neurons and glia present in various parts of the brain to stretch due to inertial loading. This causes axonal strain leading to axotomy and synapse loss and consequently neuroinflammation and neurodegeneration. To mimic this aspect of neuronal injury, the cell stretch injury model was developed. Ellis et al. (1995) developed the first model of stretch injury by seeding rat cortical astrocytes on commercially available plates having a 2-mm thick silastic well bottom. A cell injury controller was used to apply a rapid positive pressure with known amplitude and duration (in milliseconds). This pulse caused a transient stretching of the Silastic bottom thereby inducing a biaxial stretch of the cells adhered to it. By controlling pulse amplitude and duration, mild, moderate and severe stretch corresponding to 10%, 33% and 50% Silastic membrane displacement could be reproduced. Further modifications by Cargilland and Thiabault (1996) and Morisson et al. (1998a) have resulted in higher strain magnitudes and modifiable strain rates, thus achieving better reproducibility and clinically relevant physical simulations.

As mentioned by Kumaria and Tolia (2008), a major disadvantage of using primary neuronal culture is the harvesting of tissue from embryonic mice or rats, which might limit the modeling of CTE to cultures of developing rather than adult neuronal cells. Another disadvantage of using a monolayer of cells in such models, especially in the stretch injury model, is the lack of tissue architecture. Unlike the single layer of neurons or glia used in these models, the brain architecture is much more complex. To overcome these problems, 3D matrix gels and ex vivo brain slice cultures are currently being used to understand the effect of trauma on tissue architecture. Morrison et al. (2006) designed a stretch injury model using organotypic hippocampal slices where a pulse pressure applied directly to the tissue adhering to the Silastic membrane results in 93% and 86% tissue strain in the x and y dimensions, respectively.

38.6.2 In Vivo Models of CTE

CTE is a complex disease involving primarily mechanical shearing and compression, leading to inflammation, edema and hemorrhaging of brain tissue at the site of injury and possibly the opposing side (e.g., coup-contrecoup). Following this primary injury, various cellular and systemic events also occur, taking place over months or years, leading to permanent brain tissue damage and consequently neurological disorders. While in vitro models provide a simplified model of CTE and can be used to study the molecular and cellular signaling cascades following injury, they do not provide knowledge about systemic events that have been known to occur acutely or even over a prolonged period of time. Also, it is not possible to maintain primary cell cultures or cell lines for a protracted period following injury due to the finite growth of primary cultures or the occurrence of overgrowth of confluent cell lines. Hence, to study the long-term effects of CTE, animal models are widely used to recapitulate the pathobiology of brain trauma, giving researchers the opportunity to understand the biomechanics of injury as well as to test for the efficacy of new drugs (Xiong et al. 2013). As shown in Fig. 38.2, depending on the type of brain trauma the researcher wants to study, four animal models have been developed for addressing the biomechanical aspects of head injury as well as the complex molecular cascades: a) fluid percussion injury (FPI), b) controlled cortical impact (CCI) injury, c) weight-drop impact acceleration injury and d) blast injury.

Fluid percussion injury (FPI) model: In this model, the experimental animal is anesthetized and placed in a stereotaxic frame after the scalp has been incised and the temporal muscles gently moved away from the skull. Once the animal has been secured in the frame, a lateral or medial craniotomy is made exposing the underlying dura mater. Usually a plastic cap is glued to the exposed dura to prevent fracturing of the skull. The FPI device, which is a cylindrical tube containing sterile isotonic saline, fills the interstitial space of the exposed part of the brain. As the pendulum strikes the opposite end of the fluid-filled cylinder, a compression or pressure pulse is delivered to the other end connected to the dura mater causing a brief displacement and deformation of the underlying brain tissue. The severity of this trauma depends upon the force with which the pendulum strikes the cylinder. The FPI model recapitulates the inertial-loading or ADI-induced head trauma without skull fracture. FPI leads to hemorrhaging, edema, neuroinflammation and long-term neurodegeneration similar to the symptoms seen in TBI in humans. One advantage of this model is that the experimenter can study the effect of impact on different regions of the brain depending on where the craniotomy is performed. Thus a researcher can induce



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Fig. 38.2 Experimental set ups for the animal models of traumatic brain injury. **(a)** | The fluid percussion injury (FPI) device uses rapid injection of a fluid pulse into the epidural space. **(b)** | The controlled cortical impact (CCI) model uses an air or electromagnetic driven piston to penetrate the brain at a known distance and velocity. **(c)** | The penetrating ballistic-like brain injury (PBBI) involves the transmission of projectiles with high energy of a metal rod or expansion of the

probe's elastic balloon. **(d)** | In the Feeney weight-drop model, a free weight is released directly onto the exposed dura. **(e)** | In the Marmarou weight-drop model, a metal disk is placed over the skull to prevent bone fracture. **(f)** | The blast brain injury caused by primary injury of blast or other mechanisms, e.g., thoracic effect. [Reprinted in its entirety from Xiong et al. (2013) with permission from Nature Publications]

midline FPI (in which the craniotomy is performed around the vertex) (McIntosh et al. 1987), sagittal FPI or parasagittal (lateral) FPI (McIntosh et al. 1989). Lateral fluid percussion injury (IFPI) is commonly used for its ability to produce primary focal trauma along with secondary diffuse neuronal injury. Besides cortical injury, other parts of the brain, including the thalamus and hippocampus, also suffer some percentage of neuronal loss. Over a protracted period of time (months), progressive neurodegeneration occurs in these regions resulting in cognitive, behavioral as well as motor deficits (Schmidt and Grady 1993; Creed et al. 2011; Hamm et al. 1994; Floyd et al. 2002). Since many CTE-related incidences, especially contact sport-related injuries, falls and minor automobile accidents, show a similar injury profile, the FPI model is widely used. In fact, besides using rodents (Perri et al. 1997; Daugherty et al. 2004) as experimental animals, many labs have also used pigs (Armstead and Kurth 1994; Lafrenaye et al. 2015), rabbits (Hartl et al. 1997) and cats (Unterberg et al. 1988; Sullivan et al. 1976). However, this model cannot replicate moderate to severe skull fracture injuries.

Controlled cortical impact (CCI): Using a pneumatic or electromagnetic impact device to generate a controlled impact on the exposed dura of the animal, CCI at one point was considered a superior model to FPI due to its ability to accurately control parameters such as velocity, time and force of impact (Romine et al. 2014). Since the force of impact is controlled, the risk of rebound injury and hence skull fracture is reduced compared to that in the FPI and IFPI models. The impact velocity is typically in the range of 0.5 to 10 m/s. CCI was first developed by Lighthall (1988) where he used a stroke-constrained pneumatic impactor at contact velocities of 2.0–4.0 m/s to produce 2.0–5.0 mm focal cerebral deformations in ferrets. Depending upon the magnitude of impact, mild focal lesions to severe large contusions and secondary diffuse neurodegeneration could be produced. The pathobiology of CCI induces similar sequelae of events as seen in closed-head clinical injuries such as cortical contusion, edema, increased intracranial pressure and hemorrhage as well as systemic and cellular changes and coma (Xue et al. 2015; Whalen et al. 1998; Lighthall et al. 1990). Immediately following cortical impact, the neural tissue below the exposed dura shows edema and cerebral hemorrhage, and the animal may experience temporary movement deficits after recovering from anesthesia. Moderate to severe impacts can lead to hippocampal and thalamic degeneration as well as the animal becoming comatose (Lighthall et al. 1990). Long-term effects of CCI have shown cognitive decline, especially short-term memory loss as well as behavioral changes. Despite the widespread use of rodents as *in vivo* CTE models, obvious differences exist between rodent and human brains. Mainly, brain geometry, degree of lateralization, white to gray ratio and the craniospinal angle differ

between quadrupedal rodents and bipedal humans (Laurer and McIntosh 1999; Fink 1979). Recently, neonatal pigs and piglets have been used in CCI experiments since the tissue density of the pig brain more closely approximates the human brain than does the rodent brain (Mytar et al. 2012; Pesek et al. 2014). Neonatal pigs have been used instead of adult pigs mainly due to space and cost constraints. Hence the findings from neonatal pigs might be more applicable to pediatric rather than adult CTE.

Penetrating CTE model: This model was developed to mimic head injury caused by penetrating objects such as bullets or shrapnel from explosives. This model favors larger animals such as sheep, dogs and rhesus monkeys over rodents since the target area in mice and rats is too small (Crockard et al. 1977; Tan et al. 1998; Finnie 1993). Also, the high projectile speeds result in high mortality rates in rodents. Recently, Plantman et al. (2012) developed a non-fatal rat model of penetrating CTE. According to the authors, the biomechanics of this model enable high rodent survival despite the penetrating injury.

Blast injury models of CTE: With the increased occurrence of armed conflict in many parts of the world, blast-related head trauma is becoming a major concern among both military personnel as well as civilians caught in the crossfire. Upon detonation of an explosive, a high-pressure shock wave blows outward at supersonic speed from the epicenter of the blast. Any individual present in the path of the oncoming blast wave experiences excessive pressure fluctuations along with high velocity wind accompanying the blast wave. Besides traumatizing gas-filled organs such as lungs, intestines or ears, a high-velocity blast wave can also cause brain trauma resulting directly from the shockwave passing through the brain and indirectly as the pressure wave gets propagated from the thoracic cavity via the cerebral vasculature. Edema, hemorrhage and neuroinflammation are seen acutely post-blast (DeWitt and Prough 2009; Kocsis and Tessler 2009). If unchecked, the neuroinflammation leads to loss of white or grey matter leading to neuropsychiatric disorders, cognitive decline and neurodegeneration. Misfolding of proteins such as tau, alpha-synuclein or β -amyloid is also seen in the postmortem brains of some war veterans, leading to the speculation that successive mild blast injuries or a moderate-to-severe blast injury can increase the susceptibility of the patient to accelerated age-related neurological disorders such as Alzheimer's or Parkinson's disease (DeKosky et al. 2013; McKee and Robinson 2014). To recreate different scenarios involved in blast-related injuries, different blast models have been applied.

Open-field blast injury: Earlier studies were conducted mainly on large animals subjected to open-field blasts since they replicated real-life scenarios with large animals being similar in size and tissue density to humans (Richmond et al. 1967; White et al. 1965; Cernak et al. 2011). However the

cost of maintaining these animals, the large amount of explosives needed as well as the lack of reproducibility of the injury profile prompted some researchers to adopt rodent models. Recently, modifications in experimental parameters and explosive devices have led to the development of rodent (Rubovitch et al. 2011) and primate (Lu et al. 2012) animal models of open-field blast injury.

Shock-tube models: A shock-tube comprises two chambers separated by a diaphragm, with the smaller “driver” chamber pressurized with compressed gas, either air or a lighter inert gas, while the larger “driven” chamber houses an anesthetized animal. If the intent of the experiment is to focus only on neurotrauma, then the animal is placed in a protective body shield with only its head exposed to the shock wave. The pressure in the driver chamber receiving the compressed gas increases until the diaphragm ruptures, propagating a shock wave. The shock wave intensity can be altered by varying diaphragm thickness. Various shock-tubes have been developed across many labs, however, as Risling and Davidsson (2012) have expressed, we need a well-documented and calibrated system characterizing all shock tubes to facilitate efforts at replicating results across different labs (Elias and Annas 1987). Currently, a sophisticated shock-tube is being used at the Applied Physics lab at Johns Hopkins University (Cernak et al. 2011). This shock-tube is capable of delivering complex shock-wave profiles as well as real-time measurement of various parameters such as overpressure impulse, total pressure, etc. Clemedson and colleagues (1953, 1954) were one of the first groups to study the effects of blast injury on animals. Initially their research focused on understanding the pathology of blast-induced

respiratory and vasculature defects in pigs. Later, they began to study the relationship between blast overpressure and neurotrauma (Clemedson 1956; Clemedson et al. 1957). Currently, along with large animals, mice and rats are also increasingly being used as experimental animals in various blast models of CTE (Budde et al. 2013). Due to the forces that the blast wave (shock wave plus blast wind) delivers to the animal, small animals used in these experiments suffered high mortality rates. With the introduction of Kevlar vests (flak jackets) that protected the thorax and abdomen in humans, researchers became interested in studying the effects of blast waves on the brain alone (Long et al. 2009; Goldstein et al. 2012). Since blast waves can produce not only head trauma but also systemic injuries that contribute to neurotrauma (DeWitt and Prough 2009), shielding the subject’s body prevented the introduction of compounding factors such as blast-induced hypoxemia and hypotension affecting cerebral blood flow. In an effort to model primary blast injuries induced solely by the shockwave without the confounding mechanical injuries imposed by the blast wind, Shah et al. (2012) developed an open-ended shock tube (Fig. 38.3). The animal is positioned outside the shock tube, just beyond and off-axis from its open end. The magnitude and duration of the blast overpressure experienced by the animal is controlled by adjusting its angle and distance from the shock tube’s open end.

Acceleration/deceleration injury (ADI): Advancing our knowledge of concussive injuries would not be complete without animal models designed to either control for or recapitulate the biomechanical forces that impart angular or rotational acceleration on the neck and head (Gullotti et al.

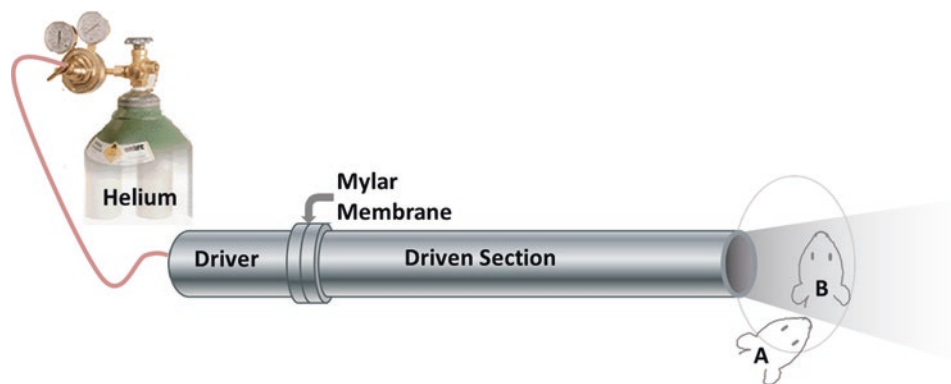


Fig. 38.3 Compressed Gas Open-ended Shock Tube. This is a computer-controlled system that can be triggered remotely. The 0.3-m long driver section is rapidly pressurized with helium gas until the mylar membrane ruptures, propelling a shock wave down the 3-m driven section. Pressure transducers located in and outside the tube record the internal burst pressure and compression wave parameters outside the tube where the animal is positioned. The shock tube was designed to simulate open-field blast waves for the laboratory study of primary traumatic brain injuries by positioning the animal outside and (a) off-axis to expose animal to a shock wave devoid of blast wind.

The intensity of the shock wave experienced by the animal depends on Mylar thickness, the animal’s distance from the shock tube’s open end and its angle from the tube’s central axis. If necessary, exposure to rotational forces (bobblehead effect) could be introduced by positioning the animal (b) within the blast wave (shock wave & blast wind) at a pre-specified distance from the shock tube. However, this variant would necessitate much lower mylar rupture pressures to avoid overly severe or lethal injuries. [Shock tube schematic adapted in part from Budde et al. (2013)]

2014). As mentioned in the introduction, Goldstein et al. (2012) modeled this ‘bubblehead effect’ in a shock tube by exposing mice to the blast wind. Only mice whose heads were unrestrained exhibited CTE pathology. Recently, Stemper et al. (2015) devised a rotational injury rat model in which the rat’s head rests inside a helmet that can be rapidly accelerated purely in a coronal plane of rotation when an impactor rod drops onto a laterally extended moment arm attached to the rat helmet. This model has the advantage of isolating the effects of one type of rotational force contributing to brain shear seen in many human mild TBIs.

38.7 Therapeutics

As described in previous sections, multiple preclinical models of experimental TBI are being used to cause static or dynamic brain trauma to help us characterize and understand TBI pathobiology and to evaluate potential therapeutic approaches. No single animal model of CTE replicates all of the pathologic events that contribute to progressive chronic neurodegeneration. Due to multidimensional cascades of secondary brain injury leading to impaired sensorimotor, cognitive, biochemical, neurochemical and histological deficits, the pathophysiology of TBI is multi-faceted and rich with pharmacological targets, thereby offering a multitude of therapeutic options. Several neuroprotective agents targeting the neuroinflammatory and neurodegenerative pathways have been tested in preclinical animal models. The therapeutic targets include AMPA-receptor antagonists, anti-inflammatory agents, antioxidants, apoptosis inhibitors, bradykinin receptor antagonists, immunophilin ligands, calcium channel blockers, necrosis inhibitors, neurotrophic factors, neurosteroids, NMDA receptor antagonists, nitric oxide modulators, oxygen carriers, polyethylene glycol, thyrotropin-releasing hormone analogs and others. Several studies have demonstrated the neuroprotective efficacy of progesterone for TBI in preclinical animal models (Schumacher et al. 2016; Lin et al. 2015). In clinical trials, the positive outcomes from Phase 2 safety trials led to multicenter, randomized and placebo-controlled Phase 3 trials, named ProTECT III and SyNAPSE. With more than 2000 TBI patients enrolled, the negative outcomes from these Phase 3 trials came as a big disappointment (Howard et al. 2015). More than 30 human phase III prospective trials of targeted drug therapies that showed promise in preclinical TBI models have failed to generate favorable results under clinical settings (Kabadi and Faden 2014; Xiong et al. 2009), in part due to inadequate single-target therapy. Therefore, any future effective therapy would most likely need to target multiple pathways, which could be partially accomplished by combination therapy. Still, major methodological hurdles exist between preclinical and clinical modeling and evalua-

tion due to a complex and heterogeneous human TBI/CTE population, thereby contributing to the failure of preclinical drugs in clinical trials. Currently, clinical trials are ongoing for at least 25 drugs developed for TBI. Furthermore, at least 50 new drug candidates are undergoing or have completed the preclinical phase and await entry to clinical phase. Recently, preclinical studies revealed E64D, a cathepsin B inhibitor (Hook et al. 2015), and Mdivi-1, a Drp1 mitochondrial division inhibitor 1 (Wu et al. 2016), as promising lead drug candidates and are being considered for clinical trials.

Future therapies: Recently, consensus panels funded by the NINDS/NIBIB defined the neuropathological criteria for CTE as an accumulation of abnormal hyper-phosphorylated tau in neurons and astroglia, non-specific p-tau immunoreactive pretangles, NFTs, extracellular tangles and supportive non-p-tau pathologies including TDP-43 and β -amyloid in immunoreactive neuronal cytoplasmic inclusions and dot-like structures in the NFTs. Therefore, clinical success might be achieved with novel neuroprotective therapies targeting tau phosphorylation and NFT formation.

Another major challenge in developing therapeutics for TBI/CTE is overcoming the blood–brain barrier (BBB) while delivering drugs. Researchers are encapsulating their therapeutics into nanoparticles to carry significant drug loads through the BBB to combat TBI/CTE. We lack FDA-approved therapeutics for mitigating TBI-induced neurodegeneration, thus stem cell therapy, deep brain stimulation (DBS) and transcranial magnetic stimulation (TMS) are all being evaluated in preclinical and clinical trials for treatment of CNS trauma. Recently, Rezai et al. (2015) demonstrated for the first time that DBS was safe, suggesting its potential effectiveness for making behavioral and emotional adjustments, which in turn improved functional independence years after severe TBI. Repetitive TMS (rTMS) is a non-invasive alternative that can influence brain plasticity, cortical reorganization and neuronal excitability. When used as a therapeutic intervention, rTMS has produced positive outcomes in people with motor disorders and psychiatric conditions suggesting that rTMS may be a promising treatment for TBI (Herrold et al. 2014). Stem cell therapy is another innovative treatment strategy against TBI/CTE currently being tested in animal models (Ahmed et al. 2015) to add trophic support to the injury response of CNS repair and regeneration.

Since there are currently no approved therapeutic products, the sector is teeming with opportunities for new entrants as well as stalwarts of the pharmaceutical industry. Some of the notable companies developing therapeutics for TBI/CTE are ALSP Inc, Cognosci, Medicortex, Amarantus BioScience Holdings, Aldagen, NeuroScience Pharmaceuticals and Targacept (<http://www.persistencemarketresearch.com/market-research/traumatic-brain-injury-therapeutics-market.asp>, accessed 29 December 2015). However, it is important that modelling preclinical CTE for future drug discovery

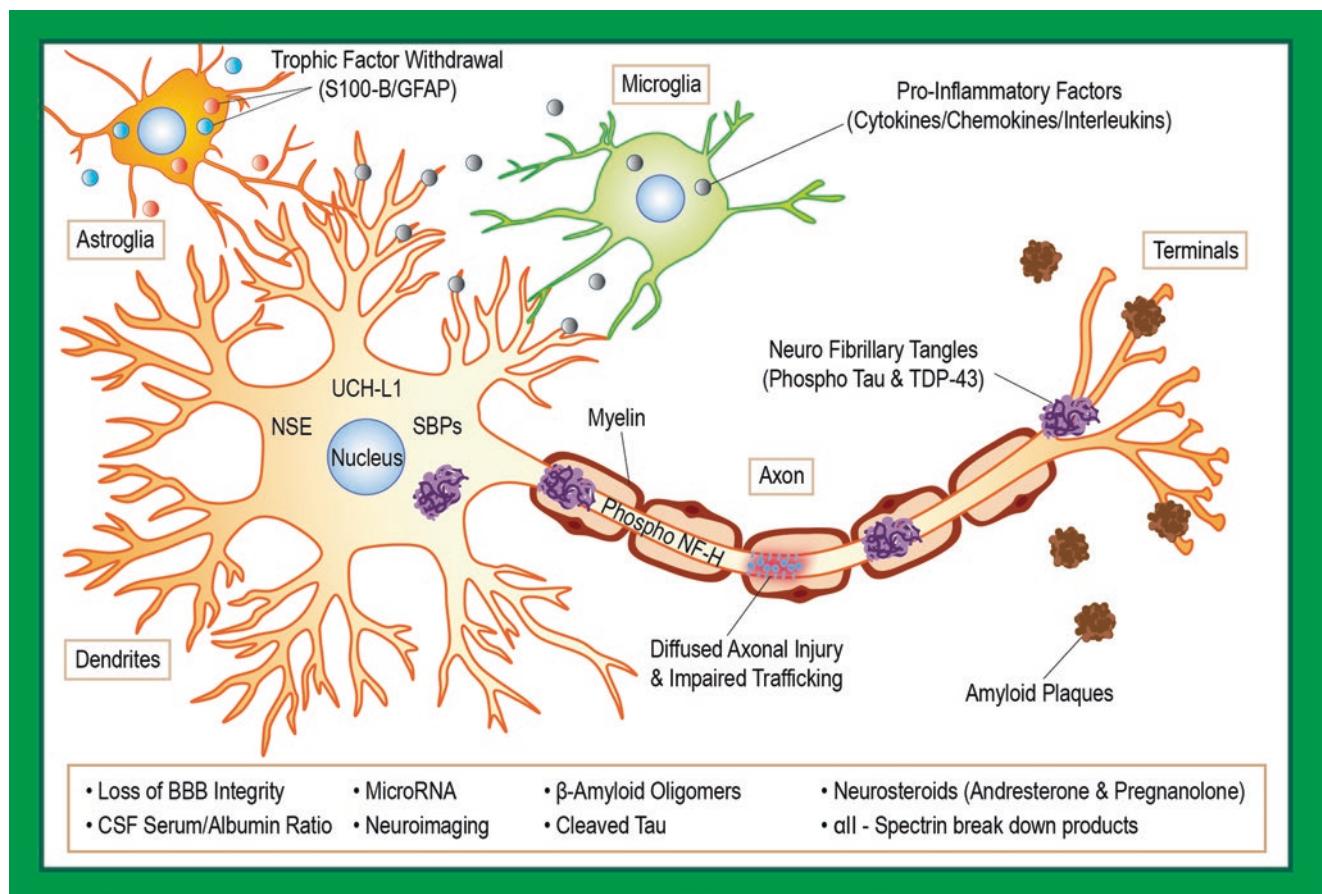


Fig. 38.4 Potential biomarkers for TBI/CTE. Within the neuronal cytoplasm, potential biomarkers include NSE, SBPs and UCH-L1. NF-H becomes phosphorylated in axons. Phospho-tau and TDP-43 are biomarkers of neurofibrillary tangles. Overexpression of amyloid precursor protein and amyloid-β could be markers for amyloid plaques. Astroglia will respond to injury by releasing S100-B and GFAP. Gliosis and injury-induced neuroinflammation upregulates the expression of interleukins and cytokines/chemokines. The ratio of cerebrospinal fluid

(CSF) to serum albumin can be a biomarker for integrity of the blood–brain barrier. Other potentially useful markers for brain injury response are cleaved tau, neurosteroids, spectrin breakdown products, microRNAs, and of course, neuroimaging. Abbreviations: GFAP, glial fibrillary acidic protein; NF-H, neurofilament heavy polypeptide; NSE, γ-enolase; SBPs, spectrin breakdown products; TDP-43, TAR DNA-binding protein 43; UCH-L1, ubiquitin carboxyl-terminal hydrolase isoenzyme L1. [Adapted in part from Zetterberg et al. (2013)]

must address the challenges associated with it and should take into consideration the length of the inter-injury interval and the number, severity, mechanism, age, gender and genetic predisposition at time of impacts (Turner et al. 2015; Goldstein et al. 2012).

38.8 Biomarkers

Biomarkers of neuronal, axonal and astroglial damage could be used to diagnose mild repetitive TBI and aid in medical counselling of at-risk individuals, such as military personnel and concussed athletes. At present, we lack validated biomarkers unique to CTE. Several researchers are actively

pursuing research to find biomarkers to develop diagnostic tests for CTE that could be used during early stages of the disease process (reviewed by Goldstein et al. 2014; DeKosky et al. 2013; Ojo et al. 2016; Yokobori et al. 2013). Currently, physicians are using a battery of neurological and brain-imaging tests. Eventually, the hope is to use a range of neuropsychological tests for determining the pattern of cognitive or behavioral decline as well as brain imaging and biomarkers to diagnose CTE. Furthermore, serial and longitudinal measurements of biomarkers in conjunction with brain imaging during the acute, subacute and chronic phases following primary TBI insult will help in understanding the pathogenesis and clinical course of CTE (McKee et al. 2016). A list of potential biomarkers of TBI/CTE is shown in Fig. 38.4.

38.8.1 Neuroimaging

Neuroimaging of the living brain has become fundamental in the effort to develop diagnostic and prognostic tools for managing TBI. Multiple imaging techniques are currently being evaluated to understand and evaluate the effects of promising therapeutic interventions for TBI in athletes, civilians and military veterans (reviewed by Wilde et al. 2015; DeKosky et al. 2013; Mitsis et al. 2014; Dani et al. 2015; Zhang et al. 2016). Computed tomography (CT), followed by MRI, remains the imaging modality of choice for initial assessments due to its ease of access, rapid acquisition and for its sensitivity (Currie et al. 2016). Structural and functional neuroimaging techniques such as quantitative fluid attenuated inversion recovery (FLAIR), positron emission tomography (PET), susceptibility weighted imaging (SWI), volumetric analysis, diffusion tensor imaging (DTI), magnetization transfer imaging (MTI), magnetoencephalography (MEG), task-based and resting state functional MRI (fMRI), arterial spin labeling (ASL) and magnetic resonance spectroscopy (MRS) are also being evaluated as diagnostic and prognostic tools for TBI/CTE (reviewed in Wilde et al. 2015). Each of these imaging techniques comes with its own advantages and limitations while providing some in vivo evidence of neurological damage such as non-specific atrophic changes (Wilde et al. 2015). Other non-invasive tests include electroencephalography event-related potentials (ERPs), quantitative EEG and single photon emission computerized tomography (SPECT). SPECT is being developed to distinguish different dementia types, such as CTE from AD or other neurodegenerative diseases. FDDNP is the only currently available radiotracer to image NFTs, besides amyloid aggregates, for visualizing AD pathology in living humans. Recently, PET scans performed with the FDDNP ligand revealed higher FDDNP levels in athletes compared to controls in areas that produce tau deposits following trauma. More recently, Pittsburgh compound B62, [18]-florbetapir, the new tau-binding ligand [18F]-T807, and THK523 compounds have been validated against amyloid or tau pathology in live patients by PET (Mitsis et al. 2014; Dani et al. 2015). Better PET ligands for amyloidopathy, tauopathy and synucleinopathy are being developed and evaluated in patients. Since the A β deposits and NFT distribution are different in CTE and AD, the PET ligands should successfully diagnose the different disease conditions.

38.8.2 Fluid Biomarkers

Biomarkers of neuronal, axonal and astroglial damage have been used to diagnose several neurodegenerative disorders, including AD, PD, ALS, etc. These same markers could help diagnose CTE, predict clinical outcomes of patients with head

trauma and provide information for medical counselling of at-risk individuals, such as military personnel and concussed athletes (Zetterberg et al. 2013; DeKosky et al. 2013; Blennow and Zetterberg 2015; Agoston and Elsayed 2012). CSF markers are preferred over peripheral blood markers, owing to their increased proximity to the brain. Biomarkers that are highly expressed within the CNS are also detectable in blood. Although potential peripheral blood markers are currently below detection limits due to sensitivity of the standard assays, detection and quantification of a number of potential CNS markers in peripheral blood is bound to steadily increase as ultrasensitive assays become available.

As shown in Fig. 38.4, several CSF and blood biomarkers of brain injury have been proposed, including proteins that indicate BBB integrity and neuroinflammation, as well as axonal, neuronal and astroglial damage (Zetterberg et al. 2013; DeKosky et al. 2013; Blennow and Zetterberg 2015). Studies have also shown an increase in the CSF:serum albumin ratio in patients with severe TBI associated with a neuroinflammatory response both in the CSF and serum (Zetterberg et al. 2013). Since an acute inflammatory response occurs within the CNS after severe TBI, studies have found increased levels of inflammatory proteins IL-6, IL-8 and IL-10 to a greater extent in CSF than in the serum of TBI patients. Increased levels of tau and neurofilament light polypeptide (NFL), the two well established biomarkers of acute axonal injury, have been found in the CSF and serum of people with possible CTE including boxers. Another marker of acute neuronal injury, γ -Enolase, is also known to increase in the serum and CSF of people with severe head trauma. The S100 protein, a member of the Ca²⁺-binding protein family that helps to regulate intracellular Ca²⁺ levels, is made up of S100-A and S100-B subunits. S100-B and GFAP (marker of astrocytes) protein levels are increased in the CSF and serum of athletes due to astroglial injury. Studies have also shown increased ventricular CSF and serum levels of APP, A β 40 and A β 42 during the first week after head trauma in patients with severe TBI but not with mild TBI. Calpain- and caspase-3-cleaved spectrin breakdown products and UCH-L1 (ubiquitin carboxyl-terminal hydrolase isoenzyme L1), a deubiquitinase, were recently shown to be potential biomarkers for TBI (Zetterberg et al. 2013; DeKosky et al. 2013; Blennow and Zetterberg 2015; Marx et al. 2016). In their studies, the CSF levels of both spectrin breakdown products and UCH-L1 were significantly higher in patients with severe TBI. Levels of these two markers also contributed to clinically relevant prognostic information in addition to that obtained by routine clinical assessments (Zetterberg et al. 2013; Blennow and Zetterberg 2015; Marx et al. 2016). Small non-coding RNAs in peripheral blood mononuclear cells have also been suggested as potential biomarkers for mild TBI (Zetterberg et al. 2013; Pasinetti et al. 2012). More recently, phosphorylated tau and

TDP-43 are being evaluated as biomarkers for TBI and CTE (Smith et al. 2013; Sierra-Rio et al. 2016). Phosphorylated tau and TDP-43 are primary constituents of NFTs and inclusions, the two most prominent neuropathological characteristics of CTE. Increased levels of total tau have been found in CSF after acute TBI, but no data have yet been examined in patients with probable CTE. The prevalence of chronic pituitary dysfunction, caused by the tearing of axons in the pituitary stalk, may be as high as 30–80% in patients 24–36 months after TBI (Dusick et al. 2012; Guerrero and Alfonso 2010). Abnormalities in pituitary hormone levels leading to chronic pituitary dysfunction correlate with the presence of long-term cognitive symptoms after TBI and so abnormal hormone levels could serve as a CTE biomarker and could be treated by hormone replacement therapy. Defining unified neuropathological criteria for the diagnosis of CTE will also facilitate the discovery of novel diagnostic biomarkers (Mez et al. 2015; McKee et al. 2016). Development of ultra-sensitive assays for detection of CNS biomarkers in the blood, clinical studies of serial, advanced brain imaging and longitudinal studies of biomarkers in chronic or progressive symptoms after TBI may help in understanding the pathogenesis and clinical course of CTE.

38.9 Review Questions

1. What are the main gross pathological features of CTE?
2. Briefly describe the major microscopic pathological features of CTE.
3. Please describe the unique features to definitively diagnose CTE.
4. Describe the “Bobblehead Effect” and its relevance to CTE.
5. What is meant by “verbal autopsy” and explain its importance to our current understanding of CTE?
6. What are some of the limitations or potential biases encountered when attempting to define a disease based on the examination of donated brains and verbal autopsies?
7. Why might people with a history of repeated concussions or TBI differ in the degree to which they manifest motor disturbances, behavioral/mood changes, or cognitive impairment?
8. How might researchers determine whether the neuropathology linked to CTE causes the clinical syndrome (behavioral/mood and cognitive) associated with CTE and to rule out comorbidities?
9. What patterns of tau and A β deposition might help discriminate CTE from other neurodegenerative disorders?
10. What major signaling pathways in the brain’s response to TBI/CTE are being investigated for their pharmacotherapeutic targets?
11. Give at least two reasons why drugs targeting TBI/CTE have failed in clinical trials.
12. Describe at least one pharmacological hurdle to the efficacious treatment of TBI/CTE, and then identify the non-pharmacological alternative treatments currently being developed for neurotrauma.

38.10 Answers

1. Grossly, there are usually no identifiable pathological abnormalities in early-stage CTE, but sometimes a cavum septum pellucidum and mild enlargement of the frontal and temporal horns of the lateral ventricles may be observed.
2. Histologically, CTE is characterized by extensive regional occurrence of p-tau-positive NFTs and NTs. In addition, the presence of p-tau-positive astrocytic tangles has been described in the McKee case series.
3. Although examinations of the brains of AD patients and two boxers with CTE revealed a similar tau isoform ratio and phosphorylation state between two distinct diseases, the abnormal distribution and localized nature of the tau pathology are unique to CTE and distinguish it from other degenerative tauopathies.
4. During head trauma, the brain may be subjected to the compressive and shearing forces of rapid acceleration and deceleration. Goldstein et al. (2012) hypothesized that the rapid acceleration-deceleration forces of concussive impact trauma (e.g., the “bobblehead effect”) were key to CTE pathogenesis in both sports- and blast-induced TBI.
5. Since neuropathological evidence is required to confirm CTE, our understanding of how CTE might manifest itself as motor, cognitive, or affective symptoms has emerged retrospectively from ‘verbal autopsies’, involving interviews of the families of those neuropathologically diagnosed with CTE post-mortem.
6. You must recognize the limited external validity inherent in such a small non-random sample from a CTE brain bank comprised of athletes’ brains donated by families who may have felt compelled to donate based on the symptoms they had witnessed.
7. Depending on an individual’s unique history of TBIs and their frequency, the resulting physical brain injuries can range from mild to severe and be either localized contusions in specific brain regions or extensive and diffuse. Other factors include the age range of exposure, genetic susceptibility, pre-existing mental traits, substance abuse, education and other lifestyle choices.
8. Given the current lack of prospective longitudinal and cross-sectional data, this will require both animal and

human studies of CTE based on randomized before-after control-impact studies employing protein-based assays, biofluid-based biomarkers and neuroimaging.

9. Distinct p-tau accumulations localized around the depths of cortical sulci and perivascular areas. Also, A β plaques are not consistently observed in CTE cases (<50%), but when present, they are less dense and predominantly diffuse. Finally, the tau found in CTE is characterized as high molecular weight tau.
10. The therapeutic targets include AMPA-receptor antagonists, anti-inflammatory agents, antioxidants, apoptosis inhibitors, bradykinin receptor antagonists, immunophilin ligands, calcium channel blockers, necrosis inhibitors, neurotrophic factors, neurosteroids, NMDA receptor antagonists, nitric oxide modulators, oxygen carriers, polyethylene glycol, thyrotropin-releasing hormone analogs and others.
11. Due to inadequate single-target therapy. Therefore, any future effective therapy would most likely need to target multiple pathways.
12. One major challenge in developing therapeutics for TBI/CTE is overcoming the blood-brain barrier while delivering drugs. Researchers are encapsulating their therapeutics into nanoparticles to carry significant drug loads through the BBB to combat TBI/CTE.

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Abstract

The term “neuroimmune system” emphasizes the fact that nervous and lymphoid tissues constitute a unified system that functions in the maintenance of homeostasis. The conventional division between the two systems has blurred, as well as the distinction between neuropeptides on the one hand, and immune cytokines on the other. Two lines of research have altered our perspective on neuroimmune interactions: (a) the identification of conventional neuropeptides and their receptors, especially those related to the hypothalamic-pituitary-adrenal (HPA) axis, in most lymphoid tissues, and (b) the large body of evidence that cytokines, historically associated with immune system communication, play vital roles in nervous function. The role of the HPA axis in coordinating responses to stressors, and the role of the immune system in mediating the “sickness” response, underscores this reciprocal relationship between the CNS and the immune system. In spite of this nascent state of knowledge, the evidence for hyper-reactivity of the HPA axis and the alterations in cytokine levels observed in both major depressive disorder (MDD) and schizophrenia serve as a foundation for further research establishing a causative relationship between alterations in immune system function and these debilitating psychiatric disorders.

Keywords

α -MSH • β -endorphin • β -lipotropin • β -MSH • γ -MSH • ACTH • Alpha-1 adrenergic receptors • Alpha-2 adrenergic receptors • Anxiety • Autoimmune disease • Autoimmune T cells • B cells • Beta adrenergic receptors • Corticotropin releasing hormone (CRH) • Cytokines • Depression • Glucocorticoids • Hypothalamic-pituitary-adrenal axis (HPA) • Interleukin-6 (IL-6) • Inflammation • Interferon- α (IFN- α) • Leukocytes • Leucine-enkephalin • Lymphocytes • Melanocortin-1 receptors • Methionine-enkephalin • Monocytes • Morphine • Muscarinic acetylcholine receptors • Neurodegeneration • Neuropeptides • Neuroprotection • Nicotinic acetylcholine receptors • Nociception • ORL-1 receptor • Orphanin FQ • Preproenkephalin • Proenkephalin • Proopiomelanocortin (POMC) • Reactive oxygen species (ROS) • Regulatory T cells • Schizophrenia • Stress • Thymocytes • Tumor necrosis factor alpha (TNF- α) • Urocortins

39.1 Introduction

The earliest evidence for neuroimmune interaction and regulation is the classical immune conditioning experiment of Ader and Cohen in 1975 (Ader 1987). Since then, it is well established that the central nervous system (CNS) and the immune system interact and regulate one another's function

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(Madden and Felten 1995; Carr and Blalock 1989). This interaction can be particularly important at times of extraordinary stress, whether due to psychological factors or to a physical response to an injury or an infection. The CNS regulates immune system function with classical neurotransmitters acting through the central nervous system and peripherally through the sympathetic nervous system; and with neuropeptides also acting through both the central and peripheral nervous systems. The immune system, in turn, alters CNS function, particularly through the release of cytokines. While this reciprocal regulation between the CNS and the immune system is designed to maintain homeostasis, dysfunction in one can lead to aberrant function in the other, producing a pathological state.

Interactions between the immune and nervous system are complex and attempts to decipher these are still incomplete. One of the first empirical findings in support of an interaction between these systems was the discovery of receptors for neurohormones and neuropeptides on cells of the immune system (Hadden et al. 1970; Wybran et al. 1979; Hazum et al. 1979; Landmann et al. 1981). This implied that immune cells can and do respond to the endogenous ligands for these receptors. Conversely, the demonstration of many cytokine receptors and the cytokines themselves in the CNS suggests not only that these small proteins have a functional role in the CNS, but that cytokines released by the immune system may be able to regulate CNS function (Adler and Rogers 2005; Cartier et al. 2005). Subsequent studies demonstrated that immune cells also synthesize and release many neurohormones and neuropeptides. This provides a mechanism for local control of the physiological effects of these transmitters, in addition to distant control mediated through the peripheral and central nervous systems. This sharing of ligands and receptors in the nervous and immune systems led to the suggestion that this constituted a complete biochemical information circuit and that this interactive communication system provided the basis for the immune system acting as a sensory system and the brain playing an immunoregulatory role (Blalock 1994).

In this chapter we will provide an overview of how the hypothalamic-pituitary-adrenal (HPA) axis, the CNS and the immune system are integrated and regulate each other's activity. While there are many neurotransmitters and neurohormones that act directly and indirectly through the CNS on the immune system, such as serotonin, norepinephrine and gamma-aminobutyric acid (GABA), we will focus on neuropeptides and cytokines found to regulate the immune response. In particular, we will examine HPA axis regulation of CNS function by release of peptides and glucocorticoids in response to stress and inflammation. We will consider opioid-like peptides as well as corticotrophin-releasing hormone (CRH) and related peptides that are synthesized in the immune system as well as the CNS and how they regulate

immune function. We will also discuss cytokines released by the immune system and how they impact CNS function. Finally, we will provide a brief overview of how the neuro-immune system plays a role in psychiatric illnesses such as depression and schizophrenia, a topic explored in more detail in the following two chapters.

39.2 HPA Axis Regulation of CNS Function

The occurrence of physical or psychological events, or “stressors”, that threaten the well-being of an organism can lead to activation of a generalized stress response. The stress response consists of a coordinated activation of adaptive processes (collectively referred to as allostasis) that are designed to return the organism to steady-state conditions, i.e. homeostasis. This coordinated response is mediated by components of the central nervous system (CNS), the peripheral nervous system (PNS), and the endocrine system. The hypothalamic-pituitary-adrenal (HPA) axis defines the classical core of the neuro-endocrine stress response system. The HPA axis coordinates the component processes of the stress response by serving as the major communication route between the CNS, the PNS and the endocrine system. The HPA axis consists of three major components: the hypothalamic paraventricular nucleus (PVN), corticotrophs in the anterior pituitary gland, and the adrenal cortex. The hypothalamus is situated at the anterior-most end of the brainstem and serves as the final common pathway for regulation of visceromotor and endocrine functions by the CNS. The PVN, situated bilaterally along the midline of the anterior hypothalamus, coordinates endocrine functions via the pituitary gland and visceromotor functions via pathways to the brainstem. The pituitary corticotrophs are one of several distinct cell types in the anterior pituitary, each serving as an interface between the central nervous system and the endocrine system to control some aspect of metabolism or sexual behavior. The adrenal cortex comprises the outer layers of the adrenal gland, which plays a major role in responses to stress, through its innervation by the sympathetic nervous system and via circulating hormones.

The activation of a stress response begins with the appraisal of stressors by the distributed CNS network commonly known as the limbic system. The concept of the limbic system, which was originally defined anatomically, has taken on a functional connotation and now typically refers to CNS systems which modulate the hypothalamus. These brain areas include the hippocampus, the amygdala, and regions in medial prefrontal cortex. The components of the limbic system all influence hypothalamic output, and constitute a distributed system that regulates affective state and is critically involved in the appraisal and response of the organism to stressors. In general, the hippocampus and the anterior

cingulate / prelimbic regions of prefrontal cortex inhibit HPA axis output, while the amygdala and the infralimbic region of prefrontal cortex potentiate HPA axis activity. Thus, different regions of the limbic system modulate the output of the HPA axis in a reciprocal fashion to coordinate the temporal sequence of the stress response, controlling the initiation, propagation, and termination of distinct processes.

39.2.1 Hormones Released by the HPA Axis

Two neuropeptides, corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) are released from parvocellular neurons in the hypothalamic PVN to initiate a stress response. The terminal endings of these neurons, located in the median eminence of the hypothalamus, release CRH and AVP into the hypothalamic-hypophyseal portal vessel system, where they travel to the anterior pituitary. The two neuropeptides act synergistically on pituitary corticotrophs to activate the synthesis of pro-opiomelanocortin (POMC). This peptide, discussed in detail below, is processed to produce several peptides including adrenocorticotrophic hormone (ACTH), or corticotropin. ACTH released from corticotrophs travels via the bloodstream to act on cells in the zona fasciculata layer of the adrenal cortex, stimulating the synthesis and release of the glucocorticoids, cortisol (in humans) or corticosterone (in rodents).

In addition to regulating the synthesis and release of ACTH from the pituitary, neurons in the PVN also project to areas in the brainstem that control the output of the sympathetic branch of the PNS. Activation of PVN neurons at the onset of a stress response thus leads to increased sympathetic outflow, resulting in release of norepinephrine from sympathetic nerve terminals and epinephrine from the adrenal medulla. The effects of these catecholamines are responsible for the behavioral syndrome known as the "fight-or-flight" response.

The effects of the neuropeptides and glucocorticoids associated with the HPA axis are mediated through distinct receptor subclasses. Two classes of CRH receptors have been identified, type 1 and type 2, with distinct but overlapping distributions in the CNS (Hsu and Hsueh 2001; Reyes et al. 2001). It is currently thought that CRH acts through type 1 receptors (CRHR1), while novel ligands have been identified for the related type 2 receptors (CRHR2). These ligands, urocortin II (Reyes et al. 2001) and urocortin III (Lewis et al. 2001), play a role in the regulation of appetitive behaviors and are thought to play a role in the adaptive (slow) phase of the stress response (de Kloet et al. 2005) as discussed below. Expression of mRNA for the urocortins has been demonstrated in the hypothalamus, brainstem, and amygdala (Hsu and Hsueh 2001). Interestingly, the CNS distributions of the CRHR1 and CRHR2 receptors correspond to the CRH and urocortin terminal fields, respectively.

The effects of the glucocorticoids are also mediated through two distinct subclasses of cytosolic steroid receptors. Glucocorticoid receptors (GRs) are found throughout the brain, but GR density is highest in the hypothalamic PVN, ascending aminergic pathways, and in limbic brain regions (Herman et al. 2003). Mineralocorticoid receptors (MR) are named for their primary ligand, the steroid aldosterone, which is produced by the outer zona glomerulosa layer of the adrenal cortex and regulates sodium balance. However, MRs in the brain have a ten-fold higher affinity for glucocorticoids than GRs and thus respond to lower levels of these hormones during the initial phase of a stress response. MRs are also expressed at high levels in limbic brain regions; thus there is considerable overlap between these two receptors in key brain areas that regulate the HPA axis.

39.2.2 The Role of HPA Axis in Response to Stress

Under resting conditions, CRH and AVP are released from the hypothalamus in a pulsatile fashion with a frequency of 2–3 episodes per hour (Engler et al. 1989). The amplitude of the neuropeptide pulses normally increases in the morning, resulting in a circadian fluctuation in ACTH and cortisol levels. This daily rhythm is modulated by feeding and activity schedules, but is particularly perturbed by stressful stimuli originating internally (e.g., anxiety or systemic infection) or externally (e.g., threatening situations). Thus, acute stressors lead to activation of the stress response.

39.2.2.1 Modes of the Acute Stress Response

The stress system is characterized by two modes of operation, a fast phase that serves to mobilize and activate, and a slow phase that promotes recovery and adaptation. Each mode is associated with characteristic behavioral changes that are mediated by distinct cellular and molecular mechanisms.

The initial fast phase is manifested behaviorally by increases in vigilance and arousal, and alterations in attention. This phase is associated with the release of CRH and AVP from the hypothalamic PVN, activation of sympathetic outflow and release of catecholamines from the adrenal medulla, and rising blood levels of cortisol. The effects of CRH in the fast phase are mediated through type 1 receptors (CRHR1), which are responsible for activation of the HPA axis and the sympathetic nervous system. Levels of cortisol rise rapidly due to release of ACTH from the pituitary, and serve in the periphery to mobilize metabolic resources and alter immune system responses (discussed below). The effects of cortisol on brain structures, and thus behavior, in the fast phase are mediated primarily by the higher-affinity MRs, which respond to the rising levels of cortisol at the initiation of a stress response. An important aspect of MR-induced

changes involves their role in the appraisal of environmental stimuli in this phase of the response. The alterations in neuronal function mediated by the interaction of cortisol with MRs lead to increases in arousal level, heightened vigilance and alertness, and enhancement of attention.

The slow phase of the stress system is characterized by processes that promote recovery from, and adaptation to, the stressful conditions that prompted the response. At the level of the hypothalamus, this phase is probably mediated by the urocortins acting through CRHR2 receptors (Reul and Holsboer 2002; de Kloet et al. 2005). In contrast to the fast phase, the slow phase is associated with activation of the parasympathetic nervous system, which promotes the appetitive and metabolic functions which help to restore homeostasis. As cortisol levels peak and then slowly subside during the slow phase, the lower-affinity GRs are activated and lead to transcriptional changes in target organs that promote recovery. In the brain, GR activation-induced transcriptional changes may be crucial for the consolidation of memory traces involving the association of stressors with sensory stimuli (in the amygdala) (Roosendaal and McGaugh 1997) and environmental context (in the hippocampus) (Oitzl and de Kloet 1992).

At all levels of the HPA axis, feedback mechanisms exist that control the output of neuropeptides and circulating hormones. These mechanisms regulate the levels of these factors during normal diurnal cycles, but also act to limit the magnitude of the stress response and terminate its effects. These feedback mechanisms consist of both "short" loops, where each factor acts to inhibit its own secretion, and "long" loops, whereby ACTH acts on PVN neurons to inhibit the secretion of CRH, and cortisol acts on the PVN and the pituitary to inhibit the secretion of CRH and ACTH, respectively (de Kloet et al. 2005).

39.2.2.2 Modes of the Chronic Stress Response

It is important to distinguish between the response of the HPA axis to acute stressors, and the response to chronic stress. While the HPA response to acute stressors can clearly be seen as an adaptive response that serves to maintain or re-establish homeostasis, the response to chronic stress can lead to maladaptive conditions, especially in "vulnerable" individuals, that may initiate or facilitate CNS disease processes, as discussed below.

39.2.3 Role of HPA Axis in Inflammation

The activation of the stress systems affects all tissues of the organism, and the peripheral immune system is no exception. These effects are mediated through at least two pathways: via the HPA axis and by virtue of the innervation of lymphatic tissues by autonomic nerve fibers, especially from

the sympathetic nervous system. All lymphoid tissues, primary (bone marrow and thymus) as well as secondary (spleen, lymph nodes, and gut-associated lymphoid tissue) are innervated by sympathetic nerve fibers. As discussed above, most lymphoid cells express catecholamine receptors, including B-lymphocytes, CD4- and CD8-positive T cells, dendritic cells, monocytes, and macrophages. The HPA axis also directly affects peripheral immune functions via receptors for CRH, ACTH and cortisol.

39.3 Neuropeptides as Modulators of the CNS and Immune Function

There is differential expression of neurotransmitter and neurohormonal receptors among the various cell types of the immune system. This suggests that particular neurohormones produce specific and differential effects on immune cells, and hence on immune function. It also suggests that the pharmacology of immune cells will vary from cell type to cell type and that these pharmacological differences represent potential drug treatment strategies in coping with various pathological states. There is still a great deal of work to be done in this field since there is debate in the literature on receptor expression, i.e., which cells consistently express which subset of receptors, and whether and how receptor expression is regulated by the changing milieu.

For example, functional activities of β 2-adrenergic, prostaglandin and histamine receptors are increased in T lymphocytes activated by IL-2 (Dailey et al. 1988). This takes place in an apparently coordinated manner even though the prostaglandin receptors are clearly dominant in regulation of signal transduction in resting cells. The change in functional activity is presumably due to up-regulation of receptor expression. These and other studies indicate that receptor expression is a dynamic aspect of immune system function and it that implies alterations in receptor function are important for immune system activities, in this particular case cellular proliferation.

39.3.1 Peptides Derived from Proenkephalin

Two of the most abundant endogenous opioid peptides, methionine-enkephalin (Met-enk; Tyr-Gly-Gly-Phe-Met) and leucine-enkephalin (Leu-enk; Tyr-Gly-Gly-Phe-Leu) are derived from the precursor protein, proenkephalin (PENK). PENK is a 267 amino acid precursor that contains 6 copies of Met-enk, two of which are extended forms (Tyr-Gly-Gly-Phe-Met-Arg-Phe and Tyr-Gly-Gly-Phe-Met-Arg-Gly-Leu) and one copy of Leu-enk. This ratio is essentially the same as the ratio of these peptides found in brain and adrenal chromaffin cells. The fact that there are two distinct

peptides closely related in structure and function proved to be a major obstacle that slowed their discovery (Kosterlitz and Hughes 1977).

The first two opioid peptides were isolated from pig brain and shown to be active in bioassay systems by Hughes and Kosterlitz in 1975 (Hughes et al. 1975). It was some time later, though, that the precursor proteins for these small peptides were discovered. The first of these to be identified was proopiomelanocortin (POMC). It was not until 1982 that PENK was identified and sequenced in multiple species (Udenfriend and Kilpatrick 1983). Hypothalamic neurons containing POMC have been extensively studied in their role regulating food intake.

As mentioned above, PENK contains not only both pentapeptide enkephalins, but also a hepta- and octapeptide enkephalin. This structure is maintained across species (Noda et al. 1982; Comb et al. 1982; Gubler et al. 1982). The processing of PENK into smaller peptides, even for an extended period of time, maintains this assortment of four distinct peptides (Fleminger et al. 1983), indicating that all are active products of PENK and that the longer peptides are not simply alternate precursors for Met-enk. Interestingly, while a great deal of attention has been paid to the role of the pentapeptides, Met-enk and Leu-enk, knowledge of the physiological role of the hepta- and octapeptides is still very limited.

There is evidence PENK is not processed in the same way in all tissues and the suggestion has been made that alternative processing may occur in immune system cells containing PENK, compared to processing in neurons. This would open the possibility that differential processing may lead to release of enkephalin-like peptides from the immune system that would have a different spectrum of activities, at least to some extent, than those derived from neural tissues. This, in turn, would provide the opportunity for differential regulation of components of the immune system, depending on the source of the enkephalin-like peptide release.

39.3.1.1 Expression of Enkephalins in the Immune System

PENK is distributed widely throughout the brain (Wamsley et al. 1980; Finley et al. 1981; Harlan et al. 1987), indicating involvement of the enkephalins in a variety of physiological functions. The first suggestion that peptides derived from PENK were involved in immune system function came from the work of Wybran (Wybran et al. 1979), which demonstrated specific binding of both Met-enk and morphine to lymphocytes. Early studies also demonstrated that the enkephalins are concentrated in leukocytes compared to other blood components, supporting the idea that immune cells can synthesize the opioid peptides. Since these initial observations, a large body of evidence has provided more detail supporting the idea that the enkephalin peptides are

produced by and have important actions in cells of the immune system.

Determination of which PENK products are formed by different immune cells has been difficult because of the lack of antibodies specific to the various peptides. For instance, many studies use readily available antibodies to Met-enk, but the possibility is strong that these antibodies can also react with larger peptides containing Met-enk. In addition, the variations in results reported in the literature are probably due to differences in cell types as well as the inducing signal. For instance, Th2 cytokines, such as IL-4, IL-6 and TGF- β , produce many-fold increases in PENK mRNA in human thymocytes. On the other hand, Th1 cytokines have variable (IL-1 β) or no (IFN- γ) effect (Kavelaars and Heijnen 2000). Further, Th2 cytokines lead to a 4- to 5-fold increase in Met-enk-like protein in thymocytes, whereas Th1 cytokines produce no such effect. It appears, moreover, that the induced Met-enk itself is not released by thymocytes; rather, larger peptides containing Met-enk are released.

Similar results have been found in T cells. PENK is synthesized in T cells and both synthesized and processed into smaller peptides in macrophages and activated monocytes, but not in inactivated monocytes. Paradoxically, in many cases it has been very difficult to demonstrate release of PENK products. For example, T cells do not appear to secrete detectable levels of PENK-derived peptides whereas monocytes do. On the other hand, under some conditions T cells have been shown to secrete intermediate products of prepro-enkephalin, the precursor to PENK. These intermediate products appear to inhibit proliferative capacity via unidentified, i.e., non-opioid, binding sites. The larger peptides eventually are processed into Met-enk (and Leu-enk) in some cases, whereas in others the larger peptides are probably the active products themselves (Dillen et al. 1993; Hiddinga et al. 1994). There are many questions yet to be answered in this area. To compound the problems of determining if the products of PENK are released and are active, the quantity of material provided by the tissues is sometimes small enough to make consistent quantitative data difficult to obtain. This leads to further confusion and contradictions in the literature.

39.3.1.2 Function of the Enkephalins in the Immune System

Met-enk, the most abundant of the enkephalin peptides, produces effects on its target cells that are both concentration-dependent and modified by the presence of other stimuli. The concentration-dependent effects can be bimodal, with low concentrations producing effects opposite those of high concentrations (Oleson and Johnson 1988). An example is the differing effects of varying concentrations of enkephalins on the production of reactive oxygen species (ROS) by polymorphonuclear leukocytes.

ROS production is enhanced by low enkephalin concentrations and suppressed by high concentrations (Roschetti et al. 1988; Marotti et al. 1992). In a similar manner, Met-enk inhibits antibody production by B cells whereas slightly larger peptides also derived from PENK stimulate B cell antibody production (Hiddinga et al. 1994; Das et al. 1997). One possible explanation of these paradoxical data is that opioid peptides may play a role as modulators of immune system function, fine tuning the current state of activity, rather than being the primary drivers or determinants of function. As such, the effects of these peptides would be highly dependent on the current status of the immune system, whether activated or inactive, and on which other regulatory factors were present and what their overall effects are. In this sense, the endogenous opioids would act more to maintain homeostasis rather than to specifically activate or inhibit the immune system.

The exact role of Met-enk and related peptides derived from PENK remains controversial since there are many studies with seemingly contradictory results. Many of these studies were carried out in vitro and how well the results translate to in vivo conditions is not clear at present. In addition, relatively few of these studies used experimental conditions that were the same or even similar to those used in other studies, making comparison and resolution of differences difficult.

39.3.2 Peptides Derived from Prodynorphin

A second major source of endogenous opioid peptides is derived from prodynorphin (pDYN), the product of a single gene that contains four exons (Gein 2014) with seven splice variants in humans. The processing of the pDYN peptide appears to be differentially regulated in CNS regions (reviewed in Schwarzer 2009). Of several peptide fragments generated, the major components appear to be dynorphin A and dynorphin B, separately derived from pDYN. Dynorphins make up a family of opioid peptides that appear to act primarily at kappa opioid receptors in the central and peripheral nervous system, with dynorphin A (amino acids 1-17) having the highest affinity for this opioid receptor (James et al. 1984) and is produced and contained within immune cells. Evidence suggests that opioids modulate innate and acquired immune responses (McCarthy et al. 2010). The endogenous opioid system consists of three peptide precursors that encode for dynorphins, enkephalins, β -endorphins and proopiomelanocortin (POMC) (Kieffer and Gavériaux-Ruff 2002). In addition, opioid peptides generated from proenkephalin A act as cytokines that are capable of regulating granulocytes and mononuclear cell function (Sharp et al. 1998).

39.3.2.1 Expression of Dynorphins in the CNS and Immune System

Within the central nervous system, dynorphins are primarily localized to the hippocampus, amygdala, striatum and the spinal cord. Through the nervous system, dynorphins control memory, learning, emotions and reactions to stress. However, dynorphins, like other opioids, are locally synthesized in various tissues including tissues of the immune system (Gein 2014). Dynorphins are known to be present within immune cells and nerve fibers with inflamed tissues (Hassan et al. 1992). Aside from dynorphin expression, opioid receptor expression has been shown on a variety of immune cells, which allow for direct and dynamic effects on immune function (Bidlack 2000). Specifically, k-opioid and δ -opioid receptors are found to be present on lymphocytes and there is further evidence for both neural and non-neural opioid receptors on immune cells (Bidlack 2000). Depending on the site of their production, dynorphins have different physiological actions, indicating that dynorphins have a wide variety of functions regarding the human immunological response.

39.3.2.2 Function of Prodynorphin Derivatives in the CNS and Immune System

The immunomodulatory function of dynorphins is determined by the expression levels of kappa receptors. The dynamic effects are thought to be due to the complicated pro-opioid system and its multiple receptors. Through k-opioid receptors, dynorphins regulate proliferation, as well as the production of antibodies. Dynorphins also regulate anti-inflammatory activity as well as pro-inflammatory activity. In addition, dynorphin peptides are capable of directly modulating lymphocytes and other cells involved in immunity (Bidlack et al. 2006). Dynorphins are thought to contribute to the pathophysiology of depression, schizophrenia, epilepsy and addiction (Schwarzer 2009). Stress, which is known to be associated with aversive mental states, is known to induce the release of dynorphins, rapidly activating k-opioid receptors. Prolonged k-opioid receptor activation can lead to characteristic depressive disorders (Knoll and Carlezon 2010). The use of dynorphin antagonists has been shown to alleviate depressive behaviors (Shirayama et al. 2004), providing evidence that therapeutic effects may be induced by preventing the over-activation of k-opioid receptors by dynorphin.

39.3.3 Peptides Derived from Proopiomelanocortin (POMC)

Another major source of endogenous opioid peptides as well as non-opioid peptides is proopiomelanocortin (POMC).

This large peptide contains the sequences of, and is processed into, a number of smaller peptides, including ACTH, β -endorphin, β -lipotropin, α -MSH, β -MSH and γ -MSH. Which products are derived from POMC depends upon the cell in which the processing takes place. In the anterior lobe of the pituitary POMC is processed into ACTH and β -lipotropin. In the neurointermediate lobe the major products are β -endorphin, α -MSH and γ -MSH. POMC was the first of the peptide precursors for which such cell-dependent processing was demonstrated.

39.3.3.1 Expression of POMC in the Immune System

The discovery by Blalock and colleagues that POMC was also produced by immune cells was a major event in the developing recognition of interactions between the immune and nervous systems (reviewed in Blalock 1999). These studies indicated that not only do neurohormones derived from the nervous system alter immune system activity, but also similar or identical hormones can be released from immune cells to regulate the nervous system as well as the immune system itself.

Similar to what was found with the PENK-derived enkephalins, peptides derived from POMC frequently have biphasic dose-response curves, i.e., low doses stimulate immune system function whereas high doses usually suppress function. Again, it appears the system is designed to maintain homeostasis in response to a wide range of conditions.

The finding that the immune and neuroendocrine systems both express receptors for opioids and for ACTH and that both systems can synthesize and release peptides active at these receptors, led to the suggestion that the immune system functions as a sensory organ (Blalock 1984, 1999) and that this forms the basis for the interaction between the two systems. It is well known that the nervous system responds to a variety of stimuli and, when appropriate, releases neurotransmitters and hormones that enable an appropriate reaction to these stimuli or stresses. This can include changes in immune system function. Blalock proposed that the immune system also responds to particular stimuli, in this case environmental changes that would not be readily detected by the nervous system, such as the presence of bacteria or viruses. In response, the immune system releases a variety of compounds, including peptide hormones that will alter both immune and nervous system function.

39.3.3.2 Function of POMC Derivatives in the CNS and Immune System

Beta-Endorphin

A locally induced inflammation followed by cold-water swimming, a stress that is known to activate intrinsic opioid

systems, produces an antinociceptive effect localized to the area of inflammation (Stein et al. 1990a). Through a series of experiments it became apparent that the agent producing this effect was β -endorphin and that it was localized to the area of inflammation; i.e., it was not a systemic effect of the peptide. This provided strong evidence that localized release of endogenous opioid peptides in areas of inflammation could play an important role in regulating inflammation and nociception. Subsequent experiments using this same model demonstrated significantly increased levels of both β -endorphin and Met-enk localized to the area of inflammation, although it appeared that β -endorphin was responsible for the antinociceptive effect (Stein et al. 1990b). These studies indicated that localized release of these opioid peptides from immune cells was responsible for the reduction in pain. Further support for the involvement of immune cells in this antinociceptive action came from suppression of the immune system with cyclosporin A, which resulted in the loss of the antinociceptive effects of the endogenous opioids. Interestingly, Met-enk did not play an important role under this experimental paradigm and it was not clear whether Met-enk was released from immune cells, even though it was definitely present in the cells. The paradoxical synthesis of two opioid peptides and the release of only one to produce antinociception leaves many unanswered questions, such as why is Met-enk synthesized in the first place? This and many other questions remain.

α -MSH

The tridecapeptide, α -MSH, is also derived from POMC. While originally discovered through its effects on the skin, α -MSH can alter immune system function. Understanding the role of α -MSH in neuroimmune modulation is still in the early stages (Luger et al. 2003). α -MSH acts through melanocortin-1 receptors (MC-1R), which are expressed by immune cells, including macrophages and monocytes. Both β -MSH and γ -MSH, also products of POMC, act through other melanocortin receptors and these receptors have not been found on immune cells to date. This indicates that α -MSH may be the only of these three to be involved in regulating immune function.

Most studies to date suggest that α -MSH modulates or reduces inflammation, down-regulating proinflammatory cytokines and other proinflammatory molecules (Luger et al. 2003). These effects are produced, at least in part, by inhibition of NF κ B activity. There is some evidence that α -MSH may have biphasic effects in this regard since low concentrations increase antibody production by B cells whereas higher concentrations reduce production.

As discussed above, several other peptides may be derived from POMC in a cell-specific manner, dependent upon processing within each cell type. The role of ACTH in nervous

system-immune system interactions is discussed elsewhere in this chapter. At present a neuroimmune function for the other POMC peptides has not been demonstrated.

39.3.4 Corticotropin Releasing Hormone

Corticotropin Releasing Hormone (CRH; corticotropin releasing factor; CRF) plays a major role in CNS regulation of the immune system. In particular, CRH released from the hypothalamus is the first point in HPA axis regulation of immune function. As a critical player in the response to stress, the HPA axis is a primary point of coordination in the neural-immune interaction. This is discussed in Sect. 39.2 of this chapter. CRH also plays an important role in many CNS functions, including regulation of the autonomic nervous system and endocrine function. CRH is involved in the central response to anxiety and stress, and hence affects immune system function via that route. There is also convincing evidence that CRH can play a role in depression. The central role of CRH has been reviewed (Dunn and Berridge 1990; Heim and Nemeroff 1999). Beyond these well-known roles for CRH, this neuropeptide hormone is also synthesized in immune cells and so appears to play an even more widespread role than originally thought.

Early studies demonstrated a role for CRH in augmenting function of immune cells, such as natural killer cells (Carr et al. 1990). The localization and the synthesis of CRH in the immune system and in immune cells were initially suggested by finding mRNA for CRH in leukocytes (Stephanou et al. 1990) and significant concentrations of CRH in areas of inflammation (Karalis et al. 1991). This suggested CRH was produced locally, i.e., in the periphery in areas of inflammation, probably in part by immune cells. Further studies supported these ideas. In Lew/N rats, which are deficient in hypothalamic CRH responses to inflammatory stimuli, there are high levels of CRH in inflamed joints (Crofford et al. 1992). These data suggested that CRH plays the role of an autocrine or paracrine inflammatory factor. The obvious paradox is that CRH plays a powerful anti-inflammatory role as the primary CNS activator of the HPA axis, yet in local peripheral sites it has just the opposite effect. Thus, CRH expression and function with respect to inflammation is site specific.

CRH had been shown to have important effects in areas of inflammation, such as augmentation of analgesia by endogenous opioid peptides. It has been further demonstrated that the effects were blocked by local administration of a CRH antagonist, a CRH antibody or by antisense oligonucleotides directed against CRH. This implies that local production and release of this hormone by immune cells do, in fact, occur and are physiologically important (Schafer et al. 1996). It was found that systemic administration of these same various

antagonists of CRH action, an approach directed against CRH released from a distant site, was either ineffective or several orders of magnitude less potent than local administration, bolstering this argument.

It proved difficult to definitively demonstrate CRH synthesis from immune cells, although numerous studies provided evidence this does happen (Aird et al. 1993; Ekman et al. 1993). Eventually it became clear that regulation of CRH synthesis and release in immune cells differs from that in hypothalamic neurons. While immune cells may synthesize and release much smaller concentrations of CRH and other neuroimmune peptides, and although their release may require *de novo* synthesis, an inherently slow process, the fact that immune cells release these hormones locally in the target area compensates for both of these factors to some extent. These data indicating site and tissue specific effects of CRH, sometimes even contradictory effects, point to the complex interrelationship between the nervous, endocrine and immune systems, an interaction that has yet to be deciphered completely.

39.3.5 Tachykinins

Tachykinins are a family of neuropeptides that are intensively studied. In fact, tachykinins are the largest family of neuropeptides present within mammals and amphibians. They make up a group of structurally related peptides all derived from *tac* genes, in alternating sequences (Steinhoff et al. 2014). *Tac* genes encode preprotachykinins, precursor proteins, which get chopped into smaller peptides, forming tachykinins. Tachykinins interact with three types of G protein-coupled receptors known as neurokinins. In mammals, substance P, neurokinin A and neurokinin B are the most well-known and studied tachykinins (Pinto et al. 2004). However, new tachykinins are still being discovered. Hemokinin 1, for example, is preferentially expressed in peripheral tissues and may have functions outside of the well-defined functions of tachykinin peptides as neurotransmitters. It has been suggested that aside from neurotransmitter like actions, tachykinins can have profound influences on inflammatory responses, effecting immune cell function in many different ways (Zhang et al. 2006).

39.3.5.1 Expression of Tachykinins in the CNS and Immune system

Tachykinins show broadly distributed expression within human tissues. It has been observed that tachykinin peptide expression is mainly found within neuronal tissues (Otsuka and Yoshioka 1993; Severini et al. 2002); however recent findings have also identified tachykinin expression in a variety of peripheral tissues. Substance P and neurokinin A, for example, are found to be expressed throughout the CNS

and within primary afferent neurons that innervate peripheral tissues. Substance P and neurokinin A have also been found to be expressed in non-neural tissues, like endothelial cells, immune cells and inflammatory cells (Linnik and Moskowitz 1989; Lecci and Maggi 2003). Further, neurokinin B has been discovered to be expressed solely in the CNS, while its function remains largely unknown (Moussaoui et al. 1992; Patacchini et al. 2000). Tachykinin expression has also been reported in pulmonary arteries and veins, suggesting its potential role in lung physiopathology (Pinto et al. 2004). More recently, hemokinin 1 expression in peripheral tissues has led to the great importance of determining new tachykinin peptide expression and function. Understanding the interactions of tachykinins and immune cells may provide advancements in new therapies for inflammatory and immune-related diseases.

39.3.5.2 Function of Tachykinins in the CNS and Immune System

Tachykinins participate in physiological regulation of the nervous, immune, gastrointestinal, urogenital, respiratory and dermal systems. They play a role in inflammation, nociception, proliferation and epithelial secretion (Steinhoff et al. 2014). In addition, regulation of neuronal survival and degeneration has also been suggested as roles of tachykinins.

Due to the variety of expression levels throughout the mammalian body, it has been found that different tachykinin expression levels, in different areas, can lead to different physiological regulations. For example, activation of substance P receptors in the spinal cord regulates synaptic transmission in response to noxious stimuli, while activation of central receptors for substance P regulates cardiovascular and respiratory functions, and is responsible for emetic reflex activation (Marriott 2004). Recently, tachykinins have been discussed as targets for a variety of different therapeutic drugs for a wide variety of disorders. It has been shown that substance P and neurokinin A elicit the production of cytokines through malignant glial cells within brain tumors. In addition, increased proliferation rates of malignant glial cells have been correlated with substance P and neurokinin A levels (Marriott 2004). These findings suggest that tumor proliferation and growth could be due to the interaction of substance P with receptors capable of producing cytokines downstream. On the other hand, substance P and neurokinin A play a role in the secretion of ions and fluid, as well as motor activity of the gastrointestinal tract. For this reason, they have been implicated in gastrointestinal disorders and have recently become ideal targets for pharmacotherapy of gastrointestinal disorders (Holzer 1998). These results suggest that through targeting tachykinins, therapeutic drugs for a variety of disorders may prove beneficial. In addition, it is important to note that when tachykinins are not tightly regulated, a variety of disorders can arise.

39.4 The Role of the Neuroimmune System in Psychiatric Disorders

39.4.1 Overview of the Immune System in Major Depression

In recent years there has been increased investigation of potential links between immune function and depression. Two large meta-analyses of multiple studies found that patients with major depression show reliable changes in immune function (Herbert and Cohen 1993; Irwin 1999), including lowered proliferative response of lymphocytes to mitogens, lower NK cell activity and changes in white blood cell populations. Data suggest that major depressive disorder (MDD) can alter immune function and this is most likely to occur in patients with severe depression (Herbert and Cohen 1993; Irwin 1999; Maes 1999; Frank et al. 2002). While the initial thought was that immune function was depressed in MDD, more recent evidence has suggested that many aspects of the immune system are likely to be activated. Both stress and major depression, two disorders that share many features, can be characterized by activation of some immune capacities and suppression of others (Raison and Miller 2001).

The mechanisms for these interactions are not well defined, although numerous hypotheses have been advanced. It should be noted at the outset that attempts to determine the relationship between depression and altered immune function are confounded by the multiplicity of factors known to be associated with both and that may alter the interrelationship. Examples include age, gender, sleep status, the likelihood that depression represents a complex of disease states with varying involvement of the immune system, and the frequent presence of other psychiatric or physical disorders which can affect both psychological status and immune function. A detailed review of this topic has been published (Irwin 2001). Indeed, it has been pointed out by Irwin that current data suggest immune changes in MDD specifically correlated with the disorder are also seen with stress and other psychiatric disorders, suggesting some common characteristic(s) shared by these problems (Irwin 2001).

In addition, the likelihood that depression represents a complex of disease states with varying involvement of the immune system supports the idea that there is a subgroup of depressed patients who have associated alterations in immune function. Indeed, data suggest that different types of depression display distinct, and even opposite, changes in neuroimmune functions (Antonijevic 2006). As a result of multiple issues, there are contradictory, or at least inconsistent, findings in the literature. This is characteristic of most types of studies of depression, again probably because depression represents multiple disease states.

There is increasing evidence that aberrations in immune system function induce or at least support the development

of MDD. Activation of some aspects of the immune system is generally associated with MDD, even in patients who are otherwise quite healthy. Many studies support this idea (Raison et al. 2006). Proinflammatory cytokines, such as IL-6 and TNF- α , are increased in plasma and in the CNS. There also are increases in the level of chemokines and adhesion molecules.

Several studies have shown increased plasma levels of IL-6 in patients with MDD. In a more extensive study, Alesci and colleagues found that IL-6 levels were increased in MDD patients throughout the circadian cycle (Alesci et al. 2005). There was a 12-h shift in the circadian rhythm of IL-6 plasma levels and its complexity was reduced. Even though IL-6 is a known activator of the HPA axis, cortisol levels were not consistently changed in MDD patients compared to controls. Additionally, it was found that IL-6 levels, with their altered rhythm, correlated significantly with mood ratings. IL-6 also induces a “sickness” behavior very similar to depression. These data suggest a direct relationship between IL-6 and depression.

A related example is the effects of interferon- α (IFN- α) on mood and, specifically, its ability to produce significant depression (Trask et al. 2000; Raison et al. 2006). IFN- α is used to treat several serious disorders, including malignant melanoma and hepatitis C. A major side effect of prolonged or high dose therapy with this agent is major depression, even to the point, though rare, of suicidality. Estimates are that this occurs in as many as 30–50% of patients undergoing IFN- α treatment. A history of psychiatric symptoms is considered a relative contraindication to the use of IFN- α , and a psychiatrist should closely monitor treatment of such patients using this drug. Further indications that the syndrome produced by IFN- α is depression are that it is responsive to treatment with antidepressants and that there are indications of changes in serotonin and possibly norepinephrine metabolism centrally. Alterations in both of these neurotransmitters are associated with depression. It is also interesting that IFN- α is a potent inducer of IL-6, which may be associated with the induction of depression.

As indicated by this brief overview there is considerable evidence for a close relationship between changes in the immune system and depression, particularly MDD. Whether there is a clear cause and effect relationship between these is not known in most cases, although there are strong data in the case of IL-6 and IFN- α , as noted. It will be important to determine more clearly the exact relationship between depression and immune system alterations. There is also a need to determine which of the immune alterations that are found are clinically important (Irwin 2001).

39.4.1.1 Impact of HPA Axis on Major Depression

The hyper-reactivity of the HPA axis that occurs with chronic stress is also often observed in depressed patients (Owens and Nemeroff 1991). In animal models, intracerebroventricular

injection of CRH induces anxiety and a depression-like phenotype (Heinrichs and Koob 2004), and CRHR1 antagonists decrease the signs and symptoms of depression. In contrast, the urocortins, thought to act via the CRHR2 system and potentially involved in the adaptive phase of the stress response, seem to have anxiolytic properties (Heinrichs and Koob 2004). The hypercortisolemia resulting from chronic stress perturbs monoaminergic systems as observed in depression, and causes dysregulation of anxiety and aggressive behavior. Using neuroendocrine tests in longitudinal studies, it has been shown that depressed patients do not respond well to anti-depressant treatment if HPA axis disturbances persist; further, patients showed higher rates of relapse when HPA axis disturbances returned (Zobel et al. 2001). In line with these clinical studies, it is interesting to note that chronic treatment of rats with the anti-depressant amitriptyline resulted in changes in limbic MR and GR that occurred in parallel with HPA axis normalization (Reul et al. 1993). In sum, these observations make a strong case for a major role for the HPA axis in the etiology of depression. An important aspect of research in depression addresses the role of genetic predispositions and developmental events as causative factors. Based on studies of twins, it is estimated that depression has a heritability of 40% (Sullivan et al. 2000).

39.4.1.2 Neuropeptides on Major Depression

A large body of research has aimed at identifying neuropeptides that are dysregulated within patients diagnosed with MDD. Of this body of research, three neuropeptides have been readily identified as being changed in individuals with the disorder, those being substance P, corticotrophin-releasing hormone and vasopressin. Substance P is thought to play a role in the pathophysiology of depression and may be a key target of antidepressants (Bondy et al. 2003). In addition, Bondy et al. (2003) found a subpopulation of individuals with MDD to have elevated serum levels of substance P, suggesting elevated levels of substance P may play a role in the pathophysiology in a subpopulation of patients with the disorder. In addition to substance P, CRH has been identified as an abnormal neuropeptide within the disorder. CRH induces release of ACTH, which is potentiated by vasopressin (Raadsheer et al. 1994). As noted above, CRH has activated the HPA axis (Raadsheer et al. 1994, 1995). Further, studies have identified increased levels of CRH expressing neurons and mRNA levels within the hypothalamic paraventricular nucleus (Raadsheer et al. 1995). Although elevated mRNA levels have been observed, the same has also been reported in cerebral spinal fluid of patients with MDD (Nemeroff 1988). The hyperactivity of CRH has been suggested to be causally related to the symptomology present within the disorder (Raadsheer et al. 1995; Holsboer 2003). Taken together, depression can be characterized by hyperactive CRH systems and HPA axis hypoactivity (Kasckow et al. 2001). Arginine vasopressin, another commonly associated neuropeptide, has

been associated with increased HPA axis activity in MDD (Bondy et al. 2003). Elevated plasma concentrations of arginine vasopressin have been identified in patients with the disorder (Müller et al. 2000; van Londen et al. 1997) and it has been suggested that chronically active vasopressin may play a role in depressive symptomology by influencing the HPA axis (Bondy et al. 2003; van Londen et al. 1997; Müller et al. 2000). Further, antagonizing vasopressin receptors may have a promising role in treatment for major depression (Pariante and Lightman 2008). Targeting of neuropeptide dysregulation in patients with bipolar disorder may prove a promising candidate for treatment of the disorder.

39.4.1.3 Impact of Immune System Cytokines in Major Depression

A large number of studies have demonstrated increases in several pro-inflammatory cytokines in patients diagnosed with MD. It is interesting to note that all of these cytokines are associated with activation of the HPA axis. Thus, elevations in IL-6 have been consistently associated with clinically diagnosed depression (Alesci et al. 2005; Lanquillon et al. 2000; Maes et al. 1997; Musselman et al. 2001; Sluzewska et al. 1995). The circadian pattern of IL-6 secretion is shifted 180° out of phase in depressed patients (Alesci et al. 2005), and IL-6 levels are significantly correlated with severity of depression (Musselman et al. 2001). Prior to administration of antidepressant medication, levels of IL-6 were elevated in patients who did not respond to the treatment, whereas responders showed normal levels before treatment, suggesting that IL-6 levels could be used to predict treatment outcome (Lanquillon et al. 2000). Depression has also been associated with increases in IL-1 (Owen et al. 2001; Thomas et al. 2005) and TNF- α (Hestad et al. 2003; Tuglu et al. 2003). These studies support a role for pro-inflammatory cytokines in MDD, and suggest a novel strategy for treatment of the disease (Raison et al. 2006).

39.4.2 Overview of the Neuroimmune System in Schizophrenia

The involvement of the immune system in schizophrenia (SZ) is an even more complicated issue compared to involvement with depression. There have been suggestions for many decades that schizophrenia is associated with immune dysfunction (Vaughan et al. 1949). However, it is only recently that substantial evidence relating to this idea has begun to appear and this is even less definitive than data supporting a link between the immune system and depression. Analogous to depression, there are multiple problems with dissecting a role for the immune system in schizophrenia, including the multiplicity of the disease etiology and the difficulty in controlling for the many factors that are frequently associated with schizophrenia and that also alter immune function themselves.

These include age, gender, sleep status, the likelihood that schizophrenia represents a complex of disease states, and the frequent presence of other psychiatric or physical disorders which can affect both psychological status and immune function. The difficulties with such studies have been set forth by Keller and colleagues (2013).

Nevertheless, despite the difficulties, there are intriguing findings that have strengthened the hypothesis that neuro-immune interactions are altered in schizophrenia patients. One argument for an association between immune function and schizophrenia is that epidemiological data show a linkage between prenatal viral infections and subsequent appearance of schizophrenia. There have also been studies that show an acute exacerbation of schizophrenia can occur in response to immune system dysfunction. These have been reviewed (Keller et al. 2013). The most extensively examined idea is that schizophrenia is associated with autoimmune diseases or that schizophrenia may, in certain instances, be associated with a lack of normal autoimmune function (Gaughran 2002; Jones et al. 2005; Kipnis et al. 2006).

Autoimmune diseases are associated with some schizophrenia-like symptoms. There are also associations between the appearance of several autoimmune diseases and schizophrenia, although these are both positive and negative correlations. It has been proposed that there is a genetic linkage between schizophrenia and autoimmune diseases. Again, there are both positive and negative associations. Many of these correlations have been refuted by later data. The extensive literature documenting multiple changes in immune system markers and function in the schizophrenic population has been reviewed extensively (Gaughran 2002).

An interesting hypothesis advanced recently is that schizophrenia is, at least in some cases, due to loss of a specific aspect of autoimmunity due to prenatal loss of a specific subset of T cells (Kipnis et al. 2006). This idea is based on a series of studies from the Schwartz lab that indicate autoimmune T cells in the CNS play a fundamental physiological role, exerting a protective effect on neurons when they are subjected to stress. The hypothesis is that there is a prenatal loss of the relevant autoimmune clones so that when stress appears later in life, the neuroprotective effects are lost or at least diminished, and ultimately neurodegeneration occurs, leading to schizophrenia. This idea is consistent with a number of characteristics of schizophrenia. The dopamine hypothesis posits abnormally high levels of dopamine in schizophrenia. Dopamine release is increased centrally in response to stress and this normally suppresses regulatory T cells, leading to increased activity in protective autoimmune T cells. In schizophrenia, however, there is a loss of autoimmune protection, consistent with a lack of protective autoimmune T cells. This would result in lack of neuroprotection and greater neurodegeneration. It is generally accepted that schizophrenia is a developmental disorder. There are also considerable data indicating schizophrenia is often associated with maternal

inflammation or viral infection. Both of these could lead to loss of immune cells prenatally, perhaps to a specific loss of protective autoimmune clones. There is a great deal of work to be done to support or disprove this hypothesis, but it does bring together several aspects of schizophrenia and it provides a framework for investigating a potential role of autoimmunity in schizophrenia.

39.4.2.1 Impact of HPA Axis on Schizophrenia

Hyperactivity of the HPA axis has been implicated in the pathology of schizophrenia (Walker et al. 2008). Others have suggested that the hyper-activation of the HPA is due to the hyper-secretion of CRH. In line with this hypothesis, elevated levels of cerebrospinal fluid CRH have been observed in patients with SZ. Others have observed abnormal cortisol levels in patients with SZ (Kaneko et al. 1992), and augmentation of these levels may exaggerate psychotic symptoms (Walker et al. 2008). Interestingly, antipsychotics have been shown to reduce HPA activity, alleviating symptoms associated with the disorder (Walker et al. 2008). In addition, Kaneko et al. (1992) have even been able to link HPA dysfunction with negative symptoms of the disorder. Although it is well known that the HPA axis is hyperactive in individuals with schizophrenia, further research is needed to identify how the HPA axis dysregulation gives rise to symptoms seen within the disorder.

39.4.2.2 Neuropeptides on Schizophrenia

Similar to other disorders, neuropeptides within schizophrenia have found to be dysregulated. Perhaps the most commonly studied neuropeptide in regards to schizophrenia, neuropeptide Y levels have been shown to be reduced in patients with SZ (Frederiksen et al. 1991; Gabriel et al. 1996; Itokawa and Yoshikawa 2003). In addition, neuropeptide YY has also been shown to be reduced in SZ (Widerlöv et al. 1988). In combination, neuropeptide Y and neuropeptide YY may be useful trait markers in identifying patients with the disorder (Widerlöv et al. 1988). Through the use of post-mortem studies, neuropeptide Y, arginine vasopressin, neuropeptide YY and galanin levels have all found to be decreased in the temporal cortex of patients with schizophrenia (Frederiksen et al. 1991). Other studies have also found decreased levels of somatostatin in temporal and frontal lobes (Gabriel et al. 1996) and mRNA levels were decreased in the dorsolateral prefrontal cortex (Morris et al. 2008). Similarly, decreased levels of somatostatin-like immunoreactivity in the cerebrospinal fluid have been observed (Bissette et al. 1986; Reinikainen et al. 1990). Interestingly, vasoactive intestinal polypeptide levels are decreased in patients with schizophrenia (Vacic et al. 2011; Gabriel et al. 1996), and are suggested to be a potential target for development of antipsychotic drugs (Vacic et al. 2011). It is of note

that antipsychotic treatment has been shown to have beneficial effects on neuropeptide Y and somatostatin cortical levels increasing levels back to normal (Sakai et al. 1995), suggesting that antipsychotics may have therapeutic effects by targeting neuropeptides. Much work remains to be done in order to identify all neuropeptides dysregulated within the disorder, however therapeutic effects may prove beneficial by targeting dysfunctional neuropeptides of the disorder.

39.4.2.3 Impact of Immune system Cytokines in Schizophrenia

While the etiology of schizophrenia is essentially unknown, it is generally accepted that the disease complex is caused by the abnormal development of limbic system structures that are assembled mainly in the third trimester of human gestation (Weinberger 1987; Waddington 1993). Additionally, the hypothesis is gaining support that the disease is precipitated by environmental stressors around the period of final maturation of these limbic structures. This “two hit” idea has led to research directed at two potential targets for cytokine action: the effects of cytokines on the development of the nervous system, and the acute effects of cytokine administration in generating psychotic symptoms.

A number of environmental factors have been correlated with the development of schizophrenia including birth trauma, maternal viral infection, and season of birth (reviewed in Nawa et al. 2000). Prolonged labor results in abnormally elevated serum IL-6 levels (De Jongh et al. 1997), suggesting a possible link between immune system activation and the perinatal complications associated with the development of schizophrenia. Given the role of proinflammatory cytokines in repair of tissue damage and infection, it is interesting that TGF- α and IL1- β can suppress the normal expression of brain-derived neurotrophic factor (BDNF), a neurotrophin that is a key regulator of neuronal and synaptic development, in the hippocampus (Lapchak et al. 1993). Conversely, IL1- β causes up-regulation of nerve growth factor (NGF), another factor that regulates growth and development of central neurons, in microglial cells (Heese et al. 1998). These findings compel further research into the interactions between cytokines and the brain-derived trophic factors that may lead to abnormal development of brain regions that ultimately result in the manifestation of schizophrenia in the mature brain.

Several reports of persistent psychosis following the administration of interferon- α (Tamam et al. 2003; Thome and Knopf 2003; Telio et al. 2006) or high doses of IL-2 (Denicoff et al. 1987) demonstrate that cytokines could conceivably mediate some aspects of cognitive impairment observed in schizophrenic patients. These studies are interesting in light of the fact that evidence for abnormal levels of cytokines in schizophrenic patients is accumulating

(Malek-Ahmadi 1996; Prolo and Licinio 1999). However, further studies are needed to show a causative relationship between cytokine levels in patients and the precipitation of psychotic episodes.

39.4.3 Bipolar Disorder

39.4.3.1 Impact of HPA Axis on Bipolar Disorder

The HPA axis remains less studied in bipolar disorder (BD) than in major depression and schizophrenia, however it is suggested that patients with BD show similar HPA axis dysfunction (Watson et al. 2004). Recent studies provide robust evidence demonstrating HPA axis abnormalities in BD. Due to HPA axis dysregulation, ACTH and cortisol levels have been reported to be dysregulated in patients with BD (Daban et al. 2005). Hypercortisolism is thought to play a central role in the pathogenesis of cognitive deficits and depressive symptoms of BD, which may result from neurocytotoxic effects of heightened cortisol levels. In fact, increased ACTH and cortisol levels may precede manic episodes giving rise to these symptoms. It has also been reported that patients with BD have a higher degree of HPA axis dysfunction than those with unipolar disorder (Rybakowski and Twardowska 1999). Compared to individuals with unipolar disorder, patients with BD exhibit significantly higher concentrations of cortisol in acute episodes and during remission (Rybakowski and Twardowska 1999). Interestingly, HPA dysregulation has not been linked to particular episodes of depression or during mania within BD (Schmider et al. 1995).

Pituitary gland volume has also become an indicator of HPA axis dysfunction (Sassi et al. 2001), and has become an indicator of HPA axis dysfunction in patients with BD. Patients with BD have shown a consistent decrease in pituitary gland volume compared to healthy individuals.

Due to the consistent findings of HPA axis dysregulation in patients with BD, it has become a trait marker for BD and may be indicative of pathophysiological processes within the illness. Further, HPA axis manipulation (i.e. drug targeting) has been shown to have therapeutic effects within patients with the disorder (Daban et al. 2005). Interestingly, therapeutic approaches targeting HPA axis dysregulation may prove beneficial for BD treatment regimes.

39.4.3.2 Neuropeptides on Bipolar Disorder

Early reports suggest alterations in the neuropeptides vasopressin (Gold et al. 1978), somatostatin (Gerner and Yamada 1982), and endorphins (Verebey et al. 1978) and in patients with BD compared to healthy individuals. Studies have also suggested decreased levels of neuropeptide Y in the prefrontal cortex of individuals with bipolar disorder (Caberlotto

and Hurd 1999; Kuromitsu et al. 2001). The decreased levels of NPY seen in patients with bipolar disorder have been shown to be similar to individuals with schizophrenia (Kuromitsu et al. 2001), suggesting a common pathology between the two disorders. Much research is still needed to identify other neuropeptide differences between BD and healthy individuals before treatment can be conducted.

39.4.3.3 Impact of Immune system Cytokines in Bipolar Disorder

Evidence suggests a bidirectional link between inflammation and mood disorders (Rosenblat et al. 2014). This is supported by studies showing that emotional, psychological or physical stress could induce stress responses (Miller et al. 2013; Raison and Miller 2013). Indeed, elevated IL-1, IL-6, TNF- α and CRP have been observed following the application of psychological stress alone (Steptoe et al. 2007). In patients with bipolar disorder, numerous studies have correlated elevation of inflammatory markers (prostaglandin E2 (PGE2), CRP, TNF- α , IL-1- β , IL-2, IL-6) with increased incidence of mood symptoms and mood episodes (reviewed in Rosenblat et al. 2014). Two recent meta-analyses of cytokine changes in bipolar patients found consistency in elevated soluble IL-2R, TNF- α , and soluble IL-6R compared to control patients (Munkholm et al. 2013; Miller et al. 2013).

One postulated mechanism to explain how pro-inflammatory cytokines can induce depressive symptoms is through regulation of tryptophan metabolism. Tryptophan is a precursor of serotonin, melatonin, and other bioactive metabolites. Impairment of serotonin neurotransmission underlies one theory explaining the etiology of mood disorders including major depression and bipolar disorder (Arango et al. 2002; Barton et al. 2008; Rosa-Neto et al. 2004; Anderson et al. 2014). Indeed, depletion of brain tryptophan levels can induce depressive symptoms in individuals who are sensitive to major depression (Young et al. 1985; Booij et al. 2002; Smith et al. 1997; Delgado et al. 1990). Moreover, inhibition of tryptophan hydroxylase, the first step in the pathway of serotonin synthesis, can induce a relapse of depression in remitted depressive patients (Smith et al. 1997; Delgado et al. 1990). Limiting tryptophan availability in the brain by shunting of tryptophan down non-serotonin-producing metabolic pathways may also limit the capacity to produce serotonin (Jaronen and Quintana 2014). In addition to metabolism by tryptophan hydroxylase, tryptophan is also metabolized by indolamine 2,3-dioxygenase (IDO) to kynurinine. IDO activity is upregulated, at least in part, by inflammatory cytokines such as interferon- α , IFN- γ (the major inducer), IL-2, TNF- α and IL-6 (Capuron et al. 2001; Anderson and Maes 2015). Kynurinine, kynurenic acid and quinolonic acid are all tryptophan metabolites, and

each has been shown on their own to induce depressive symptoms (Maes et al. 2011). Proinflammatory cytokines, such as IL-1, IL-6 and TNF- α , have also been shown to activate the HPA axis leading to increased circulating cortisol levels. Cortisol, in turn, drives higher IDO activity and tryptophan depletion (Hoes and Sijben 1981; Maes et al. 2011; Wolf 1974). Finally, the elimination of serotonin from the brain is initiated by its conversion to 5-hydroxyindoleacetic acid, a process that is enhanced by IL-6 and TNF- α (Wang and Dunn 1998; Zhang et al. 2001). Many studies have examined how cytokines vary in bipolar disorder compared to healthy individuals. A meta-analysis, consisting of 30 studies evaluating levels of cytokines in patients with BD have revealed consistent elevations in pro-inflammatory, anti-inflammatory and regulatory cytokines (Modabbernia et al. 2013). Increases in IL-6 and TNF- α serum levels have been consistently observed in patients with BD (Kupka et al. 2002; Kim et al. 2007; Ortiz-Domínguez et al. 2007; Brietzke and Kapczinski 2008). In contrast, other cytokines have been revealed to have different levels during the different manic and depressive phases of BD. During manic episodes, elevated serum levels of IL-6 (Kim et al. 2007; Ortiz-Domínguez et al. 2007), TNF- α (Kupka et al. 2002; Kim et al. 2007; Ortiz-Domínguez et al. 2007; Brietzke and Kapczinski 2008) and IL-4 (Ortiz-Domínguez et al. 2007) have been observed; During depressive phases of the illness, IL-6 and TNF- α show elevated levels, while IL-2 show decreased serum levels (Ortiz-Domínguez et al. 2007). These studies suggest that serum levels of cytokines may vary depending on the phase of the illness. The most consistent finding in regards to cytokine levels is the elevated levels of TNF- α seen within both phases of the illness. These studies suggest that therapeutic targets on the TNF- α pathway may prove beneficial in the treatment of bipolar disorder. In addition, inflammatory cytokines have been implicated in reducing anti-depressive levels of serotonin in the brain and increasing pro-depressive levels of alternate tryptophan metabolites.

39.4.4 Post-Traumatic Stress Disorder (PTSD)

39.4.4.1 Impact of HPA Axis on PTSD

Posttraumatic stress disorder is often experienced after traumatic experiences (i.e. war or life-threatening experience) and is generally accompanied with acute and chronic alterations in stress responses. Traumatic stress responses include intense fear, helplessness and horror (Brewin et al. 2000). Further, it is known that 8–18% of individuals exposed to traumatic events develop PTSD (Breslau et al. 1998; Kessler et al. 1995; Sledjeski et al. 2008). Individuals who develop PTSD show symptoms such as hyperarousal and symptoms are thought to be accompanied by or lead to alterations in biological stress responses (de Kloet et al. 2006).

Like other disorders (e.g. anxiety disorder and major depressive disorder), individuals with PTSD show alterations in stress response and hypothalamic-pituitary-adrenocortical (HPA) function. However, inconsistencies have been seen in studies linking HPA function and PTSD (Morris et al. 2012). The HPA axis is one of the three systems activated in response to stress (Teicher et al. 2002). Exposure to stress triggers emotional responses, including activation of the limbic system that initiates HPA activity. Some studies have reported PTSD patients to have high levels of CRH in cerebrospinal fluid (CSF) (Baker and Shalhoub-Kevorkian 1999; Bremner et al. 1997), while diurnal plasma cortisol levels are decreased (Yehuda et al. 1994). The combination of low diurnal plasma cortisol levels and high levels of CRH in CSF has been attributed to enhance HPA-axis feedback regulation.

Recently, researchers have posited an “attenuation hypothesis” of HPA axis functioning in PTSD. This hypothesis states that low cortisol levels seen in individuals with PTSD may be explained by allostasis, the body’s adaptive efforts to maintain homeostasis. In normal individuals, stress leads to HPA axis activation and release of cortisol to allow for adaptive coping that may have protective effects of neuroendocrine processes and blunt the impact of physiological and psychological stressors. In the case of PTSD, it is hypothesized that stress and trauma are prolonged, unpredictable or overwhelming and the demand of the HPA axis may be excessive, leading to allostatic overload. This allostatic overload may decrease the body’s ability to mediate stress and increase the vulnerability for PTSD pathology (Jones and Moller 2011).

The most significant observation in regards to PTSD is the low cortisol levels consistently found in patients. Due to these findings, novel therapeutic approaches to treat PTSD are being developed to target low levels of cortisol (Yehuda 2006).

39.4.4.2 Neuropeptides on PTSD

Neuropeptide Y dysregulation following exposure to stress is thought to give rise to behavioral consequences (i.e. problems regulating emotion) (Heilig 2004) and is thought to act as a biological mediator of these negative actions (Yehuda 2006). Recently, there has been increased evidence for the role of neuropeptide Y (NPY) as a protective stress factor. Recently, many studies have implicated decreased levels of NPY in patients with PTSD (Sah et al. 2009; Rasmusson et al. 2000; Yehuda 2006). Decreased levels of NPY have been observed in plasma (Rasmusson et al. 2000; Sah et al. 2014) and in cerebrospinal fluid (Sah et al. 2009) of patients with PTSD. Rasmusson et al. (2000) have suggested this decrease in plasma NPY to contribute to PTSD symptoms (hyperarousal, exaggerated alarm reactions and anxiety), and may mediate the noradrenergic system hyperactivity. In

addition, Sah et al. (2014) have also suggested that NPY may regulate PTSD symptomology. For these reasons, many studies have proposed that NPY may be a promising clinical target and diagnostic measure. Interesting, recently derived anti-depressants, used to treat PTSD, have been shown to increase levels of NPY (Yehuda 2006), which helps alleviate some symptoms seen within the disorder.

39.4.4.3 Impact of Immune System Cytokines in PTSD

Within patients with PTSD, increased levels of pro-inflammatory cytokines have been observed (Hoge et al. 2009; Tucker et al. 2014; Spivak et al. 1997; von Känel et al. 2007; Baker et al. 2001). Some studies have shown increased levels of IL-1 β , IL-6 and TNF- α in PTSD by isolated peripheral blood mononuclear cells; this suggests evidence for low grade inflammation in PTSD. Other have shown increases in plasma IL-1 β (Tucker et al. 2014; Spivak et al. 1997), IL-6 (Maes 1999) and TNF- α (von Känel et al. 2007; Gola et al. 2013). In addition, elevated levels of IL-6 have been detected in cerebrospinal fluid of patients with PTSD (Baker et al. 2001). Interestingly, some studies have correlated increased levels of TNF- α with PTSD symptom severity (Gola et al. 2013; Gola et al. 2013; Gola et al. 2013). It has been shown that pro-inflammatory cytokines, like IL-6, TNF- α and others are indicative of HPA disruption and physical response (Biffi et al. 1996; Hisano et al. 1997; Hirano et al. 1990). Interactions between cytokines and the HPA axis have effects at the pituitary, allowing for physiological and psychological disturbances following traumatic experiences and injuries, like those seen in PTSD (Sutherland et al. 2003). Much evidence suggests elevated cytokine levels in patients with PTSD and may suggest potential treatments of the disorder. However, much research remains to be conducted to understand the full effects of cytokine dysregulation in PTSD.

39.4.5 Anxiety Disorder

39.4.5.1 Impact of HPA Axis on Stress and Anxiety Disorders

Homeostasis is dependent on the maintenance of the complex dynamic equilibrium of the constantly changing external and internal factors present in everyday life. This proper balance is however dysfunctional in cases of increased and prolonged stress and anxiety. In fact, early life stress, such as neglect and childhood abuse, has been linked to later life anxiety disorders (Arborelius et al. 1999). HPA hyper-activation has been shown in patients with anxiety and stress disorders (Holmes et al. 2003). Some studies have shown increased levels of cortisol to correlate with high anxiety levels (Granger et al. 1994; Coplan et al. 2002), in turn increasing basal HPA axis activity (Feder et al. 2004). Further, increased levels of CRH have

been observed in human and animal models of anxiety disorder (Dunn and Berridge 1990).

39.4.5.2 Neuropeptides on Stress and Anxiety Disorders

Similar symptoms of anxiety and depression commonly co-exist. Therefore, changes in neuropeptides are similar to those seen in major depression. Like other disorders previously discussed, substance P, CRH, vasopressin, NPY and galanin neuropeptide changes have been shown to alter anxiety and depressive behaviors (Holmes et al. 2003). Studies have shown reduced concentrations of NPY in cerebrospinal fluid and plasma in depression and anxiety (Westrin et al. 1999; Widerlöv et al. 1988). Further, NPY injections in animal models have been shown to have anxiolytic effects (anxiety alleviating) (Griebel 1999), while CRH injections have anxiogenic-like (anxiety causing) effects (Radulovic et al. 1999). These observations may correlate with the previous findings of decreased NPY levels and increased CRH levels in individuals with high stress and anxiety. Currently, clinical trials are ongoing testing the efficacy of substance P antagonists. Preliminary studies show promising results for future treatment of stress, anxiety and depression (Holmes et al. 2003). It could be hypothesized that other neuropeptide antagonists could prove beneficial for treatment of these disorders.

39.4.5.3 Impact of Immune system Cytokines in Stress and Anxiety Disorders

Little work has been dedicated towards identifying the role of cytokines in anxiety and stress disorders. Of studies conducted, changes have been reported in production of TNF- α , IL-6, IL-1 receptor antagonist, INF- γ and IL-10 in stress. In addition, increased levels of INF- γ and decreased levels of IL-10 and IL-4 have been reported in anxiety disorders (Maes 1999). Further, preliminary results suggest an increase in IL-2 levels in patients with anxiety disorder (Rapaport and Stein 1994). Overall, it can be concluded that stress and anxiety disorders lead to changes in TNF- α , IL-6, INF- γ , IL-10 and IL-4. These changes in cytokines may lead to the pathophysiology of stress and anxiety disorders and in turn may prove valuable as beneficial treatment candidates.

39.5 Review Questions

1. Name three of the four peptides for which proenkephalin is the precursor.
 - (a) methionine-enkephalin
 - (b) leucine-enkephalin
 - (c) methionine-enkephalin-Arg-Phe
 - (d) methionine-enkephalin-Arg-Gly-Leu

Answer: Any combination of the 3 listed above

2. The major physiological role of the enkephalins in regulating immune function appears to be
 - (a) stimulation of all immune cells
 - (b) inhibition of all immune cells
 - (c) maintenance of homeostasis
 - (d) increased expression of regulatory T cells
 - (e) decreasing reactive oxygen species production

Answer: (d) increased expression of regulatory T cells

3. Name the three products derived from proopiomelanocortin (POMC) for which evidence is the strongest supporting involvement in regulating immune system function.

Answer: β -endorphin; α -MSH and γ -MSH

4. The site of action for α -MSH is
 - (a) mu opioid receptors
 - (b) melanocortin-1 receptors
 - (c) glucocorticoid receptors
 - (d) melatonin receptors
 - (e) ACTH receptors

Answer: (b) melanocortin-1 receptors

5. Local release by immune cells of which neuropeptide plays the most important role in nociception produced in areas of inflammation by the immune system?
 - (a) methionine-enkephalin.
 - (b) α -MSH
 - (c) ACTH
 - (d) β -endorphin
 - (e) urocortin-1

Answer: (d) β -endorphin

6. Which neuropeptide is anti-inflammatory when activating the HPA axis but is proinflammatory when released locally by immune cells?
 - (a) ACTH
 - (b) CRH
 - (c) β -endorphin
 - (d) nociceptin
 - (e) γ -MSH

Answer: (b) CRH

7. Which cytokine has been found to induce major depressive disorder in a high percentage of patients when used to treat malignant melanoma or hepatitis C?
 - (a) IL-6
 - (b) TNF- α
 - (c) IL-4
 - (d) interferon- α
 - (e) IL-10

Answer: (d) interferon- α

8. The neuropeptide synthesized and released from the CNS, particularly the hypothalamus, as well as from immune cells is
 - (a) ACTH
 - (b) urocortin-1

- (c) CRH
- (d) TNF- α
- (e) insulin

Answer: (c) CRH

9. The proinflammatory cytokine found to have increased levels in plasma and CNS in major depressive illness and whose levels correlate significantly with mood rating is
 - (a) IL-6
 - (b) TNF- α
 - (c) CRH
 - (d) IL-2
 - (e) β -endorphin

Answer: (a) IL-6

10. Hyper-activation of the HPA axis in schizophrenia is thought to be due to hyper-secretion of what neuropeptide?
 - (a) CRH
 - (b) IL-6
 - (c) IL-2
 - (d) ACTH
 - (e) IL-10

Answer: (a) CRH

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Abstract

Autism spectrum disorders (ASD) are characterized by deficits in sociability and communication, as well as severe anxiety and stereotypic movements. Moreover, over 50 % of children with ASD experience atopic symptoms indicative of mast cell (MC) activation (asthma, eczema, food allergies and/or intolerance) which correlate with the presence of brain auto-antibodies. ASD pathogenesis is unknown preventing the development of effective treatments. As a result, more than 70 % of children with ASD are prescribed psychopharmacologic agents that often have little benefit and serious adverse effects. Increasing evidence indicates that local brain inflammation is involved in ASD. In particular, there is activation and proliferation of microglia, which communicate with MCs, located perivascularly primarily in the thalamus and hypothalamus, as well as the lining of the ventricles. MCs are stimulated by corticotropin-releasing hormone (CRH) and neurotensin (NT) secreted from the hypothalamus under stress; these peptides can also induce each other's surface receptors leading to autocrine and paracrine effects. Stimulated MCs release inflammatory and neurotoxic mediators that disrupt the blood-brain barrier (BBB), activate microglia and cause focal inflammation. CRH and NT are significantly increased in serum of ASD children compared to normotypic controls. Addressing the "allergic" and anxiety symptoms would not only improve the health of the patients, but could also potentially reduce the core symptoms of ASD. Use of the natural anti-inflammatory flavonoid luteolin, in an olive fruit oil preparation to increase oral absorption, appears to provide significant benefit and should be investigated further. Treatment approaches targeting brain inflammation could have a great benefit.

Keywords

Autism spectrum disorders (ASD) • Corticotropin-releasing hormone (CRH) • Mast cells (MCs) • Microglia • Neurotensin (NT)

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40.1 Introduction

Autism spectrum disorders (ASD) are pervasive neurodevelopmental disorders characterized by varying degrees of deficiencies in communication and social interactions, as well as the presence of stereotypic behaviors (Fombonne 2009; Lai et al. 2014). Many ASD children regress at about age 3 years implying the importance of some environmental (Vijayakumar and Judy 2016) and epigenetic trigger (Herbert 2010). Although these could include vaccination (Delong 2011), infection (Hsiao et al. 2012), trauma (Blenner et al. 2011), toxin exposures (Deth et al. 2008) and stress (Lanni et al. 2012) none have, to date, been shown causal for disease. The prevalence of ASD has been increasing globally (Perou et al. 2013; Elsabbagh et al. 2012). Recent results indicate that as many as 1 in 68 children have ASD (Developmental Disabilities Monitoring Network Surveillance Year 2010 Principal Investigators Centers for Disease Control and Prevention (CDC) 2014). The diagnosis and definition of ASD (Erturk et al. 2015) has evolved since the original description of Dr. Kanner (Volkmar and McPartland 2014). The American Psychiatric Association’s Diagnostic and Statistical Manual of Mental Disorders (DSM-5) changed the classification in 2013 and abandoned the previous designations of autism disorder, pervasive development not otherwise specified (PDD-NOS) and Asperger’s syndrome for ASD with mild, moderate and severe symptoms (Volkmar and McPartland 2014). The lack of uniformity of symptoms across ASD and the lack of reliable biomarkers (Ruggeri et al. 2014; Subramanian et al. 2015) has made diagnosis even more challenging. Moreover, the existence of subgroups within ASD (Table 40.1) and the lack of agreement of the use of intelligence have even prompted patients and families to reject the stereotypic and categorical term “autistic” (Volkmar and McPartland 2014). Certain interactive instruments such as Childhood Autism Rating Scale (CARS) and Aberrant Behavior Checklist (ABC) may be suggestive of ASD, but diagnosis typically depends on the use of Autism Diagnostic Observation Schedule (ADOS-G) or ADI-R or fulfillment of DSM-V criteria (Volkmar and McPartland 2014; McPheeters et al.

2016). ASD are more common in boys than girls (approximately 4:1), but there is no plausible explanation for this difference. In spite of the fact, that ASD have been considered to affect individuals for life, recent studies have shown that many cases may be preventable or reversible (Beaudet 2012).

ASD have emerged as one of the most complex behavioral disorders, which in spite of great advances a possible etiology, remains poorly understood (Yoo 2015a; Ziats et al. 2015). Studies have shown that for “strict” autism probandwise concordance for male twins was 0.58 for monozygotic and 0.21 for 31 dizygotic pairs; this penetrance increased for ASD to 0.77 and 0.31 respectively leaving room for epigenetic influences (de et al. 2016; Loke et al. 2015). In spite of the discovery of numerous gene mutations in ASD (Yoo 2015) they do not account for more than 5% of the cases with the exception of Fragile X, Tuberous Sclerosis and Rett Syndromes. This risk may be further increased if children have mutations leading to decreased phosphatase and tensin homolog (PTEN) (Aldinger et al. 2011), which is an upstream inhibitor of the mammalian target of rapamycin (mTOR) (Aldinger et al. 2011).

These problems, coupled with the lack of biomarkers and specific pathogenesis, have increased the estimated annual economic burden of ASD from \$242 billion in 2014 (Buescher et al. 2014) to \$268 billion in 2015 and \$416 billion in 2025 (Leigh and Du 2015). To make matters worse, there are no reliable animal “models” of ASD (Ruhela et al. 2015), creating an urgent need for representative experimental animal models for ASD (Ruhela et al. 2015). In fact, mouse “models” are increasingly considered unreliable with respect to human inflammatory diseases (Seok et al. 2013).

There may be preventable forms of ASD (Beaudet 2012), but it is critical that one identifies and targets a specific pathogenetic mechanism in order to succeed (Ghosh et al. 2013). It is quite encouraging that the European Union started a unique program, “European Autism Interventions-A MultiCentre Study for Developing New Medications (EU-AIMS) Initiative (Ecker et al. 2013).

40.2 Perinatal Conditions Associated with Increased ASD Risk

Increasing evidence indicates that brain inflammation is important in the pathogenesis of neuropsychiatric disorders (Beumer et al. 2012; Hagberg et al. 2012; Jones and Thomsen 2013; Munkholm et al. 2013; Schmidt et al. 2007; Theoharides and Zhang 2011). Inflammation during fetal and neonatal life has been associated with subsequent neuropsychiatric disorders (Hagberg et al. 2012). Exposure to mold was associated with decreased cognitive function in six-year old children (Jedrychowski et al. 2011) and cognitive dysfunction has been linked to toxigenic fungi exposure (Gordon et al. 2004).

Table 40.1 Autism spectrum disorders subgroups

ADD/ADHD
Allergies/Food intolerance
Gastrointestinal symptoms
Hyperactivity/disruptive behavior
Mitochondrial dysfunction
PANDAS ^a
PTEN mutations
Seizures

^aPediatric autoimmune neuropsychiatric disorders associated with streptococcal infections

Prenatal exposure to common environmental triggers affected brain lipid composition and increased the risk of ASD (Wong et al. 2015). A recent study reported associations between higher ASD prevalence and proximity to industrial facilities emitting air pollutants (Dickerson et al. 2015). In fact, a cross-sectional study reported significant correlation between hair mercury content and ASD (Fido and Al-Saad 2005; Geier et al. 2012). Chemical intolerant mothers were three times more likely to report having a child with ASD (Heilbrun et al. 2015); moreover, these mothers reported that their children were more prone to allergies, food cravings and sensitivity to odors (Heilbrun et al. 2015).

Exposure to air pollutants (Suades-Gonzalez et al. 2015) and interactions between chemical irritants and pathogens from sewage sludges (biosolids) may also be important (Lewis et al. 2002). Use of antidepressants, especially serotonin-specific reuptake inhibitors (SSRIs) (Boukhris et al. 2015; Sorensen et al. 2013) during pregnancy was associated with higher risk of delivering children with ASD (Rais and Rais 2014), while other studies showed an increased association with ADHD, but not ASD (Clements et al. 2015). Prolonged oxytocin use to induce labor was associated with increased risk of delivering children who developed ASD (Gregory et al. 2013). Use of acetaminophen during pregnancy was also associated with increased risk of ASD but only the type accompanied by hyperkinetic symptoms (Liew et al. 2015).

An analysis of 30,942 cases of ASD concluded that very young or old parents, and primarily the bigger the age difference between the couples, was associated with higher risk of having children with ASD (Sandin et al. 2015).

ASD was associated in women with childhood sexual or physical abuse (Roberts et al. 2015; Weisman et al. 2015), and in 0–9 years old boys with ritual circumcision (Frisch and Simonsen 2015). In both setting, the precipitating factor may have been stress. Prenatal stress has been linked to increased risk of ASD (Beversdorf et al. 2005; Ronald et al. 2010). A meta-analysis showed a strong correlation between the presence of anxiety disorders and ASD (van Steensel et al. 2011). ASD patients were prone to stress (Gillott and Standen 2007; Vasa and Mazurek 2015) and anxiety was also strongly correlated with repetitive behaviors with children with ASD (Rodgers et al. 2012). Children with ASD, especially those with GI problems; had significantly higher rate of both anxiety and sensory over-reactivity (Mazurek et al. 2013). Anxiety in children with ASD was consistent with sympathetic over-arousal and parasympathetic under-arousal (Kushki et al. 2013). Heightened response to stress and comorbid anxiety disorders may be due to maladaptive psychological responses in children with ASD (Hollocks et al. 2014).

Paternal stress altered sperm micro RNA and reprogrammed the offspring stress response (Rodgers et al. 2013).

Amazingly, prereproductive stress in female rats alters of corticotropin-releasing hormone (CRH) expression in ova as well as CRH-1 receptor expression in the brain of the offspring (Zaidan et al. 2013). Prenatal negative life events increased cord blood immunoglobulin E (IgE) (Peters et al. 2012) and the risk of atopy (Sternthal et al. 2009; Andersson et al. 2016). Moreover, neonatal maternal deprivation stress induced long-term interactions in rats between colonic nerve-MCs (Barreau et al. 2008).

Acute stress can exacerbate inflammatory disorders, such as migraines (Theoharides et al. 2005) and multiple sclerosis (Karagkouni et al. 2013; Mohr et al. 2000). Stress activates MCs through CRH and leads to BBB disruption in rats (Esposito et al. 2002). CRH is also responsible for disruption of the gut-blood barrier through MC stimulation (Vanuytsel et al. 2014). Restraint stress resulted in activation of brain MCs and elevation of rat MC protease levels in cerebrospinal fluid (CSF), effects abolished by pretreatment with polyclonal antiserum to CRH (Theoharides et al. 1995) or pretreatment with the CRH-1 receptor antagonist Antalarmin (Theoharides et al. 1995). CRH can actually be secreted from MCs (Kempuraj et al. 2004) and could have autocrine and paracrine effects.

40.3 Immune Dysregulation

It is now recognized that ASD are associated with some immune dysfunction (Derecki et al. 2010; Onore et al. 2012; Rossignol and Frye 2012a; Theoharides et al. 2012a; Zimmerman et al. 2005), autoimmunity (Ashwood and Van de Water 2004; Theoharides et al. 2013a), and/or an neuro-immune component (Theoharides et al. 2009). In fact, recent reviews stress neuroinflammation in the pathogenesis of ASD (Estes and McAllister 2015; Volkmar and McPartland 2014; Gottfried et al. 2015; Theoharides and Zhang 2011; Kern et al. 2015).

Maternal and autoimmune diseases, especially asthma and psoriasis have been reported to increase the risk of ASD in the offspring (Chen et al. 2016). Circulating auto-antibodies directed against fetal brain proteins have been reported in about 30% of mothers of children with ASD and ASD patients (Braunschweig and Van de Water 2012; Rossi et al. 2011; Wills et al. 2009) implying BBB break neonatally. Another study reported that 42% of 3-year old children with ASD had plasma autoantibodies against GABAergic cerebellar neuronal proteins (Rossi et al. 2011). A recent paper described a strong statistical correlation between the presence of brain autoantibodies and allergic symptoms (Mostafa and Al-Ayadhi 2013).

The markers of inflammation identified in the brain and CSF of many ASD patients include tumor necrosis factor

(TNF), IL-6 and monocyte chemoattractant protein-1 (MCP-1) (Li et al. 2009b), the latter of which also is chemotactic for MCs (Theoharides et al. 2010b). In fact, elevated MCP-1 in amniotic fluid was strongly correlated with increased risk for infantile autism (Abdallah et al. 2012). A recent review reported that MCP-1 was elevated in archived neonatal blood specimens (Zerbo et al. 2014). Maternal immune activation (MIA) in mice led to increased serum IL-6 and IL-17, and contributed to immune dysregulation and autism-like behaviors in the offspring (Dahlgren et al. 2006; Hsiao et al. 2012; Smith et al. 2007). Another study showed that MIA also led to increased levels of IL-1 β , IL-33, MCP-1 and VEGF in the fetal brain (rrode-Bruses G and Bruses 2012). We had shown that acute stress significantly increases serum IL-6 in mice that was entirely dependent on MCs as it was absent in MC-deficient W/W^v mice (Huang et al. 2003). We later showed that the MIA model of autism could not develop in these mice (unpublished). Nevertheless, a recent study reported that TNF and IL-1 β were not increased, while IL-8 was elevated in the plasma of children (mean age 9.3 \pm 0.7 years) with ASD (n=15) compared to healthy controls (n=20) (Tonhajzerova et al. 2015). Two studies showed increased TNF (Zimmerman et al. 2005) levels in CSF of patient with ASD (Chez et al. 2007). We recently reported that TNF and IL-6 are increased in a subgroup of patients with ASD who improved significantly concomitant to lower cytokine levels after treatment with a flavonoid containing supplement (Tsilioni et al. 2015).

Other studies showed that stimulated peripheral blood mononuclear cells (PBMCs) from patients (n=23) with ASD produced twice as much TNF as those from controls (n=13) (Jyonouchi et al. 2001). This finding was confirmed later where stimulated PBMCs from patients with ASD (n=66) produced more TNF and IL-13 than controls (n=73) (Ashwood et al. 2011). It was uniquely interesting that these PBMC produced more TNF also in response to common dietary proteins such as gliadin, cow's milk protein and soy (Jyonouchi et al. 2002). Given that TNF is produced following activation of NF κ -B, it is important that NF κ -B DNA binding activity was twice as much in peripheral blood from patients with ASD than controls (n=29) (Naik et al. 2011). Moreover another study identified signaling through NF κ -B was prominent in interacting gene networks constructed from brains of ASD patients (Ziats and Rennert 2011).

IL-6 and TNF could disrupt the BBB (Theoharides and Doyle 2008) and cause "focal encephalitis" in specific brain areas, thus contributing to the pathogenesis of ASD (Theoharides et al. 2008; Theoharides 2013).

Epidemiological studies have shown that allergic diseases are associated with psychological and behavioral problems in preschoolers (Tsai et al. 2013). In particular, one study showed that infant atopic eczema was associated with attention-

deficit-hypersensitivity disorder (ADHD) (Genuneit et al. 2014) and another large epidemiological study reported a strong correlation between eczema and both ADHD and ASD (Yaghmaie et al. 2013). Another study of 14,812 atopic subjects younger than 3 years old and 6944 non-atopic subjects (followed from 1997 to 2010) showed a dose-dependent relationship between atopy and greater risk of ASD and ADHD (Chen et al. 2014a). Many ASD patients suffer from food allergies (Jyonouchi 2010) and "allergic-like" symptoms (Angelidou et al. 2011; Kogan et al. 2009), indicating MC activation (Kempuraj et al. 2010; Theoharides et al. 2012a). The suggestion was, therefore, made that a subtype of ASD may be "Allergy of the brain" (Theoharides 2013). A recent publication actually reported neurochemical changes and autistic-like behavior in a mouse model of food allergy (de Theije et al. 2014). A recent case control study among members of Kaiser Permanente Northern California (1980–2003) reported that allergies and autoimmune disorders were diagnosed more often, with psoriasis occurring more than twice as often in ASD patients than controls (Zerbo et al. 2015). Asthma was also 35 % more common in ASD (Heilbrun et al. 2015).

Interestingly, children with mastocytosis, characterized by an increased number of MCs (Theoharides et al. 2015c) appear to have a five to tenfold higher risk of developing ASD than the general population (Theoharides 2009).

40.4 Mast Cells and Microglia

During healthy brain development, microglia may "prune" neural circuits through a complement-dependent manner (Schafer et al. 2012) and provide neuroprotection (Chen and Trapp 2015). Microglia are innate brain macrophages that sensitize the brain for damage; then both establish and resolve inflammation (Shemer et al. 2015). However, abnormal microglia activation and proliferation could lead to focal inflammation and "choking" of normal synaptic traffic (Felger et al. 2015). Neuroglial activation and proliferation has been reported in brains of patients with ASD (Morgan et al. 2012; Rodriguez and Kern 2011; Vargas et al. 2005). A recent study of the transcriptomes from 104 human brain cortical tissue samples from patients with ASD showed an inverse relationship between increased M2-microglia activation and decreased neuronal activity (Gupta et al. 2014). As a result of these studies, microglia are now considered an important component of the pathogenesis of ASD (Takano 2015) and other neuropsychiatric disorders (Kritas et al. 2014b; Theoharides et al. 2015e). MCs (Nelissen et al. 2013) and MC-microglial interactions are increasingly involved in neuroinflammatory diseases (Ohnmacht and Voehringer 2010; Skaper et al. 2012; Theoharides et al. 2013b) and MC-derived tryptase can activate microglia (Zhang et al.

2012c), as does histamine (Rocha et al. 2014; Zhu et al. 2014; Hakim-Rad et al. 2009). In fact, suppression of brain MC degranulation inhibited microglia activation and inflammation of the brain (Dong et al. 2016).

MCs derive from bone marrow progenitors, mature in tissues and are critical for allergic reactions (Theoharides et al. 2015d). MCs are also implicated in immunity (Kalesnikoff and Galli 2008) and inflammation (Theoharides et al. 2010a), but may have additional immunomodulatory functions (Galli et al. 2008; Kalesnikoff and Galli 2008).

MCs are located perivascularly in close proximity to brain neurons especially in the leptomeninges (Rozniecki et al. 1999), as well as thalamus and hypothalamus (Pang et al. 1996) where they contain most of the brain histamine (Alstadhaug 2014). Increasing evidence indicates that brain histamine is involved in the pathogenesis of neuropsychiatric diseases (Haas et al. 2008; Shan et al. 2015) and in the disruption of the blood-brain-barrier (BBB) (Banuelos-Cabrera et al. 2014), through MC activation (Esposito et al. 2001, 2002; McKittrick et al. 2015). In fact, MCs are located adjacent to CRH-positive neurons in the rat median eminence (Theoharides et al. 1995). MCs and CRH regulate the permeability of the gut-blood (Vanuytsel et al. 2014) and blood-brain (Esposito et al. 2002) barriers. Interestingly, the presence of commensal house dust mite allergen in the human gut was recently shown to contribute to intestinal basic dysfunction (Tulic et al. 2015). MCs are typically stimulated by specific IgE and antigen (Blank and Rivera 2004), but also by bacterial and viral products through toll-like (TLR) receptors (Sandig and Bulfone-Paus 2012), which are important in the development of innate immunity (Abraham and St John 2010). We showed that stimulated MCs can secrete mitochondrial particles (Zhang et al. 2011) and mitochondrial ATP and DNA (Zhang et al. 2012a) extracellularly. These mitochondrial components could augment MC activation and inflammation (Asadi and Theoharides 2012) since they are mistaken by the body as “innate pathogen” and induces a strong auto-inflammatory response (Zhang et al. 2012a). In fact, we showed that serum of young autistic children had increased levels of extracellular mitochondrial DNA (Zhang et al. 2010). A recent review connected mitochondrial dysfunction to oxidative stress and inflammation in children with ASD (Rossignol and Frye 2012b).

Nucleic acids derived from such stimulated MCs, as well as from damaged cells or micro-organisms, can lead to chronic inflammation and autoimmunity (Rubartelli and Lotze 2007). In fact, DNA released from dying host cells mediates the adjuvant activity of aluminum (Marichal et al. 2011; McKee et al. 2013), which has replaced thimerosal in many vaccines, and has shown a significant correlation with ASD (Tomljenovic and Shaw 2011).

Neuropeptides such as substance P (SP) (Zhang et al. 2011), NT (Donelan et al. 2006) and nerve growth factor

(NGF) (Kritas et al. 2014a) also stimulate MCs, an action augmented by IL-33 (Theoharides et al. 2010c). IL-33 acts through MCs to alert the innate immune system (Enoksson et al. 2011; Moussion et al. 2008) and has recently been linked to brain inflammation (Chakraborty et al. 2010). MCs are also triggered by many other cationic substances, sometimes called “basic segregates” in what have been termed “pseudo-allergic reactions” through a recently identified MRGPRX2 receptor (McNeil et al. 2015; Grimbaldston 2015).

We reported that NT was increased in the serum of young children with ASD (Angelidou et al. 2010) and so was serum levels of CRH (Tsiloni et al. 2014a). NT and NTR immunoreactivity have been identified in the rat hypothalamus (Goedert et al. 1985). Microglia expresses the neurotensin receptor 3 (NTS3), activation of which leads to their proliferation (Martin et al. 2003). NT is a brain peptide involved in inflammation (Mustain et al. 2011). NT and CRH synergistically stimulate MCs, leading to increase vascular permeability (Donelan et al. 2006) and contribute to BBB disruption (Theoharides and Konstantinidou 2007). Microglia also expresses CRH-R1 (Wang et al. 2002). NT also induces expression of CRH-1 receptor (Zhang et al. 2012a), activation of which by CRH increases allergic stimulation of human MCs (Asadi and Theoharides 2012). NT is neurotoxic (Ghanizadeh 2010) and can facilitate *N*-Methyl-D-aspartate (NMDA)-induced excitation of cortical neurons (Antonelli et al. 2004). NT may also stimulate basal pathway neurons (Boules et al. 2014).

Stimulated MCs secrete numerous vasoactive, neurosensitizing and pro-inflammatory mediators: a) the preformed histamine, serotonin, kinins, proteases as well as (b) newly synthesized, leukotrienes, prostaglandins, chemokines (CCXL8, CCL2), cytokines (IL-4, IL-6, IL-1, TNF) and vascular endothelial growth factor (VEGF), which increase blood-brain barrier (BBB) permeability (Theoharides et al. 2008; Theoharides and Konstantinidou 2007). MCs are the only cell type that stores pre-formed TNF in secretory granules (Olszewski et al. 2007) from which it can be released rapidly (Zhang et al. 2012b) and superactivate T cells (Kempuraj et al. 2008; Nakae et al. 2006). In fact, purified brain MCs were reported to synthesize and release TNF (Cocchiara et al. 1999).

MCs can release some mediators, such as IL-6 selectively (Theoharides et al. 2007). We showed that IL-1 can stimulate selective release of IL-6 (Kandere-Grzybowska et al. 2003) and CRH can stimulate selective release of VEGF (Cao et al. 2005). IL-6 could affect brain function (Theoharides et al. 2004) and activate the HPA axis (Kalogeromitros et al. 2007). MC-derived IL-6 and TGF β drive the development of Th-17 cells (Nakae et al. 2007) involved in autoimmunity and MCs secrete IL-17, themselves (Nakae et al. 2007). IL-17 was shown to be increased in the serum of children

with ASD (Al-Ayadhi and Mostafa 2012). The maternal IL-17 pathway was recently shown to promote autism-like behavior in the offspring (Choi et al. 2016). MCs can also secrete exosomes that can deliver microRNAs (Bryniarski et al. 2013) and could be involved in brain pathology (Kawikova and Askenase 2014; Tsilioni et al. 2014b).

Stimulation of mTOR (Lee 2015), especially in subjects with low PTEN, leads to microglia and MC proliferation (Theoharides et al. 2015b). Atopic diathesis, plus increased CRH and NT released under stress could contribute to stress-induced neuroinflammatory diseases (Theoharides and Cochrane 2004) and increase the risk of developing ASD (Theoharides et al. 2016).

40.5 Treatment

40.5.1 Subgroups

Unfortunately, there are no approved effective treatments for the core symptoms of ASD (Broadstock et al. 2007; Parikh et al. 2008; Rogers and Vismara 2008). The existence of ASD subgroups (Table 40.1) makes a unified approach to treatment of ASD difficult (Angelidou et al. 2012). Moreover, placebo responses in neuropsychiatric diseases (Weimer et al. 2015) including ASD (Masi et al. 2015) tend to be high. In fact, an integrative approach (Klein and Kemper 2016) or better yet personalized treatment of ASD is probably the best approach (Molteni et al. 2014) making the need for randomized, placebo-control, trials rather anachronistic (Peck 2016).

40.5.2 Behavioral and Social Interventions

Numerous studies have shown that early behavioral intervention, especially with speech, exercise, music and occupational therapy can significantly improve children with ASD (Rogers et al. 2014; Kasari et al. 2014). In particular, two interventions that have been well established include applied behavior analysis (ABA), developmental social-pragmatic (DSP) or both (Smith and Iadarola 2015). A recent systematic review and meta-analysis concluded that there was strong support for joint attention interventions, even though it was not clear which children most benefit from which type of intervention (Murza et al. 2016).

40.5.3 Psychotropic Medications

A recent review concluded that the percentage of youth receiving outpatient mental health service increased from 9.2% in 1996 to 17.3% in 2012, with those with no or mild impairment accounting for most of the absolute increase

(Olfson et al. 2015). Most children with ASD are prescribed multiple psychotropic medications, primarily antipsychotics (Schubart et al. 2014; Spencer et al. 2013; Wong et al. 2014). A recent publication on psychotropic drug use in children with ASD in 30 countries reported heavy use of antipsychotics, followed by antidepressants and anxiolytics even though they only address aggressive behaviors and not the core symptoms of the ASD (Wong et al. 2014). As a result, there is increased polypharmacy and risk of unwanted drug interactions (Theoharides and Asadi 2012). The antipsychotic agents risperidone and aripiprazole are approved for use in children with ASD (Lake et al. 2014; Spencer et al. 2013). Additional studies have cast doubt on the benefit of psychotropic agents, especially antipsychotics (Mohiuddin and Ghaziuddin 2013; Sochocky and Milin 2013), given frequent adverse effects that include weight gain, sedation, tremor and drooling (Ching and Pringsheim 2012). Two meta-analyses showed that use of psychotropic drugs in children with ASD were not helpful (Sochocky and Milin 2013; Williams et al. 2010). In particular, a double-blind, placebo-controlled trial using the antidepressant citalopram showed there was no benefit and actually worsened ASD symptoms (King et al. 2009; Volkmar 2009). A recent review also concluded that selective serotonin reuptake inhibitors (SSRIs) are not effective in ASD and frequently have adverse effects leading to hyperactivation (Young and Findling 2015). In fact, hyperserotonemia is present in about 25% of children with ASD (Muller et al. 2016) (Table 40.3).

40.5.4 Complementary and Alternative Approaches

Complementary and alternative medicines are more frequently used in children with ASD than in children with other conditions (Marti 2014; Hendren 2013) regardless of ethnic origin (Valicenti-McDermott et al. 2014). However, most supplements and vitamins tried did not have any significant benefits (Adams et al. 2011; Frye et al. 2013; Marti 2014). In contrast, gluten-free and casein-free diets resulted in significant positive changes in communication, attention and hyperactivity (Sanders and Aziz 2012).

A number of children have been shown to have antibodies against the folate receptor alpha (FR α) or low CSF N5-methyltetrahydrofolate (MTHF) and may benefit from treatment with folinic acid (Ramaekers et al. 2016). A small size, double-blind, placebo-controlled, 16-week study showed that supplementation with arachidonic acid (40 mg) and docosahexaenoic acid (40 mg) administered as 6 capsules (240 mg daily) significantly improved the ABC-social withdrawal and social responsiveness scales (Yui et al. 2012).

A recent double-blind, placebo-controlled, study using broccoli-derived sulforaphane ($n=40$, 13–17 years old for 18 weeks) showed significant improvement in social interaction and communication (Singh et al. 2014). However, participants in this study were older and were selected for their unusual history of reduced ASD symptoms during febrile episodes (Curran et al. 2007); moreover, the apparent significance was due to an uncharacteristically low placebo effect (3.3 %).

A number of studies have shown that N-acetyl cysteine (NAC) at this doses (>600 mg/day) can reduce irritability, but does not affect the core symptoms of ASD (Deepmala et al. 2015; Nikoo et al. 2015).

40.5.5 Hormonal Therapy

A number of studies have shown that the posterior pituitary hormone oxytocin can improve eye gaze and emotion recognition (Anagnostou et al. 2014; Bakermans-Kranenburg and van IJM 2013; Preti et al. 2014). However, the current evidence remains limited (Guastella and Hickie 2016).

There is no evidence that single or multiple doses of intravenous secretin have any benefit in ASD (Williams et al. 2012). The clinical experience from the use of the NMDA inhibitor memantine is too limited at present (Hosenbocus and Chahal 2013) and can be associated with neurologic adverse effects such as anxiety, confusion, insomnia, hallucinations and reversible neurological damage, all of which could present as worsening of ASD symptoms requiring even more medication.

40.5.6 Anti-Histamine and Anti-Allergic Agents

Food elimination diets, especially for casein and gluten, may be useful for those children shown to have allergy or food intolerance to these or other food substances (Rossignol 2009). Common allergic symptoms can be largely addressed through administration of histamine-1 (H-1) and H-2 receptor antagonists. However, in the case of ASD, sedating H-1 receptor antagonists may be preferred because they enter the brain and could have a beneficial function there, too. These include diphenhydramine and/or hydroxyzine, especially since the latter is also anxiolytic. The dual H-1 and serotonin receptor antagonist cyproheptadine could be useful for patients with GI symptoms and/or headaches. H-2 receptor antagonists, such as famotidine and ranitidine, are also helpful particularly in patients with GI symptoms or gastroesophageal reflux.

Ketotifen (Schoch 2003) and rupatadine (Alevizos et al. 2013; Vasiadi et al. 2010) are H-1 receptor antagonists (actually reverse agonists), but also have mast cell activation-inhibitory effects. Rupatadine also has platelet activation factor (PAF) blocking actions (Mullol et al. 2015).

In certain cases, especially if asthma is also present, the anti-leukotriene drug montelukast may be helpful. It should be noted however, that none of these drugs have been investigated in ASD specifically.

Certain physicians have been advocating treatment with dog pin worms on the grounds that they shift the immune response in the intestine (Wammes et al. 2014); however, even though animal data were promising, clinical trials in humans have shown no benefit (Evans and Mitre 2015).

40.5.7 Anti-Inflammatory Agents

Glucocorticoids are powerful anti-inflammatory agents, but are not likely to be used in children because of serious limitations, especially growth retardation.

Recent reviews have discussed the potential use of flavonoids for the treatment of neuropsychiatric (Grosso et al. 2013; Jager and Saaby 2011) and neurodegenerative (Jones et al. 2012; Solanki et al. 2015) diseases including Alzheimer's disease (Baptista et al. 2014; Mecocci et al. 2014; Sheikh et al. 2012; Vauzour 2014), and brain "fog" (Theoharides et al. 2015a).

Flavonoids are naturally occurring compounds mostly found in green plants and seeds (Middleton et al. 2000). Many of the flavonoids can suppress TNF-dependent inflammatory pathways (Gupta et al. 2014b). Luteolin (5,7, 3', 4'-tetrahydroxyflavone) is structurally closely related to 7, 8-dihydroflavone, which was shown to have brain-derived neurotrophic factor (BDNF) activity (Jang et al. 2010b). In fact, absence of BDNF was associated with autistic-like-behavior in mice (Scattoni et al. 2013) and 7, 8-dihydroflavone reduced symptoms in a mouse model of Rett syndrome, which is associated with ASD (Johnson et al. 2012). Luteolin induced the synthesis and secretion of neurotrophic factors in cultured rat astrocytes (Xu et al. 2013). Moreover, the related flavonoids 4'-methoxyflavone and 3', 4'-dimethoxyflavone were shown to be neuroprotective (Fatokun et al. 2013).

Luteolin inhibits IL-6 release from microglia (Jang et al. 2008) and from astrocytes, (Sharma et al. 2007), microglial activation and proliferation (Chen et al. 2008; Dirscherl et al. 2010; Kao et al. 2011) and microglia-induced neuron apoptosis (Zhu et al. 2011) (Table 40.2). Luteolin inhibits MCs

Table 40.2 Useful treatment approached for ASD

• Introduce behavioral interventions
• Treat any comorbid conditions, especially allergies
• Eliminate food triggers, especially casein and gluten
• Minimize phenol containing substances, especially chocolate and acetaminophen
• Address gastrointestinal symptoms, especially constipation
• Limit sensory overload
• Reduce inflammation

Table 40.3 Categories of drugs and supplements used for ASD

Drugs
<i>OCD and Disruptive Behavior</i>
• Aripiprazole
• Risperidone
<i>Hyperactivity</i>
• Hydroxyzine
• Propranolol
Supplements
<i>Hyperactivity</i>
• N-acetyl cysteine (NAC)
• S-adenosyl methionine (S-AMe)
• Valerian/Paciflora extract
<i>Oxidative Stress</i>
• Broccoli extract
• Fish oil
• Glutathione
<i>Inflammation</i>
• Luteolin/berberin (BrainGain)
• Luteolin/quercetin (NeuroProtek)
<i>Neuroprotection</i>
• Methyl B12
• Biotin, hydroxytyrosol, selenium

(Asadi et al. 2010; Kempuraj et al. 2005; Kimata et al. 2000), MC cytokine release (Asadi and Theoharides 2012) and thimerosal-induced inflammatory mediator MC release (Asadi et al. 2010). Luteolin also protects mitochondria against methylmercury-induced damage (Franco et al. 2010) and dopaminergic neurons from inflammation (Chen et al. 2008). Luteolin also inhibits auto-immune T cell activation (Kempuraj et al. 2008; Verbeek et al. 2004).

Luteolin improved spatial memory in a scopolamine-induced model (Yoo et al. 2013) and in amyloid β -peptide-induced toxicity (Liu et al. 2009) in rats. Luteolin also attenuated diabetes-associated cognitive decline in rats (Liu et al. 2013b), cognitive dysfunction induced by chronic cerebral hypoperfusion in rats (Fu et al. 2014; Hagedorn et al. 2010) and high fat-diet-induced cognitive dysfunction in mice (Liu et al. 2014). Furthermore, luteolin (Jang et al. 2010a; Liu et al. 2009; Yoo et al. 2013) increased memory and inhibited autism-like behavior in a mouse “model” of autism (Parker-Athill et al. 2009).

Luteolin further inhibits release of the excitatory neurotransmitter glutamate, (Lin et al. 2011), while it activates receptors for the inhibitory neurotransmitter γ -amino butyric acid (GABA) independent of GABA, suggesting it may also have an anxiolytic effect (Hanrahan et al. 2011). In fact, benzodiazepines that act by activating GABA receptors were shown to bind to MCs (Miller et al. 1988). Flavonoids can also inhibit acetylcholinesterase (Boudouda

Table 40.4 Beneficial effects of luteolin

• Reduces oxidative stress
• Inhibits inflammation
• Inhibits mast cell activation
• Inhibits microglia activation
• Inhibits neurotoxicity
• Increases memory
• Mimics BDNF
• Inhibits acetylcholinesterase
• Inhibits demethylase
• Prevents autism-like behaviour in mice
• Improves attention and sociability in children with ASD

et al. 2015; Tsai et al. 2007), which will increase acetylcholine and improve memory (Tables 40.1 and 40.4). Moreover, polyphenols inhibit lysine-specific demethylase-1 (Abdulla et al. 2013), an action that may be relevant since ASD have been associated with methylation defects (Trivedi and Deth 2012).

The luteolin structurally related flavonol quercetin (5,7,3',4',11-pentahydroxyflavonol), also inhibits histamine, IL-6, IL-8, TNF- α and tryptase release from human MCs (Kempuraj et al. 2005; Park et al. 2008) protected against amyloid β -induced neurotoxicity (Liu et al. 2013a; Regitz et al. 2014) and improved cognition in a mouse “model” of Alzheimer’s disease (Wang et al. 2014). In fact, quercetin-o-glucuronide reduced the generation of β -amyloid in primary cultured neurons (Ho et al. 2013). Quercetin reversed acute stress-induced autistic-like behavior and reduced brain glutathione levels in mice (Kumar and Goyal 2008).

In one case-series (Theoharides et al. 2012b) and one open-label study (Taliou et al. 2013) a luteolin and quercetin containing formulation significantly improved attention and behavior in children with ASD. This dietary supplement contains 100 mg luteolin and 70 mg quercetin per softgel capsule, >98 % pure) formulated in olive fruit extract, which increases oral absorption. We recently showed that those patients who most improved with this formulation were those who had high serum TNF at the beginning and decreased at the end of the treatment (Tsilioni et al. 2015), suggesting these are distinct subgroups.

Olive fruit extract contains hydroxytyrosol, which has been reported to protect against brain hypoxia (Gonzalez-Correa et al. 2008). It also contains oleocanthal, which inhibits fibrilization of tau proteins (Li et al. 2009a) and reduces aggregation of A β oligomers (Pitt et al. 2009) implicated in Alzheimer’s disease. Moreover, olive oil (Mohagheghi et al. 2010) and olive leaf extract (Mohagheghi et al. 2011) protected the brain by reducing BBB permeability. In fact, use of olive oil increased memory in rodents (Farr et al. 2012; Martinez-Lapiscina et al. 2013; Tsai et al. 2007).

Flavonoids, especially quercetin and luteolin (they differ by one hydroxyl group) are generally considered safe (Corcoran et al. 2012; Harwood et al. 2007; Kawanishi et al. 2005; Seelinger et al. 2008; Theoharides et al. 2014).

However, dietary supplements containing flavonoids (e.g. bioflavonoids, soy flavonoids, citrus flavonoids) often state “proprietary formula” or “complex” without listing the exact type or amounts of the flavonoids. Moreover, most supplements do not list the sources of flavonoids that are commonly peanut shells and fava beans; these may lead to anaphylactic reactions or hemolytic anemia to allergic and G6PD-deficient individuals, respectively. Less than 10% of orally ingested flavonoids are absorbed (Passamonti et al. 2009; Thilakarathna and Rupasinghe 2013) and are extensively metabolized to inactive ingredients in the liver (Chen et al. 2014b), primarily through glucuronidation, methylation and sulfation (Hollman et al. 1995; Hollman and Katan 1997). Therefore, flavonoids (especially, over 1000 mg/day) must be used with caution when administered with other natural polyphenolic molecules (e.g. curcumin, resveratrol) or drugs metabolized by the liver as they may affect the blood levels of themselves or of other drugs (Theoharides and Asadi 2012).

A methylated luteolin analogue (6-Methoxyluteolin) was shown to inhibit IgE-stimulated histamine release from human basophilic KU812F (Shim et al. 2012). Moreover, we recently showed that tetramethoxyluteolin is a more potent inhibitor of human MCs than luteolin (Weng et al. 2014). Methylated flavonoids are less likely to affect liver metabolism, are more stable (Walle 2007) and have better bioavailability (Wei et al. 2014).

40.5.8 Other Immune Modulators

Intravenous immunoglobulin G (Ig) is often used when one suspects an autoimmune component and all other treatment approaches have failed (Wong and White 2015). A few case studies showed significant benefit (Wong and White 2015a; Gupta et al. 2010; Plioplys 1998; Giudice-Asch et al. 1999; Melamed et al. 2015). Nevertheless, Ig may be helpful especially in children with frequent infections or Pediatric Autoimmune Neuropsychiatric Disorders associated with Streptococcal Infections (PANDAS).

40.5.9 Chelation

A recent comprehensive review concluded that pharmaceutical chelation was not effective and was associated with potential serious adverse effects (James et al. 2015).

40.6 Conclusions

Addressing allergic symptoms and anxiety, as well as reducing brain inflammation can provide significant benefit both for the core and ancillary symptoms of ASD. Luteolin analogues with better bioavailability and BDNF activity should be investigated further.

40.7 Review Questions

1. Do patients with ASD experience nonpsychiatric symptoms?
2. Does the risk for ASD increase with any maternal or childhood conditions?
3. Is there any evidence of brain inflammation in children with ASD?
4. Could mast cell activation contribute to ASD?

40.8 Answers

1. Up to 60% of patients have intolerance to foods (e.g. gluten, casein) allergies and gastrointestinal complaints, while 20–30% of patients have mitochondrial dysfunction, seizures or repeated infections.
2. There is strong statistical association with increased risk of ASD and maternal stress, psoriasis, asthma, allergies, use of psychotropic agents and excessive use of oxytocin for labor induction. There is also strong association with prematurity, small gestational weight, food intolerance and toxic exposures.
3. Biochemical and pathological studies have shown increased expression of the cytokines IL-6 and TNF as well as, the chemokines IL-8 and MCP-1. There is also increased proliferation and activation of microglia in key brain regions.
4. Mast cells both outside and inside the brain can secrete vasodilatory, inflammatory and neurosensitizing molecules that could: (a) disrupt the blood-brain-barrier, (b) activate microglia, (c) focal inflammation especially in the diencephalon.

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Toby K. Eisenstein and Thomas J. Rogers

Abstract

Opioids and cannabinoids have been shown to modulate many different parameters of immune responses. In general, opioids and cannabinoids have been found to be immunosuppressive, both in vitro and in vivo in a variety of assays, including response of lymphocytes to mitogens, antibody formation, activation of T-cells, Natural Killer Cell activity, and phagocytosis by macrophages and neutrophils. Drugs in these two classes have also been shown to inhibit migration of cells from the circulatory system to areas of inflammation, including passage across the blood-brain barrier. Opioids and cannabinoids have both been shown to sensitize mice to a variety of infectious agents, which is commensurate with an immunosuppressed state. In specialized rodent models and in monkeys, opioids sensitized to HIV and SIV, possibly related to their ability to cause heterologous desensitization of chemokine co-receptors for these viruses. The literature covered in this chapter supports the existence of a robust neural-immune interaction.

Keywords

Cannabinoids • Chemokines • Cocaine • Cytokines • Immunosuppression • Interleukins • Mitogen • Morphine • Natural killer cell • Opioids • Th1/Th2 • Δ^9 -THC • Tolerance • Withdrawal

41.1 Introduction**41.1.1 Opioid and Cannabinoid Receptors are on Cells of the Immune System****41.1.1.1 Opioid Receptors**

There is now indisputable evidence of natural physiological connections between the neural and immune systems. Much of the evidence supporting this link has come from studies of

opioids and cannabinoids and their effects on immune responses. Opioid receptors were first demonstrated biochemically in brain in the early 1970s. Three distinct receptors were discovered in neural tissue and were designated mu, kappa, and delta (Simon 1991). Their respective natural ligands were found to be the neuropeptides, β -endorphin (predominantly mu), dynorphin (kappa) and methionine-enkephalin (delta) (Akil et al. 1984), although there is considerable cross-reactivity. Alkaloid opioids, including morphine and heroin, bind primarily, but not exclusively, to the mu receptor. Morphine is extracted from opium produced by the poppy plant, and heroin is synthetically diacetylated morphine. In vivo, the major metabolite of heroin is morphine. Most laboratory studies employ morphine because it is less lipophilic than heroin, is used therapeutically, and is easier to dissolve. Since the mu receptor is the major target of both compounds, results obtained with morphine are believed to be directly translatable to heroin abuse, although there is some evidence for unique biologically active heroin metabolites (Rossi et al. 1996).

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Credit for recognition of the potential existence of receptors for opioids on cells of the immune system is given to Joseph Wybran in the laboratory of Govaerts. In 1979 he reported modulation of the function of human T-cells, purified from normal peripheral blood, by exogenous and endogenous opioids (Wybran et al. 1979). He observed that if morphine was added to human T-cells in culture, they lost the ability to rosette with sheep red blood cells (SRBCs), a fortuitous property that was used before flow cytometry to enumerate T-cells. In contrast, when the opioid neuropeptide, met-enkephalin, was added to the T-cells, rosetting increased. The actions of both opioids were blocked by naloxone, an antagonist for all types of opioid receptors, showing pharmacological specificity of the effects. Wybran proposed that, "normal human blood T lymphocytes bear surface receptor-like structures for morphine and methionine-enkephalin". He concluded that, "Such findings may provide a link between the central nervous system and the immune system." This paper was seminal because it presented strong pharmacologic data to support the existence of opioid receptors on cells of the immune system. However, it has been very difficult to demonstrate opioid receptors routinely by binding studies using cells of the immune system, although scattered reports of positive results are published (Sharp et al. 1998; Sibinga and Goldstein 1988). The difficulties may be due to low receptor numbers on cells of the immune system, to inability to obtain enough purified subsets of immune cells to do adequate binding studies, or to a need to activate immune cells before the receptors are expressed. With the advent of cloning of the opioid receptors from brain tissue, mRNA transcripts have been detected in primary cells of the immune system or lymphoid or monocytic cell lines for all three classes of opioid receptors (Loh and Smith 1990; McCarthy et al. 2001; Sharp et al. 1998).

41.1.1.2 Cannabinoid Receptors

Cannabinoid receptors were identified and cloned several years after the opioid receptors. Cannabinoids, like the psychotropic active ingredient extracted from marijuana, Δ^9 -tetrahydrocannabinol (Δ^9 -THC), were initially believed by some to simply interdigitate into cell membranes. Subsequently, a cannabinoid receptor was cloned from neural tissue (Zhu et al. 1993). When lymphoid tissue was probed for cannabinoid receptor transcripts, a second receptor (designated CB2) was discovered that is primarily expressed in the immune system and has only 44% homology to the first receptor (designated CB1) (Munro et al. 1993). Cells of the immune system express predominantly CB2 receptors, although mRNA for CB1 had also been detected. The brain expresses predominantly CB1 receptors, although there are a few reports of CB2 expression and functional activity. mRNA for CB2 is expressed by B-cells>natural killer cells>monocytes>neutrophils>T-cells in rat, mouse, and human tissues

and cells (Bouaboula et al. 1993; Galiegue et al. 1995). Endogenous cannabinoid ligands have also been discovered, but they are not peptides like the opioids. Anandamide, or arachidonylethanolamide, is a polyunsaturated N-acylethanolamine (Devane et al. 1992), as is 2-arachidonoyl-glycerol (Di Marzo and Deutsch 1998; Mechoulam et al. 1995). Radiolabeled cannabinoids have been shown to bind to mouse spleen cells and human T-cell lines (Kaminski et al. 1992; Schatz et al. 1997). Δ^9 -THC and endogenous cannabinoids all bind to both CB1 and CB2 receptors. However, it is becoming increasingly apparent, using selective CB1 and CB2 antagonists, as well as cannabinoid receptor knock-out mice, that most of the immunomodulating effects of these compounds on the immune system are mediated via the CB2 receptor.

41.1.1.3 Summary

It is now widely accepted that immune cells express receptors for opioids and cannabinoids. This conclusion is based on an impressive body of literature demonstrating immune modulation by exogenous and endogenous opioids and cannabinoids. Evidence from in vitro experiments suggests that effects of these drugs on the cells of the immune system occur, at least partially, by binding of opioid and cannabinoid receptors on these cells. Cocaine has also been found to affect immune function, but the mechanisms have not been defined. Other commonly used and abused drugs include nicotine and alcohol, both of which have marked effects on the immune system, and are frequently part of the panoply of artificial substances used by addicts. In addition, methamphetamines can alter immune status. Due to space limitations the reader is referred to key papers on these subjects (Molina et al. 2010; Soporì 2002).

41.1.2 Mechanisms by Which Drugs of Abuse can Affect Cells of the Immune System

Testing of whether drugs of abuse can alter immune function has been carried out by in vivo administration of the drug, with assessment of in vivo or ex vivo immune function, or alternatively, by harvesting immune cells from a drug naïve (normal) host and exposing them to the drug in vitro. If a drug is given in vivo, there is the possibility that effects on the immune system are not due to a direct interaction of the drug with cells of the immune system, but that immunomodulatory effects are instead mediated by additional systems in the body. For example, opioids, cannabinoids, and other drugs of abuse, can modulate the Hypothalamic-Pituitary-Adrenal (HPA) axis, which can lead to release of immunosuppressive corticosteroids or to activation of the sympathetic nervous (SNS) system, resulting in downstream alterations in immune function. Effects on immune function could thus

be a combination of indirect and direct effects if the drug is given *in vivo*. An additional and important consideration is that both the neural system and cells of the immune system produce opioid peptides, which could lead to complicated interacting pathways, especially *in vivo* (Carr 1991). In evaluating the studies discussed in this Chapter, it should be recognized that most immune responses were assessed 2–3 h after a single injection of a drug. For studies testing longer exposure to morphine, the drug is frequently delivered by subcutaneous implantation of a pellet that slowly releases the drug over a period of a week. Alternatively, mini-pumps dispensing drugs can be implanted. In the case of morphine or other addictive opioids, these methods are used to avoid episodes of withdrawal, which have their own immunomodulatory effects on immune function. In fact, a complication of studies with opioids is that repeated administration can result in tolerance to their analgesic effects, and in many, but not all, cases tolerance to the effects of the drugs on the immune system (Eisenstein et al. 2006). There are a limited number of studies that have addressed the effects of tolerance and of withdrawal from morphine on immune responses (Eisenstein et al. 2006).

41.2 Functional Consequences of Drugs of Abuse on the Immune System

41.2.1 Effects on Innate Immunity and the Inflammatory Response

41.2.1.1 Cells and Molecules of the Innate Immune Response

It is well established that drugs of abuse are involved in a wide range of immunomodulatory activities. Among the areas in immune system function in which these alterations have been observed is innate immunity. This arm of the immune system does not exhibit specificity in recognizing foreign pathogens. It encompasses Natural Killer (NK) cells and the ‘professional’ phagocytic cells that are the first responders to inflammation or infection. Natural Killer cells are lymphocytes that are involved in recognition and killing of mammalian cells that express foreign antigens, such as tumor antigens or viral antigens, or which fail to express histocompatibility antigens of the host. They also produce cytokines (small proteins produced by immune cells that act in an autocrine, paracrine, or endocrine fashion) that can activate the mononuclear phagocytic cells. The phagocytic cells engulf microbes invading the host and include neutrophils, monocytes, and macrophages. Monocytes are formed in the bone marrow and released into the peripheral blood. They are mature, but unactivated cells. When they are stimulated in various ways they can develop into macrophages *in vivo* or *in vitro*. Macrophages have several states of activation that

are characterized by their production of cytokines and by their levels of microbicidal compounds, including reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs). Macrophages also play an important role in the acquisition and presentation of foreign antigens to lymphocytes, which triggers the adaptive immune response.

41.2.1.2 Effects of Opioids, Cannabinoids, and Cocaine on Natural Killer (NK) Cells

In the cellular repertoire of the innate immune system, NK cells have the unique ability to kill infected target cells in a manner independent of antigen recognition. Alteration in NK cell function was among the earliest, and has been one of the most consistently observed, effects of drugs on the immune system. Many studies have shown that administration of opioids to rodents results in the suppression of NK cell activity. Leibeskind’s laboratory (Shavit et al. 1986a) was the first to show that morphine given to rats subcutaneously for 4 days suppressed NK cell activity. They later concluded that this effect was not directly on the NK cells in the periphery, because N-methylmorphine, which does not pass the blood-brain barrier, did not depress NK function (Shavit et al. 1986b). Subsequently, Weber and Pert (Weber and Pert 1989) reported that injection of morphine into the periaqueductal gray region of the brains of rats resulted in suppression of NK cell activity in the spleens of these animals 3 h later. Morphine administered into 5 other brain sites was without effect. Moreover, the opioid specific antagonist, naltrexone, blocked the effects in the studies by both groups, implicating the involvement of classic opioid receptors in modulating NK cell activity. Studies have also been carried out in which morphine has been given to normal human subjects under controlled conditions. The results show that peripheral blood NK cell activity is depressed 24 h after morphine administration (Yeager et al. 1995). The exact mechanism by which engagement of opioid receptors in certain regions of the brain alters peripheral functional capacity of NK cells is not known. However, more recent work implicates a neural pathway involving dopamine in modulating NK activity (Saurer et al. 2006). Nonetheless, the mu opioid receptor (MOR) is certainly involved in the effects on NK cell activity, as mice with a genetic deletion of MOR do not respond to morphine with depressed NK cell activity (Gavériaux-Ruff et al. 1998).

The cannabinoids represent another class of abused drugs affecting immune cell function. Although fewer studies have explored the effects of cannabinoids on NK cells, the literature to date shows that the activity of splenic NK cells is suppressed following *in vivo* injection of Δ^9 -THC into rats and mice (Klein et al. 1987; Patel et al. 1985), and also after *in vitro* treatment of human peripheral blood NK cells (Specter et al. 1986). Δ^9 -THC has also been shown to block proliferation of NK cells in response to the cytokine, IL-2, by down-regulating the IL-2 receptor (Zhu et al. 1993).

There are a limited number of reports of immunomodulation by cocaine. In vivo administration of cocaine i.v. to humans elevated NK cell activity in peripheral blood, but exposure of leukocytes to the drug in vitro had no effect (Van Dyke et al. 1986). It was hypothesized that the in vivo effects occurred via release of epinephrine from the SNS (Van Dyke et al. 1986). Contradictory results have been reported for the effect of cocaine on NK activity in rats, with no effect in vivo (Bayer et al. 1996) or suppression of activity after acute or chronic in vivo administration (Pacifici et al. 2003).

41.2.1.3 Drugs of Abuse Alter Functions of Neutrophils and Monocytes/Macrophages

Opioids given in vivo have been demonstrated to inhibit ex vivo phagocytic cell activity (Tubaro et al. 1983). Opioids selective for all three classes of opioid receptors (mu, kappa, and delta), when added to mouse peritoneal macrophages in vitro, inhibited phagocytosis of the yeast *Candida albicans*, and the effects were blocked by the respective receptor selective antagonists (Szabo et al. 1993). Others have shown that mu and delta agonists inhibited Fc-mediated phagocytosis of antibody-coated sheep red blood cells by mouse peritoneal cells exposed in vitro to morphine (Tomassini et al. 2004). These results showed that the activation of each of the opioid receptors resulted in reduced phagocytic activity. More recent studies have provided a mechanism by which phagocytosis is inhibited by morphine, namely through disruption of actin polymerization resulting from an increase in intracellular cAMP, activation of phosphokinase A and inhibition of Rac1-GTPase, and p38 mitogen-activated protein kinase (MAPK) (Ninković and Roy 2012). In addition, morphine given in vivo by slow release pellet, or when added to murine bone marrow cells in vitro, blocked the maturation of stem cells into more mature macrophages (Roy et al. 1991). Morphine pellets also inhibited adhesion of leukocytes to vascular endothelium, thus reducing inflammation (Ni et al. 2000). Further work has shown the morphine also inhibited the ability of phagocytic cells to produce superoxide and peroxide (Peterson et al. 1987a), two metabolites that have strong anti-microbial activity. Peterson and colleagues (Chao et al. 1992) have shown that the inhibitory effects of morphine on phagocytic cell activity are dependent on the production of Transforming Growth Factor (TGF)- β . Reactive oxygen intermediates (ROI), such as superoxide, contribute to the microbicidal activity of phagocytes, so impairment of their production would be consonant with reduced capacity to kill ingested yeast or other microbes. Macrophages taken from mice exposed to morphine can undergo apoptosis (Nair et al. 1997; Yin et al. 1999), and evidence supports a role of ROI and nitric oxide (NO) in this process (Bhat et al. 2004; Singhal et al. 1998). Usually depression of NO is associated with decreased macrophage function, so these findings are

dichotomous with the other evidence on effects of opioids on these cells.

Morphine treatment has been reported to result in reduced recruitment of both neutrophils and macrophages to growing tumors, using a murine Lewis Lung Carcinoma model system (Koodie et al. 2014). Chronic morphine administration has also been shown to inhibit the migration of neutrophils and monocytes that are a part of the process of wound healing (Clark et al. 2007; Martin et al. 2010a, b). The attenuated recruitment of neutrophils was attributed to morphine-induced inhibition of the expression of the chemokines CXCL3 and CCL2 (Martin et al. 2010a).

Δ^9 -THC is also reported to decrease the phagocytic activity of human peripheral blood monocytes and mouse peritoneal macrophages (Lopez-Cepero et al. 1986; Specter et al. 1991a). Long-term use of Δ^9 -THC in non-human primates has also been associated with depressed macrophage function (Cabral et al. 1991). Alveolar macrophages of marijuana smokers exhibited decreased microbicidal activity against *Staphylococcus aureus*, which was associated with decreased production of NO (Roth et al. 2004). Δ^9 -THC also affects macrophage processing of exogenous protein antigens through the CB2 receptor, as shown pharmacologically and using CB2-deficient mice (Buckley et al. 2000; McCoy et al. 1999).

Cocaine, like Δ^9 -THC and morphine, has been reported to decrease the antimicrobial activity of alveolar macrophages obtained from chronic crack cocaine smokers (Roth et al. 2004) and to decrease parameters of mouse macrophage activation (Ou et al. 1989; Pacifici et al. 1993). However, in contrast to the effects of opioids, cocaine has been shown to increase the activation of neutrophils in human subjects, as evidenced by increased killing of *Staphylococcus aureus* (Baldwin et al. 1997).

41.2.1.4 Effects of Drugs of Abuse on the Production of Cytokines and Chemokines

One mechanism by which drugs of abuse modulate immune function is through effects on expression of cytokines and chemokines, or on the functional capacity of their receptors. These proteins act as messengers to direct appropriate cellular activation and differentiation (cytokines) and coordinate the migration of cells to particular sites of inflammation or infection (chemokines). Cytokines have a number of functions. They may alter the innate immune response, act as a bridge to influence the type of adaptive immune response, and can also amplify adaptive immune responses. Chemokines can participate in innate immunity, and also influence adaptive immunity by orchestrating the type of cells that migrate to a pathological site. In addition, some of the chemokines can act as co-activators of immune cell function. Pro-inflammatory and anti-inflammatory cytokines, and also chemokines, which are part of the innate immune

response, mediate generalized symptoms of inflammation including fever (IL-1), organ damage (tumor necrosis factor (TNF)- α), and migration of phagocytic cells (IL-8, CCL2), or dampen these responses (IL-10).

Morphine and other ligands for the mu receptor have been shown to modulate the production of both cytokines and chemokines, although the results have been complex. For example, treatment of stimulated peripheral blood mononuclear cells with morphine decreased IFN- γ production (Peterson et al. 1987b), but increased TGF- β expression (Chao et al. 1992). Since TGF- β can be immunosuppressive, it is possible that the up-regulation of TGF- β may be responsible for the depressed IFN- γ expression. A biphasic dose-response relationship was shown for release of pro-inflammatory cytokines from stimulated mouse peritoneal macrophages treated with morphine in vitro, with increased release of IL-6 and TNF- α and reduced production at high doses (Roy et al. 1998b). In contrast, when mice were treated with relatively high doses of morphine in vivo and their peritoneal macrophages were stimulated ex vivo, they showed increased production of IL-12 and TNF- α (Maldonado et al. 1992).

The pattern of pro-inflammatory cytokines produced has implications for the character of the adaptive immune response that will occur if the host is exposed to a foreign antigen. A T-helper type 1 (Th1) cytokine profile polarizes the immune response towards a more pro-inflammatory cellular immune response, whereas a T-helper type 2 (Th2) profile favors certain types of antibody responses (Mosmann and Coffman 1987). IFN- γ is often considered a marker of a Th1 response, and IL-4 is indicative of a Th2 response. There is evidence that morphine given in vivo biases the immune response toward a Th2 response (Roy et al. 2004). In addition, recent studies of the murine immune response to *Acinetobacter baumannii* and to pulmonary *Streptococcus pneumonia* infection show that chronic morphine administration inhibits the development of T-helper type 17 (Th17) immunity, and decreases the production of the Th17-associated cytokines IL-17 and IL-23 (Breslow et al. 2011b; Ma et al. 2010; Wang et al. 2011). The consequences of this apparent shift toward Th2-type immunity are not clear, since other studies show that antibody production is suppressed in animals treated similarly with morphine (Bussiere et al. 1993). These results will be discussed in greater detail below.

In regard to chemokines, exposure of peripheral blood mononuclear cells (PBMCs) to DAMGO, a synthetic peptide selective for the μ -opioid receptor, has been shown to increase the expression of pro-inflammatory chemokines CCL2, CCL5, and CXCL10 (Wetzel et al. 2000), and the chemokine receptors CXCR4 and CCR5, the major HIV-1 co-receptors (Choi et al. 1999; Steele et al. 2003), the latter leading to increased replication of HIV. More recent work has shown that morphine and other μ -opioid agonists induce CCL2 expression by inducing NF- κ B activation (Happel

et al. 2011). It is difficult to reconcile some of these in vitro observations with the in vivo studies cited above showing that morphine inhibits migration of neutrophils and monocytes that are a part of the process of wound healing through inhibition of chemokine expression (Clark et al. 2007; Martin et al. 2010a, b). Thus it appears that opioids can exert both pro- and anti-inflammatory effects on the immune response.

A major and important finding concerning opioids and chemokines is that there is heterologous desensitization between certain opioid and chemokine receptors (Adler and Rogers 2005). Heterologous desensitization of G-Protein Coupled Receptors (GPCRs) is a process in which engagement of one receptor leads to the inactivation of a second receptor (in the absence of the agonist for the second receptor) to function. This process is mediated by initiation of a signaling pathway which leads to the cross-phosphorylation of the second receptor (Chen et al. 2004; Szabo et al. 2002, 2003), reviewed in (Steele et al. 2002). These studies show, for example, that activation of MOR leads to the cross-desensitization of CCR1, CCR2 and CCR5, and these cross-talk interactions are bi-directional (Fig. 41.1). Heterologous desensitization of chemokine receptors by opioids blocks chemotaxis of human peripheral blood mononuclear cells (Grimm et al. 1998; Szabo et al. 2002) and also blocks HIV infection of primary human monocytes in vitro (Szabo et al. 2003). Moreover, studies conducted in vivo show that pre-treatment of rats with CCL5 or CXCL12 in the periaqueductal gray (PAG) region of the brain, blocks the antinociceptive effects of morphine similarly administered (Szabo et al. 2002). These results are consistent with cross-talk between the chemokine receptors CCR5 or CXCR4, respectively, and MOR. More recent work has shown that at least 90% of neurons in the rodent PAG co-express CXCR4 and MOR (Heinisch et al. 2011; Patel et al. 2006). These results are very important since the process of heterologous desensitization requires that the receptors must be co-expressed in order for the cross-phosphorylation to take place (Steele et al. 2002). More recent work has demonstrated cross-talk between a number of chemokine receptors and MOR in the brain, and these results have potential clinical relevance since MOR plays an important role in the sensitivity to pain. (reviewed in Parsadaniantz et al. 2015).

There are striking parallels between the effects of opioids and cannabinoids on cytokine production. Δ^9 -THC has also been shown to suppress pro-inflammatory cytokines crucial in host defense, such as TNF- α (Fischer-Stenger et al. 1993). Further studies showed a biphasic dose response to cytokine production by Δ^9 -THC on human PBMCs, but it was the opposite of that observed with morphine, with low doses inhibiting TNF- α , IL-6 and IL-8, and high doses increasing these cytokines (Berdyshev et al. 1997). In a mouse model of Legionnaires' Disease, Δ^9 -THC blocked IFN- γ , IL-12, and

Opioid-Induced Heterologous Desensitization of CCR5

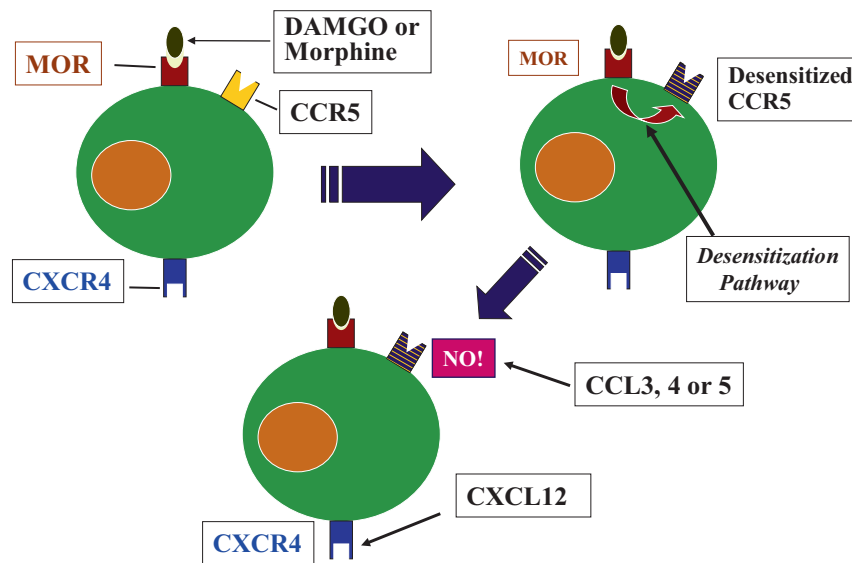


Fig. 41.1 Heterologous desensitization of chemokine receptors following activation of the MOR. The activation of MOR by either morphine or a MOR-selective agonist such as DAMGO leads to the induction of a cross-desensitization pathway which rapidly leads to the inactivation of some the chemokine receptors, including CCR5. At this point the cell is unresponsive to CCR5-selective chemokines such as

CCL3, 4 or 5. However, the MOR-induced cross-desensitization does not effect all chemokine receptors. For example, CXCR4 remains fully responsive to activation by the CXCR4-specific chemokine CXCL12. The cell is also protected from infection with an R5 strain, but not an X4 strain, of HIV-1

IL-12 receptor expression and increased IL-4 levels, reflecting a polarization of the immune response towards a Th2 phenotype (Klein and Cabral 2006; Perez-Castrillon et al. 1992). In an immune assay of graft rejection, Δ^9 -THC added into the cultures depressed IFN- γ production by human peripheral blood T-cells (Yuan et al. 2002). When lymph node cells of mice were stimulated with Staphylococcal enterotoxin B, histone methylation and acetylation patterns were consistent with up-regulation of Th2 cytokine genes and parallel down-regulation of Th1 genes (Yang et al. 2014). These results are interesting given the ability of Δ^9 -THC to induce elevated expression of the anti-inflammatory cytokines IL-10 and TGF β in tumor bearing mice (Zhu et al. 2000). Work with the endocannabinoid, anandamide, has demonstrated an inhibitory effect on the production of the pro-inflammatory cytokines IL-12 and IL-23, with a modest up-regulation of the anti-inflammatory cytokine IL-10 in microglial cells (Correa et al. 2009, 2010).

Like morphine, cannabinoids have also been shown to increase the mRNA for the chemokines CCL2, and also for IL-8, in a monocytic cell line transfected with the CB2 receptor (Jbilo et al. 1999). Further, the cannabinoids are reported to be chemotactic for macrophages (and microglial cells), which suggests that the CB2 receptor mediates activation of the cell migration machinery (Derocq et al. 2000; Walter et al. 2003). In contrast and somewhat anomalously, several reports have shown that treatment with Δ^9 -THC or CB2

agonists depressed the capacity of macrophages or microglial cells to migrate in response to certain chemokines, including CCL2, CCL3 and CCL5 (Fraga et al. 2011; Montecucco et al. 2008; Raborn et al. 2008; Romero-Sandoval et al. 2009). Treatment with high doses of CB2 agonists has been reported to attenuate the response of human T-cells to CXCL12 (Ghosh et al. 2006), and taken together these results suggest that CB2 cannabinoids can inhibit leukocyte traffic. This would be consistent with studies that have shown that CB2 agonists inhibit leukocyte adhesion to brain vascular endothelium, and reduce the migration of monocytes across the blood-brain barrier (Persidsky et al. 2015; Ramirez et al. 2012; Rom et al. 2013).

Cocaine is also reported to modulate the production of cytokines and chemokines. Analysis of in vitro cocaine treatment on cytokine and chemokine expression by mouse and human cells has revealed a decreased ability of stimulated immune cells to produce INF- γ , TNF- α , IL-1, and IL-8 (Shen et al. 1994; Watzl et al. 1992). Other studies did not agree, and reported an increase in TNF- α , IL-8, IL-2 and other chemokines (Wang et al. 1994; Zhang et al. 1998). Interestingly, in vivo infusion of cocaine showed an increase in IFN- γ and a decrease in the anti-inflammatory cytokine, IL-10 (Gan et al. 1998). The lack of concordance between in vitro and in vivo studies again suggests that drugs may have complex effects in vivo, partly because they can engage mediators from other systems that might affect immune function.

41.2.2 Effects on Adaptive Immunity

41.2.2.1 Humoral Immunity (Antibody Responses)

Morphine given by slow-release pellet has been shown to block the capacity of mouse spleen cells to mount an *ex vivo* antibody response to the artificial antigen, sheep red blood cells, and a naltrexone releasing pellet blocks the effect of the opioid (Bussiere et al. 1992). Serum antibody responses to tetanus toxoid (Eisenstein et al. 1990) and to trinitrophenylated bovine serum albumin (Weber et al. 1987) were also suppressed in mice by morphine given *in vivo*. Morphine, U50,488H (kappa agonist) and deltorphin II (delta₂ agonist) have all been shown to be immunosuppressive when administered by osmotic mini-pump. All three agonists gave inverted U-shaped dose response curves over a dose range of 0.1–10 mg/kg/day, with maximal immunosuppression between 0.5 and 2 mg/kg/day (Rahim et al. 2001). It has been suggested that part of the suppressive effect of morphine pellets on humoral immune responses is due to activation of the HPA-axis (Pruett et al. 1992). However, all of the effects of opioids on the capacity to mount an antibody response cannot be mediated, since spleen cells taken from normal animals that were never exposed to opioids *in vivo*, have depressed capacity to make antibodies to sheep red blood cells when treated with various opioid agonists *in vitro* (Eisenstein et al. 1995; Taub et al. 1991). Also, quaternary naloxone, which does not pass the blood-brain barrier, blocks the immunosuppressive effect of a kappa agonist given *in vivo* (Radulovic et al. 1995), suggesting that the drug is acting in the periphery. The suppressive effects of morphine and U50,488H *in vivo* and *in vitro* appear to result from individual effects on T-cells and macrophages (Bussiere et al. 1993; Guan et al. 1994) which are needed to cooperate with B-cells to generate antibody production. Morphine has also been shown to have an effect on the mucosal antibody response. Cultured ileal segments from mice receiving morphine and given cholera toxin orally were suppressed in their ability to produce IgA specific for cholera toxin compared with mice receiving placebo (Peng et al. 2001). The effect of chronic administration of morphine and withdrawal from the drug have been examined for effects on responses to a B-cell mitogen (LPS) (Bryant et al. 1987) and on antibody responses (Rahim et al. 2002). Exposure to morphine from slow-release pellets for 3 to 4 days led to tolerance to the immunosuppressive effects in both assays, and withdrawal led to renewed immunosuppression. Extensive investigation into the mechanisms of withdrawal-induced immunosuppression showed that there was a deficit of macrophage function (Rahim et al. 2003, 2005). In contrast, other data suggest that withdrawal biases the immune response to a Th2 cytokine phenotype (Kelschenbach et al. 2005). At present it is not known how to reconcile these observations.

Δ⁹-THC, like morphine, has been shown to suppress *in vitro* antibody formation by mouse spleen cells (Eisenstein et al. 2007; Kaminski et al. 1992, 1994). Interestingly, Δ⁹-THC is reported to increase IgG1 responses to *Legionella* in drug-treated animals, presumably by polarizing the immune response towards a Th2 phenotype (see below under T-cell responses) (Newton et al. 1994).

Cocaine has been investigated for capacity to alter antibody responses and has been found to be immunosuppressive for some antigens, but not others (Havas et al. 1987; Ou et al. 1989; Watson et al. 1983).

41.2.2.2 T-Cell Responses

Morphine given by slow-release pellet also inhibits the ability of splenic T-cells to respond to mitogens (plant products that cause nonspecific proliferation of immune cells). Thus, at 72 h after pellet implantation splenic murine T-cells had a marked inhibition of replication when exposed to Concanavalin A (Con A) (Bryant et al. 1987). Reduction in response to Con A has also been observed using peripheral blood T-cells of rats 2 h after a single subcutaneous injection of morphine (Bayer et al. 1990). Morphine has also been reported to inhibit thymocyte and lymph node derived T-cell proliferation when added to the cells *in vitro* (Roy et al. 1997; Wang et al. 2001). Other measures of T-cell function relate to adaptive immunity in the cellular arm of the immune system. Several studies have shown that chronic morphine exposure inhibits development of delayed-type hypersensitivity (DTH) in rats, mice, and pigs (Bryant and Roudebush 1990; Molitor et al. 1992; Pellis et al. 1986), and cytotoxic T cells in mice (Carpenter and Carr 1995).

Δ⁹-THC has also been shown to inhibit responses to Con A in a protocol which exposed mouse spleen cells *in vitro* to the drug (Klein et al. 1985). Further, Δ⁹-THC and CB2 selective agonists have been shown to inhibit T-cell responses to foreign tissues in an *in vitro* assay of graft rejection called the Mixed Lymphocyte Reaction (MLR) (Robinson et al. 2013). A major mechanism of this immunosuppression was found to be by inhibition of IL-2, a critical autocrine growth factor for T-cells (Robinson et al. 2013). The CB2 receptor has also been studied in murine models of a number of autoimmune diseases including Experimental Autoimmune Encephalitis (EAE), colitis, inflammatory bowel disease, and autoimmune uveoretinitis (Maresz et al. 2007; Singh et al. 2012; Storr et al. 2009; Xu et al. 2007; Zhang et al. 2009). Use of synthetic cannabinoids with selectivity for this receptor (as opposed to CB1) and use of CB2 receptor k/o mice demonstrated that CB2 activation has marked activity in depressing the T-cell responses in these autoimmune models. Other effects of the CB2 system were to depress T-cell trafficking by depressing adhesion molecule expression (Xu et al. 2007; Zhang et al. 2009) and by up-regulating the immunosuppressive cytokine, IL-10 (Storr et al. 2009).

Cannabinoids have been studied for their ability to polarize T-cells toward Th1 or Th2 responses. It has been reported that Δ^9 -THC given to mice that are subsequently infected with *Legionella* suppressed IL-12R β 2 expression by a CB1 mediated mechanism and increased GATA3 mRNA via CB2 signaling (Klein et al. 2004). The IL-12 receptor participates in Th1 responses and GATA3 biases towards a Th2 response, leading to the conclusion that cannabinoids can polarize T cell responses towards a Th2 phenotype. Evidence from exposure of activated human peripheral blood T cells to Δ^9 -THC also supports a Th2 polarization (Yuan et al. 2002). Δ^9 -THC has also been shown to suppress cytotoxic T cell activity against both allogeneic cells (Klein et al. 1991) and virus-infected cells (Fischer-Stenger et al. 1992).

An acute i.v. dose of cocaine has been reported to have an effect similar to that of morphine in suppressing proliferation of T cells in the peripheral blood of rats exposed to Con A 2 h after injection of the drug (Bayer et al. 1996). Cocaine has been shown to inhibit production of Th2 cytokines which would be expected to reduce antibody responses (Wang et al. 1994). Cocaine given orally also suppressed DTH responses in mice (Watson et al. 1983).

41.3 Effects of Drugs of Abuse on Infection

41.3.1 Overview

The majority of publications cited above concluded that the various drugs of abuse were immunosuppressive. A logical conclusion from these observations would be that exposure to the drugs would sensitize to infection. Clinical observations of intravenous drug using (IVDU) populations have long noted an increased incidence of bacterial infections (Honarvar et al. 2013; Risdahl et al. 1996). However, it has not been possible to determine whether more infection occurs in this group because of unsanitary use of needles or because the drugs render the person more susceptible. For example, there have been recent outbreaks of injection-associated anthrax and botulism in IVDUs in Europe, which have been attributed to needle contamination (Berger et al. 2014; MacDonald et al. 2013). This question was important in regard to Human Immunodeficiency Virus (HIV) infection, since in the United States, at the beginning of the epidemic, one third of all cases of HIV occurred in IVDUs (CDC 2000), and estimates suggest that approximately 20 % of global IVDUs are infected with HIV (Mathers et al. 2010; Vlahov et al. 2010). The current estimate in the United States is that 11 % of HIV cases occur in IVDUs (Spiller et al. 2015). Epidemiologic studies have been compromised because drug abusers usually abuse more than drug, and also smoke and abuse alcohol. Studies have not adequately teased apart these factors, nor controlled for drug dosage or fre-

quency of drug use. Several other reviews explore these issues in depth (Kapadia et al. 2005; Rogers et al. 2005). Laboratory studies using animal models of infections can more definitively clarify whether drugs of abuse can be cofactors in susceptibility to infection. Yet, surprisingly there are only a limited number of studies on drugs of abuse and experimental infections of any type.

41.3.2 Effects of Drugs of Abuse on Infections Other Than HIV

41.3.2.1 Effects of Morphine on Infection

There is clinical literature documenting increased incidence of infection in opioid abusers (Haverkos and Lange 1990; Hussey and Katz 1950; Louria et al. 1967; Scheidegger and Zimmerli 1989), and in a hospital setting where controlled doses of opioids were administered to burn patients (Schwacha et al. 2006). As mentioned in the introduction to this chapter, these correlations do not prove causality. In laboratory studies, morphine has been shown to sensitize mice to fungal, bacterial, parasitic and viral infections. In a seminal paper, morphine was shown to increase replication of the fungus, *Candida albicans*, apparently at least partly due to decreased phagocytosis and killing of the organism by neutrophils (Tubaro et al. 1983). Other early laboratory studies showed that morphine pretreatment led to a shock-like syndrome when mice were injected with the parasite, *Toxoplasma gondii* (Chao et al. 1990). In a murine model of *Leishmania donovani* infection, morphine was found to have a biphasic dose response effect, with low doses affording increased protection, and high doses sensitizing to infection (Singh and Singal 2007). Further, morphine and withdrawal from morphine were reported to induce sepsis through increased permeability of the intestinal epithelial barrier, and to enhance sensitivity to bacterial LPS (Hilburger et al. 1997; Roy et al. 1998a), as well as sensitize to oral *Salmonella* infection (Feng et al. 2005; MacFarlane et al. 2000). Morphine has been reported to increase the virulence of *Pseudomonas aeruginosa* in the mouse intestinal tract by a direct effect on the microbe (Babrowski et al. 2012). Epidemiology studies link use of opioid analgesics to increased risk of infection with *Clostridium difficile* (Mora et al. 2012). Thus, morphine seems to have a significant effect on intestinal microbes, possibly through its well known action as an inhibitor of gastrointestinal transit (Feng et al. 2006). Sensitization to infection could also occur by decreasing innate immune responses. As mentioned previously, morphine inhibits phagocytosis of *Candida*, which is one mechanism for potentiating infection (Szabo et al. 1993; Tubaro et al. 1983). Inhibition of neutrophil mobilization by morphine was shown to be a factor in sensitizing to infection with *Streptococcus pneumoniae* and *Acinetobacter*

baumannii infections (Breslow et al. 2011a; Ma et al. 2010). In the case of the pneumococcal infection, depression of the IL-17/IL-23 pathway by the opioid was found to be a mechanism for impaired neutrophil responses (Ma et al. 2010), but IL-17 was not found to be important in sensitization to *Acinetobacter* (Breslow et al. 2011b). For the pneumococcus, morphine was also shown to decrease macrophage signaling through Toll-like receptor-9 (TLR-9), which would dampen the protective inflammatory response (Wang et al. 2008, 2011). Several papers also support an effect of morphine on the adaptive immune response. Meningitis is a devastating manifestation of tuberculosis. Morphine has been shown to depress microglial functions that are necessary for initiating T-cell responses to *Mycobacterium bovis* (Olin et al. 2011). This opioid has also been shown to inhibit secretory antibody formation to cholera toxin in the gastrointestinal tract (Peng et al. 2001), which could be a mechanism of sensitization to gastrointestinal infections. Further, morphine was found to potentiate the development of Herpes simplex virus, type 1 encephalitis in a murine model of Herpes simplex virus, type 1 (Liroy et al. 2006), and to potentiated systemic infection with this virus by decreasing IFN- γ and IL-12 levels, and inhibiting cytotoxic T-lymphocyte responses (Mojadadi et al. 2009).

41.3.2.2 Effects of Δ^9 -THC on Infection

Δ^9 -THC has been shown to sensitize to bacterial, viral and parasitic infections. The first observations were in guinea pigs showing that the cannabinoid augmented infection with herpes simplex virus (Cabral et al. 1986). In addition, Δ^9 -THC administration has been shown to lead to greater viral replication and disease severity in murine models of Friend Leukemia Virus (Specter et al. 1991b), influenza infection (Buchweitz et al. 2007), vaccinia infection (Huemer et al. 2011) and Kaposi's sarcoma virus (KSV) (Zhang et al. 2007). At least one mechanism of viral potentiation was decreased influx of T-cells and macrophages into the lungs of influenza infected animals (Buchweitz et al. 2007). In contrast, treatment with Δ^9 -THC has been shown to depress the reactivation of KSV or Epstein Barr virus in an immortalized B-lymphocyte line (Medveczky et al. 2004). In regard to bacterial infections, Δ^9 -THC sensitized to *Listeria monocytogenes* (Morahan et al. 1979), and inhibited the adaptive immune response to *Legionella pneumophila*. It was found that if the cannabinoid was given 18 h prior to a primary sublethal infection, it sensitized to a secondary challenge with *Legionella* (Newton et al. 1994). Mechanistic studies showed that Th1 responses, which are necessary for host defense to *Legionella*, were depressed by Δ^9 -THC (see above section on cannabinoids and T-cell responses), and the antibody response was shifted from an IgG2a to an IgG1 isotype, indicative of a switch from a Th1 to a Th2 response (Klein et al. 2000; Newton et al. 2009). Δ^9 -THC has also been

reported to sensitize mice to encephalitis with the parasite, *Acanthamoeba castellanii*, by inhibiting the anti-microbial functions of microglia (Cabral and Marciano-Cabral 2004).

41.3.3 Effects of Drugs of Abuse on HIV or Related Infections

There are several papers showing that opioids up-regulate HIV replication when added to infected human peripheral blood cells or microglia in vitro (Li et al. 2002; Peterson et al. 1990, 1994). As mentioned above in Sect. 41.2.1.4. on cytokines and chemokines, the effects of opioids on HIV infection may be related to drug-induced alterations in cytokine or chemokine production or on expression of chemokine receptors (Li et al. 2002; Peterson et al. 1994; Steele et al. 2003). Proinflammatory cytokines, particularly TNF- α , can activate T-cells and lead to increased HIV replication, and chemokine receptors are co-receptors for the virus. It is to be expected that modulation of chemokine and/or chemokine receptor levels can alter HIV infectivity by increasing the pool of infectable cells or by altering availability of the number of co-receptors.

Similar immunodeficiency syndromes may result in monkeys and cats infected with SIV (simian immunodeficiency virus) and FIV (feline immunodeficiency virus), but studies with these viruses have not provided a clear indication of the influence of opioids on the development of these immunodeficiency diseases. In one study monkeys maintained on morphine had a slower progression of the SIV infection, although temporary withdrawal augmented viral load (Donahoe et al. 1993), but in two other studies, viral progression was more rapid (Chuang et al. 1997; Kumar et al. 2006). Neither acute or chronic morphine, nor morphine withdrawal, altered FIV infection in cats, but the number of animals was small and the variance in FIV load large (Barr et al. 2003).

Morphine administration has been reported to exacerbate the recruitment of leukocytes to the CNS during an inflammatory response (Olin et al. 2012). A number of studies have shown that opiate administration promotes the neuroinflammatory and neurodegenerative influences associated with HIV infection. Morphine promotes the cytotoxicity of HIV viral products by a variety of mechanisms including the up-regulation of proinflammatory cytokines, oxidative stress, and other processes (Ellis et al. 2007; Mahajan et al. 2008; Turchan-Cholewo et al. 2009). Furthermore, morphine exacerbates the response of astrocytes to HIV viral proteins, leading to an increase in the production of proinflammatory cytokines and chemokines which lead, in turn, to the recruitment of additional inflammatory cells to the CNS (El-Hage et al. 2005, 2006; Hauser et al. 2012).

Cannabinoids have also been investigated for their effects on HIV and related retroviruses. The humanized severe

combined immunodeficient mouse treated with Δ^9 -THC was found to have a 50-fold increase in viral load of an engineered HIV reporter virus (Roth et al. 2005). Studies using the SIV model have shown that chronic Δ^9 -THC depressed the progression of infection in male macaques (Molina et al. 2011). However, more recent studies from the same laboratory group showed that chronic Δ^9 -THC does not substantially alter the progression of SIV associated disease or viral replication in female macaques (Amedee et al. 2014). The basis for the disagreement in the results with the male and female animals remains uncertain at this time. Several studies have tested the effect of CB2 selective cannabinoids on HIV replication in vitro and have shown that these agonists reduced the replication of HIV in both human T-cells and macrophages (Costantino et al. 2012; Ramirez et al. 2013). Evidence was presented to support a mechanism in the T-cells whereby the CB2 agonist reduced F-actin and blocked viral integration (Costantino et al. 2012). It is interesting that a cannabinoid should be found to interfere with actin polymerization pathways, because, as noted previously in this review, a similar observation has been made for morphine (Ninković and Roy 2012). In an in vivo study, a CB2 selective agonist was found to be protective against HIV encephalitis using a humanized SCID mouse model of intra-cranial infection (Gorantla et al. 2010). The CB2 selective cannabinoid blocked T-cell infiltration into the brain by down-regulating adhesion molecules and altering the blood-brain barrier.

Cocaine has also been investigated in regard to effects on HIV infection and found to potentiate replication in in vitro systems (Bagasra and Pomerantz 1993; Peterson et al. 1992). Cocaine has also been shown to up-regulate DC-SIGN, a molecule on the surface of human dendritic cells that can act as a receptor for HIV (Nair et al. 2005). In addition, it has been reported that mice with severe combined immunodeficiency, that were reconstituted with human leukocytes and infected with HIV, demonstrated enhanced replication of the virus after cocaine treatment (Roth et al. 2002). However, a recent report using the SIV model shows that chronic cocaine administration results in minimal effects on viral replication, neuroinflammation including inflammatory cytokine mediator expression, or neurodegeneration (Weed et al. 2012). A more complete accounting of the impact of cocaine abuse on HIV pathogenesis is clearly warranted.

41.3.4 Summary

The fact that opioids, cannabinoids, and cocaine can alter replication of HIV in cell culture in vitro certainly suggests that the drugs should be able to alter HIV progression in vivo. Isolation of variables in the laboratory permits elimination of confounding factors in epidemiologic studies in humans,

such as poly-drug abuse, and also standardizes drug dosage. Thus, there is considerable evidence from laboratory studies supporting a role for drugs of abuse in potentiating HIV infection. There are, however, some inexplicable contradictions in the literature showing that opioids and cannabinoids in general inhibit immune cell migration, yet seem to potentiate HIV infection in the brain.

41.4 Synopsis

It has been clearly demonstrated that opioids, cannabinoids, and cocaine alter a variety of assays of immune function when added to cells of the immune system in vitro. Receptors and/or mRNA for the receptors for opioids and cannabinoids have been demonstrated in a variety of cells of the immune system. In many cases, especially for the opioids, pharmacological specificity of the action of the drugs has been verified using appropriate antagonists. For the cannabinoids, mice with genetic defects in CB1 or CB2 receptors have been used. These observations provide a biological basis for concluding that drugs of abuse have direct effects on immune cells. In addition, all three drug classes alter immune responses of rodents, and in some cases, humans, when treated in vivo. In vivo mechanisms of action may be harder to determine as the drugs may activate other physiologic systems in the body that may also impinge on immune responsiveness, such as the hypothalamic-pituitary axis and the sympathetic nervous system. Nonetheless, drug abusers suffer the cumulative effects of direct and indirect effects of the drugs on the immune system. Assays of immune function that have been measured include effects on the innate immune system, which encompass effects on neutrophils, macrophages and natural killer cells, and effects on adaptive immunity, which include the humoral and cellular arms of the immune system. Considerable attention has been focused on cytokine and chemokine mediators of immune function that are modulated by the drugs and on their capacity to modulate activation of the various arms of the immune system by polarization of the cytokine profile.

In general, opioids and cannabinoids have been found to be suppressive in a wide variety of assays of immune responsiveness. The literature on cocaine is smaller and less consistent. An extension of these observations is the assessment of effects of the drugs on resistance to infection, a consequence of alterations in immune function. Both opioids and cannabinoids have been shown to sensitize animals to a variety of experimental infections. Interest in the effects of the opioids on HIV progression is sparked by the epidemiological intersection of intravenous drug abuse and incidence of HIV. In vitro studies give robust results showing inhibition of HIV replication in the presence of opioids. Studies with Δ^9 -THC and cocaine, using an immunodeficient mouse strain

repopulated with human lymphoid cells, showed enhanced replication of HIV in the presence of the drugs. The implications of the research are that drug abuse increases vulnerability to infection. Together, the vast majority of the literature suggests substantial effects of drugs of abuse on immune competence in general, resulting in immunosuppression. However, there are several studies showing that opioids increase chemokine production, which would increase inflammation. At present, these aspects of the literature have not been reconciled. Taken as a large overview, the studies reviewed in this chapter reinforce the existence of physiological pathways between the neural and immune systems mediated by opioids and cannabinoids.

41.5 Review Questions

- It is observed that morphine given subcutaneously suppresses responses of spleen cells put into tissue culture with the T-cell mitogen Concanavalin A (Con A).
 - Mice with a disruption of the gene coding the mu opioid receptor (Mu Opioid Receptor knock-out mice), would not show suppression to Con A.*
 - Morphine acts mainly through the kappa opioid receptor.
 - Morphine is mainly metabolized to heroin when it is injected in vivo.
 - Methyl-morphine could not be used to determine if peripheral receptors or brain receptors are involved in the immunosuppression.
 - None of the above.
- In regard to cannabinoids:
 - They are proteins.
 - They exert their effects primarily by interdigitating into cell membranes.
 - There is evidence that they polarize the immune response towards a Th1 type phenotype.
 - There is evidence that they polarize the immune response towards a Th2 type phenotype.*
 - They have no direct effects on cells of the immune system.
- In regard to the immune system, morphine has been shown to:
 - Elevate antibody responses to various antigens.
 - Elevate responses to the B-cell mitogen, lipopolysaccharide (LPS).
 - Elevate delayed-type hypersensitivity (DTH) responses.
 - Increase phagocytosis by macrophages.
 - Depress natural killer (NK) cell activity.*
- Cocaine:
 - Has no effect on the immune system.
 - Exacerbates HIV replication in a mouse/human model of viral replication.*
 - Binds to the delta opioid receptor.
 - Depresses IL-2 production.
 - All of the above.
- In regard to infections and drugs of abuse:
 - Morphine sensitizes mice to infection with *Legionella pneumophila* (Legionnaire's Disease).
 - Δ^9 -THC sensitizes mice to *Salmonella* infection.
 - Morphine and Δ^9 -THC have direct effects on the ability of bacteria to grow in broth cultures.
 - Morphine can affect resistance to infection by altering the activity of phagocytes.*
 - Intravenous drug abusers have a rate of infection the same as the general population.
- Morphine most probably affects the progression of HIV infection by all of the following EXCEPT:
 - Changing the level of chemokine receptors.
 - Changing the level of chemokines.
 - Causing T-cells to undergo uncontrolled cell division.*
 - Mediating heterologous desensitization of chemokine receptors.
 - Altering cytokine levels.
- Which of the following statements is true:
 - Opioids, cannabinoids, and cocaine uniformly suppress the activity of NK cells.
 - The use of N-methylmorphine substantiated the direct effects of opioid-induced NK cell suppression.
 - Mice lacking the μ -opioid receptor do not have suppressed NK cell activity in response to morphine treatment.*
 - Δ^9 -THC prevents NK cell proliferation by inhibiting the secretion of TGF- β
 - Morphine inhibits NK cell activity by inducing endogenous cannabinoid levels.
- Opioids modulate phagocyte activity by:
 - increasing phagocytic uptake of bacteria.
 - decreasing apoptosis of phagocytic cells.
 - enhancing maturation of bone marrow cells into macrophages.
 - decreasing uptake of microbes.*
 - decreasing maturation of lymphocytes in the bone marrow.
- Immune cells treated with morphine:
 - Uniformly have markers of activation.
 - Display altered levels of pro-inflammatory chemokines, but chemokine receptor numbers remained unchanged.
 - Display bi-directional heterologous desensitization between opioid receptors and certain chemokine receptors.*

- (d) Clearly have inhibition of Th2 cytokine responses.
 (e) None of the above
10. Which of the following statements is true:
 (a) Δ^9 -THC induces its effects through both CXCR4 and CCR5.
 (b) *Both Δ^9 -THC and morphine have been shown to induce the expression of pro-inflammatory chemokines, such as MCP-1 and IL-8.*
 (c) Like cannabinoids and morphine, cocaine treatment increases TNF- α and IL-1.
 (d) Cocaine uniformly suppresses cytokine and chemokine production when given in vivo or applied to cells of the immune system in vitro.
 (e) Opioids have no effect on antibody formation.
11. Morphine and/or DAMGO treatment results in decreases in which of the following?
 (a) Apoptosis
 (b) Pro-inflammatory chemokine production
 (c) *Antibody responses*
 (d) TGF- β
 (e) All of the above
12. Inhibition of T cell responses is observed for which of the following drugs of abuse?
 (a) Morphine
 (b) Δ^9 -THC
 (c) Cocaine
 (d) *All of the above*
 (e) None of the above
13. The initial evidence suggesting that opioid receptors are expressed by cells of the immune system was:
 (a) *Results showing impairment of T-cells to rosette to sheep red blood cells in the presence of morphine.*
 (b) studies examining functions of the CB1 and CB2 receptors.
 (c) primate studies examining disease incidence in self administration of heroin
 (d) biochemical analyses of lymph nodes in heroin addicts.
 (e) studies showing increased HIV infection in heroin abusers.
14. All of the following statements are true EXCEPT?
 (a) In vitro studies have shown Δ^9 -THC biases the immune response towards a Th2 cytokine profile.
 (b) *Cocaine administration stimulated production of the anti-inflammatory cytokine IL-10, while simultaneously suppressing IFN γ .*
 (c) Activated human PMNs show enhanced functional activity in response to cocaine.
 (d) The effects of opioids, cannabinoids, and cocaine on immune system function are compounded by the involvement of the HPA axis.
 (e) Chemokine expression is induced by morphine administration.
15. Evaluation of the effects of drugs of abuse can be complicated by a number of factors, including:
 (a) The effects of the sympathetic nervous system on immune cell function.
 (b) The effects of the HPA axis on immune cell function.
 (c) The capacity of cells of the immune system to produce endogenous opioids.
 (d) The production of endogenous cannabinoids in the periphery.
 (e) *All of the above.*
16. The function of G protein-coupled receptors, such as the opioid and cannabinoid receptors, can be regulated by the process of heterologous desensitization. Which of the following statements is true about this regulatory mechanism?
 (a) This process occurs when the activation of one receptor leads to an increased expression of a second receptor.
 (b) This process only occurs between receptors expressed on the surfaces of adjacent cells.
 (c) This process cannot occur between receptors expressed on the same cell.
 (d) *This is a process which appears to involve transphosphorylation of G protein-coupled receptors.*
 (e) All of the above.
17. Both opioids and cannabinoids have been shown to sensitize animals to a variety of experimental infections. The impact of drugs of abuse on resistance of humans to infectious agents can be difficult to evaluate because:
 (a) Drug abuse rarely involves the administration of a single drug, and the effects of poly-drug abuse are poorly understood.
 (b) Drug abusers are exposed more frequently to pathogenic agents than non-abusers.
 (c) A number of additional factors, which are hard to control, impact on measurement of the immune competence of drug abusers, including the dose of the drug and the time since it was last taken.
 (d) The contributions of legal drug use, including nicotine and alcohol, can complicate the effects of illegal drug abuse.
 (e) *All of the above.*
18. All of the following influence the effect of opioids on susceptibility to HIV infection EXCEPT:
 (a) Opioids alter the expression of chemokine receptors that are co-receptors for HIV-1.
 (b) Opioids increase the expression of some chemokines which may promote the attraction of additional susceptible target cells for HIV infection.
 (c) Opioids increase the expression of chemokines which, for an individual cell, may block viral replication by blocking the chemokine coreceptor.

- (d) *Opioids alter the phagocytic activity of neutrophils, and this would be expected to significantly alter the kinetics of the infection.*
- (e) *Opioids would be expected to alter the expression of cytokines that may, in turn, alter the replication rate of the virus in monocytes and T-cells.*

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Part III

Therapies and Diagnostics

Kristi M. Anderson and R. Lee Mosley

Abstract

Until the 1990s, progress in developing therapy for neurodegenerative diseases was slow and there were few clinical trials. However, with the advent of animal models and the recent advances in understanding the basic pathophysiological mechanisms underlying the diseases, potential therapies to prevent, delay the onset or slow the progression of neurodegenerative disease are being identified at an ever increasing rate. High throughput technologies are being used to screen large numbers of potential therapeutic agents and new developments in the realms of immune modulation, RNA interference, viral vector delivery of gene products and stem cell therapy hold great promise for the future. There are still many unanswered questions regarding the mechanisms of disease and why beneficial therapy in animal models has not translated well into human clinical trials. Neurodegenerative diseases are rare, and so identifying enough patients to obtain studies with adequate power has been difficult. Novel phase II designs are now being used to screen greater numbers of agents and to better define correct dosing before proceeding to phase III trials. Multicenter phase III trials are being designed with adequate power, and using meaningful validated outcome measures that reduce the high dropout rates of past trials. Agents are now being tested in combination in order to detect possible additive effects. Focus is also being given to the need to better define the best symptomatic therapies in randomized controlled trials. Further research will prompt more targeted therapies that we hope will soon provide truly meaningful breakthroughs for neurodegenerative disorders.

Keywords

Alzheimer's disease • Amyotrophic lateral sclerosis • Animal models • Apoptosis • Clinical trials • Combination therapy • Dementia • Huntington's disease • Inflammation • Neurodegeneration • Neuroprotection • Parkinson's disease • Randomized controlled trials • RNA interference • Stem cell therapy • Symptomatic therapy • Therapeutics • Vaccination

42.1 Introduction

The neurodegenerative disorders, which include amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD), are clinically heterogeneous. Medications can ameliorate some symptoms, but none reverse the relentless progression of the illnesses. The past several decades have seen dramatic increases in the understanding of the complex pathophysiology

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underlying the diseases. Overlapping features at the cellular level provide clues for targets of therapeutic interventions. While the disorders can now be studied using *in vitro* and animal models, the complex biochemistry, pathology and lack of mechanism-based treatments pose a particular challenge. With few unequivocal neuroprotective agents, we await the major breakthrough that will provide truly meaningful clinical improvement. Until then, numerous symptomatic therapies improve the quality of life for those suffering from neurodegenerative diseases. This chapter outlines the current strategies for the use of symptomatic therapies and the development of neuroprotective treatment for neurodegenerative disorders.

42.1.1 Overview of Mechanism

Progressive and premature neuronal cell death is a common feature among the neurodegenerative disorders. ALS involves progressive degeneration of the motor neurons in the brain and spinal cord. AD shows selective neuron degeneration in the hippocampus and cerebral cortex. PD is caused by degeneration of pigmented dopaminergic neurons in the substantia nigra. HD has selective neuron loss in the striatum and cortex. It is increasingly recognized that the clinical syndromes may overlap, with features of two or more disorders co-existing. For instance, ALS and dementia, or ALS, parkinsonism, and dementia may occur together in the same family or in an individual. Similarly, the disorders have common pathological findings at the cellular level. While the mechanisms that lead to neuronal degeneration are still sought, in general, neurodegenerative diseases are thought to be caused by a combination of events associated with factors of age, genetics, and environment; the causality of each varying proportionately (Koo and Kopan 2004; Rowland and Shneider 2001). All cases of HD, but only rare cases of ALS and AD are due to single genetic mutations. In sporadic forms, complex genetic interactions may render an individual susceptible to some as yet unknown environmental exposure, with the combination necessary to initiate the disease. Whatever the cause, be it genetic, environmental or some combination of the two, a cascade of events at the cellular level is set in motion that ultimately results in cell death. Animal models of the genetic forms of the disorders have helped elucidate some of the underlying molecular mechanisms that contribute to neuronal demise. Common processes among the neurodegenerative diseases include oxidative stress, excitotoxic injury, altered metal homeostasis, aberrant protein aggregation, mitochondrial injury, apoptosis, and inflammation (Andersen 2004; Bossy-Wetzel et al. 2004; Ross and Poirier 2004). With better understanding of mechanism(s) of disease

etiology and progression, more effective treatments may follow. Until then, a major goal is to identify therapies that slow the course of the illnesses by targeting cellular changes that occur after the inciting event.

42.1.2 Overview of Animal Models

The inherited forms of neurodegenerative diseases have been utilized in the development of animal models. Rodent models created using transgenic and gene targeting technologies (Deng and Siddique 2000) provide the opportunity to better study disease pathology and screen potential therapeutic agents. The advent of the ALS mouse model is one example of the progress that has resulted from this technology. A mutation in the gene encoding the enzyme copper-zinc superoxide dismutase (SOD1) on chromosome 21 was identified as a causative mutation in one form of familial ALS (Rosen 1993). The first mutant transgenic mouse line was created by over-expressing high levels of mutant SOD 1 (G93A). This mouse developed a phenotypic and pathologic condition similar to human familial and sporadic ALS (Gurney et al. 1994). The murine model of ALS has since been used to screen a variety of therapeutic agents. Riluzole, the only approved agent for ALS, yielded positive results in the model that correspond very closely to the modest benefit in human clinical trials (Gurney et al. 1996, 1998).

Similarly, mouse models have been created for other neurodegenerative diseases. Transgenic mouse models of AD express mutations for the amyloid precursor protein (APP), presenilin genes, or a combination of the two (German and Eisch 2004). Insight into the molecular mechanisms of dopaminergic cell death in PD have come from the study of 6-hydroxydopamine (6-OHDA) and 1-methyl 1-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) animal models (Dawson 2000). Discovery of genes linked to familial PD have also provided the opportunity to generate animal models that better recapitulate the phenotypic and pathologic features of PD. Similarly, animal models have been created for HD through utilization of CAG triplet expansion in the HD gene. Homozygous and heterozygous knockout mice did not produce phenotypic or pathologic features of HD, but transgenic mouse models of HD produced a progressive neurological phenotype that resembles features of HD and has improved the understanding the disease process (Deng and Siddique 2000).

Many of the past and current therapeutic studies have targeted slowing neurodegenerative processes in animal models. While models of human diseases have played an essential role in understanding the mechanisms involved in neurodegeneration and in developing therapeutic strategies, the translation of findings in animal models to human therapeutics

has been fraught with difficulty. Twenty years after the approval of riluzole and following numerous clinical trials based on findings in the model, we still await the second unequivocally positive trial in ALS. This suggests that human disease processes are seemingly more complex than those otherwise transferred to rodents. Murine models may best be used for establishing the scientific justification for testing a potential agent, with the knowledge that findings in the laboratory, as yet, cannot provide certain hope of success in human trials.

42.2 Disease Specific Therapy

In certain disorders, such as AD and PD, neurodegeneration leads to deficiencies in specific neurotransmitters. Therapy has been targeted at replacing or increasing levels of the deficient neurotransmitter in patients. Disease specific therapy that targets neurotransmitter replacement or inhibition of neurotransmitter loss does not alter the underlying progression of neurodegeneration, but may improve symptoms, at least temporarily.

42.2.1 Alzheimer Disease

Changes in the cholinergic system in AD were first studied by Whitehouse *et al.* in 1981, who found atrophy of cholinergic nerve cells in the substantia innominata (Whitehouse *et al.* 1981). It was thought that replacing or increasing acetylcholine in patients with AD could stabilize cholinergic transmission. The cholinergic hypothesis of AD has been the basis of many research efforts and the accumulated evidence has launched the development of a variety of compounds to improve cholinergic neurotransmission (Lancot *et al.* 2003; Scarpini *et al.* 2003). Currently, the cholinergic deficiency is considered to be a secondary event resulting from nerve cell death due to other causes (Albers and Beal 2000).

42.2.2 Cholinesterase Inhibitors

Cholinesterase inhibitors, developed with the goal of stabilizing cholinergic transmission, block the enzyme that degrades acetylcholine in the synaptic cleft and lead to increased levels of acetylcholine in the brain. Three cholinesterase inhibitors currently are available in the United States: donepezil, rivastigmine and galantamine (Table 42.1). Donepezil, a piperidine derivative, is a selective inhibitor of acetylcholinesterase that noncompetitively and reversibly blocks acetylcholinesterase.

Individually, the cholinesterase inhibitors improve memory in patients with AD. Although all three drugs inhibit acetyl-

Table 42.1 Disease specific and neuroprotective therapy in alzheimer disease

Medication	Dosage
<i>Cholinesterase Inhibitor</i>	
Donepezil (Aricept)	5–10 mg QD
Rivastigmine (Exelon)	3–6 mg BID
Galantamine (Razadyne)	8–12 mg BID
<i>NMDA Antagonist</i>	
Memantine (Namenda)	10 mg BID

cholinesterase, they differ in additional mechanisms of action, pharmacokinetics, and side effects (Hogan and Patterson 2002). Both rivastigmine and galantamine have additional mechanisms of action; galantamine may induce a stronger response by acting as an allosterically potentiating ligand of nicotinic acetylcholine receptors, making the remaining nicotinic acetylcholine receptors more sensitive. Additionally, it is believed to increase the presynaptic release of acetylcholine and prolong postsynaptic neurotransmission (Greenblatt *et al.* 1999; Scott and Goa 2000). Rivastigmine, a slow reversible inhibitor of acetylcholinesterase and the only of the three drugs that also inhibits butyrylcholinesterase, may be more useful in later stages of disease when butyrylcholinesterase may provide greater functional importance (Hogan *et al.* 2004). Despite these differences, the primary known differences are the side effect profiles, titration schedules and the dosing regimens (Table 42.1). The most common side effects of treatment with cholinesterase inhibitors are initially gastrointestinal and include nausea, vomiting and diarrhea. Chronic use has been thought to increase the likelihood of gastric acid secretion and possible risk of gastrointestinal bleeding, especially for those with history of peptic ulcer disease or chronic use of non-steroidal anti-inflammatory drugs (NSAIDs). A recent retrospective study of over 97,000 cholinesterase inhibitor users and non-users showed the gastrointestinal bleeding event rate for users was 10.6 events per 1000 person-years compared to 9.6 events per 1000 person-years for non-users and was insignificant as determined by Cox proportional hazards model (Thavorn *et al.* 2014).

A meta-analysis of the efficacy and safety of these agents revealed that despite many clinical studies showing short-term benefit, there is insufficient evidence to conclude long term efficacy (Lancot *et al.* 2003). Head to head studies performed shed some light as to which cholinesterase inhibitor to use as initial therapy for patients with AD. Interpretation of the study results should be made with caution, but the conclusion was that galantamine should be favored over donepezil (Hogan *et al.* 2004). Ultimately, which cholinesterase inhibitor is used as a first-line drug must be made based on a patient by patient basis and many variables taken into account.

Table 42.2 Disease specific therapy in Parkinson' disease

Medication	Dosage
<i>Dopamine Precursor</i> Levodopa	300–3000 mg per day
<i>Dopa decarboxylase inhibitor/</i> <i>Dopamine precursor</i> Carbidopa/Levodopa	25 mg/300 mg–200 mg/2000 mg per day
<i>Dopamine Agonist</i> Bromocriptine Ropinirole Pramipexole Cabergoline Rotigotine transdermal patch	10–30 mg TID 3–8 mg TID 0.5–1.5 mg TID 2–10 mg per day 2–6 mg per day
<i>COMT Inhibitor</i> Entacapone Tolcapone	200–400 TID–6×/day 100–200 TID
<i>NMDA Antagonist/Anticholinergic</i> Amantadine	100–300 per day
<i>Anticholinergic</i> Trihexyphenidyl Benztrapine	2 mg TID–QID 1–4 mg QD–BID
<i>MAO Inhibitor</i> Selegiline	5 mg BID

42.2.3 Parkinson Disease

As in AD, knowledge of the neurotransmitter deficiency underlying PD (*i.e.*, dopamine) has been the basis for the development of therapy. Early studies showed that cerebral dopamine was concentrated in the striatum and that levodopa, the precursor to dopamine, could reverse the akinetic effects of the dopamine-depleting agent reserpine in experimental animals (Carlsson et al. 1957, 1958). Eventually, the identification of striatal dopamine depletion as a key neurochemical finding in parkinsonian brains lead to treatment with levodopa in humans and to the subsequent advent of compounds that mimic the effects of dopamine or prolong its action (Table 42.2).

42.2.3.1 Levodopa

Levodopa is the most effective drug for the symptomatic management of PD. Levodopa, which usually produces an excellent initial response in alleviating the motor symptoms of PD, is particularly effective for bradykinesia and tremor (Fahn 2000). However, side effects such as motor fluctuations and dyskinesias may limit its long-term usefulness for individual patients (Fahn 2000; Jankovic 2000). Up to 50 % of all patients treated for 5 years or more, develop instability in their motor response (Koller et al. 1999; Schrag and Quinn 2000). As a consequence, levodopa is usually given later in PD, particularly in younger patients who may require many years of anti-Parkinson therapy. In this group, dopa-sparing agents (see below) are usually prescribed until symptoms

begin to interfere with independence. Levodopa is often used as the first drug of choice in the elderly for whom low dose monotherapy is preferable to avoid side effects, and who may not be expected to develop motor fluctuations after years of therapy due to already advanced age. Immediate and controlled release preparations of levodopa are available.

Carbidopa, a peripheral aromatic-L-amino-acid decarboxylase (AADC) inhibitor, enhances the therapeutic benefits of levodopa (Opacka-Juffry and Brooks 1995). Levodopa is converted to dopamine by AADC, an enzyme that functions both inside and outside the brain. Peripherally produced dopamine directly stimulates the medullary vomiting center, which is not protected by a blood brain barrier, producing anorexia, nausea and vomiting. Carbidopa in doses less than 150 mg per day does not enter the brain and so blocks only the peripheral metabolism of levodopa by AADC (Cedarbaum et al. 1986). Co-administration of carbidopa and levodopa therefore has two main effects; to reduce levodopa-related gastrointestinal side effects, and ensure the majority of levodopa is converted to dopamine in the brain. Dosages of 75 mg/day of carbidopa are necessary to completely block peripheral AADC activity. Additional doses can be given to those who are particularly sensitive to levodopa-induced nausea. Other major side effects of levodopa therapy include orthostatic hypotension, chorea, dystonia, myoclonus, akathisia and hallucinations. Studies have not yet shown conclusively whether levodopa alters the underlying rate of neurodegeneration (Fahn 2005).

42.2.3.2 Catechol-O-Methyltransferase Inhibitors

Another strategy to enhance dopamine response utilizes the inhibition of catechol-O-methyltransferase (COMT), which metabolizes dopamine once converted from levodopa either within or outside the brain. Agents that block the degradation of dopamine by inhibition of COMT effectively prolong the half-life of dopamine and may increase peak concentration. The two currently available COMT inhibitors, entacapone (Holm and Spencer 1999; Rinne et al. 1998) and tolcapone (Tolcapone Study Group 1999; Kurth and Adler 1998), reduce motor off time when given with levodopa, but the efficacies of the two drugs have not been directly compared. Tolcapone has a theoretical advantage over entacapone in that it inhibits both peripheral and central COMT, and has a longer duration of action necessitating administration only three times per day. However, tolcapone can cause explosive diarrhea and abnormal liver function. Fulminant liver failure has occurred, albeit rarely, and therefore frequent monitoring of liver function is necessary in those prescribed tolcapone (Assal et al. 1998). Entacapone usually does not cause diarrhea or abnormal liver function and can be administered with levodopa up to eight times a day.

42.2.3.3 Dopamine Agonists

Dopamine agonists exert their effect by activating dopamine receptors and bypassing the presynaptic synthesis of dopamine. They are often used as part of a dopa-sparing strategy, in which agonists are prescribed early in PD, postponing the need for levodopa and the motor complications associated with exogenous dopamine administration. Activation of the dopamine-2 (D_2) receptors is important in mediating the motor effects of dopamine agonists, but to achieve optimal physiologic and behavioral responses, both D_1 and D_2 receptors must be stimulated (Brooks 2000).

Bromocriptine was the first dopamine agonist developed to stimulate D_2 receptors and inhibit D_1 receptors (Perachon et al. 1999). Dosage should be increased slowly to prevent orthostatic hypotension and gastrointestinal side effects. Because the compound is an ergot derivative, rare associations with retroperitoneal or pericardial fibrosis, including cardiac valvular disease have been reported (Van Camp et al. 2004).

In 1997, two additional dopamine agonists, ropinirole and pramipexole, were approved. These agents are nonergolines, and therefore may carry a lower risk of ulcerative, vasoconstrictive, or fibrotic complications (Shaunak et al. 1999). Pramipexole has been promoted as a D_3 agonist (Bennett and Piercey 1999), but a study that measured the affinities of bromocriptine, pramipexole, pergolide, and ropinirole for human recombinant dopamine D_1 , D_2 , and D_3 receptors found that all four compounds had high affinity for the D_3 receptor (Perachon et al. 1999). The observation that D_3 receptors are located primarily in the mesolimbic system may explain the possible antidepressant effect of pramipexole and ropinirole (Corrigan et al. 2000), but may also explain the occurrence of hallucinations noted particularly with higher doses of dopamine agonists.

Pramipexole has been shown to be safe and effective as monotherapy early in PD (Parkinson Study Group 1997, 2000) and in mild to moderate PD (Shannon et al. 1997). Ropinirole has also been shown to be effective in early PD (Korczyn et al. 1999; Rascol et al. 1998). In later stages of PD, dopamine agonists are usually prescribed with levodopa to achieve optimal therapeutic effects and to help moderate the motor fluctuations associated with levodopa (Pinter et al. 1999). Possible side effects of all dopamine agonists include nausea, peripheral edema, somnolence, hallucinations, and compulsive behavior (Driver-Dunckley et al. 2003).

Rotigotine, licensed in 1998, is commonly used for the treatment of PD and restless leg syndrome. Rotigotine is also the first transdermal patch for the treatment of PD. After being withdrawn in 2008 for problems with the delivery system, rotigotine was reintroduced in 2012 (Waters 2013). Pilot tests of 24 month duration to evaluate the efficacy and tolerability of newly formulated rotigotine in PD patients proved beneficial. Rotigotine not only improved UPDRS scores, but was well-tolerated and improved patient compliance (Moretti et al. 2014a, b).

42.2.3.4 Anticholinergic Agents

The manipulation of non-dopaminergic neurotransmitters can also be used as disease specific therapy in PD. Anticholinergic agents, one the first effective therapies for PD signs, having been prescribed by Charcot in the 19th Century, are particularly helpful in tremor reduction. The exact mechanism of anticholinergic medications in PD remains unclear. They likely counteract an imbalance between striatal dopamine and acetylcholine that results from degeneration of dopaminergic neurons (Katzenschlager et al. 2003). Medications with anticholinergic properties include amantadine, benztropine and trihexyphenidyl, among others. Amantadine may be the most commonly used anticholinergic medication in PD. In addition to its anticholinergic properties, amantadine promotes the release of dopamine and blocks the N-methyl-D-aspartate (NMDA) receptor, which may relieve levodopa-induced dyskinesias. Benztropine has anticholinergic properties and also blocks the reuptake of dopamine.

A meta-analysis of nine double-blind cross over trials found that anticholinergic therapy is effective as monotherapy or as an adjunct to other anti-Parkinson drugs (Katzenschlager et al. 2003). The most common side effects include dry mouth, constipation, and urinary hesitancy. These agents may occasionally exacerbate narrow angle glaucoma. Neuropsychiatric and cognitive adverse effects limit their use in the elderly.

42.3 Neuroprotective Therapy

A neuroprotective agent alters the underlying pathophysiology of a disease, and therefore delays the onset or slows the progression of neurodegeneration. Much of the current research into the treatment of neurodegenerative diseases focuses on neuroprotective therapy; agents are being tested that impact processes identified at the cellular level.

Glutamate is a major excitatory neurotransmitter in the central nervous system. Theoretically, increased glutamatergic neurotransmission may lead to excitatory neurotoxicity or excitotoxicity. The only two currently approved neuroprotective agents in the United States, riluzole for ALS and memantine for AD, may act to prevent glutamatergic excitotoxicity.

42.3.1 Riluzole

Riluzole, which may block the presynaptic release of glutamate, was initially developed as an anti-epileptic medication. However, due to evidence of excitotoxicity-mediated neurodegeneration in epilepsy and potential excitotoxicity involvement in ALS, riluzole was tested as a neuroprotective agent in the latter. It was approved for use in ALS after two double-blind, placebo-controlled trials showed modest improvement in survival from 3 to 3.25 years (Bensimon et al. 1994; Lacomblez et al. 1996). *Post hoc* analyses

revealed that patients who took riluzole experienced a slight prolongation in the time to progress from mild to severe disability; however, this effect was not apparent to patients, family or physicians. A meta-analysis of four randomized controlled trials indicated that 100 mg/day of riluzole prolongs survival by approximately 2 months (Miller et al. 2002). Nausea, fatigue, vertigo, and somnolence are the most common side effects of riluzole. Serum transaminase elevations may also occur but rarely to levels that are clinically meaningful.

Once riluzole was shown to be effective for ALS, and excitotoxicity was thought to be a component of neurodegeneration in general, studies of riluzole began in PD, HD, and AD. The neuroprotective effects of riluzole have been shown in the 6-OHDA and MPTP models of PD (Barneoud et al. 1996; Benazzouz et al. 1995), and in a small human trial, riluzole reduced levodopa-induced dyskinesias (Merims et al. 1999). In a separate pilot trial, riluzole extended the “on” state compared to placebo, but the difference was not statistically significant. A placebo-controlled trial in patients with HD showed that riluzole ameliorated chorea intensity (Huntington Study Group 2003). Phase III trials for the treatment of Parkinson’s and Huntington’s disease with riluzole failed to show beneficial outcomes and were discontinued (Bensimon et al. 2009; Landwehrmeyer et al. 2007). Large trials with adequate power are still needed to determine whether riluzole is neuroprotective in disorders other than ALS.

42.3.2 Memantine

Memantine, a non-competitive NMDA antagonist, has medium affinity for the phencyclidine binding site of the NMDA receptor. Memantine may block glutamate-mediated excitotoxicity, but leaves the activation of the NMDA receptor during physiological neurotransmission unchanged (Molinuevo et al. 2004). Two randomized placebo-controlled clinical trials showed positive effects in later stages of AD (Reisberg et al. 2003; Tariot et al. 2004). These studies led to the approval of memantine for the treatment of moderate and severe AD. Additional clinical data are needed to determine whether memantine can be used as monotherapy or combination therapy in early AD. Common side effects include headache, dizziness, and confusion. Initial pilot trials have already been conducted in HD and PD (Beister et al. 2004; Merello et al. 1999) and showed that memantine treatment was beneficial and associated with lower axial motor symptoms and dyskinesia scores (Moreau et al. 2013). Phase II/III clinical trials in ALS patients showed memantine was safe and well-tolerated, but did not show any benefits to patients; however, the positive effects of memantine on neuronal survival cannot be entirely ruled out at this time (de Carvalho et al. 2010).

42.3.3 Selegiline

Selegiline (L-deprenyl) is a monoamine oxidase (MAO) inhibitor used in PD. In doses under 10 mg/day, selegiline irreversibly binds MAO-B, preventing the enzyme from degrading dopamine, but does not inhibit MAO-A, thereby avoiding the potential hypertensive crisis, or the “cheese effect”, seen with inhibitors of both MAO-A and MAO-B (Yasuhara et al. 2004). The DATATOP (Deprenyl and Tocopherol Antioxidative Therapy of Parkinsonism) study conducted in 800 patients with early untreated PD assessed the neuroprotective effects of selegiline 10 mg/day, alpha-tocopherol (vitamin E) 2000 IU/day or a combination of the two (NINDS NET-PD Investigators 2006; Parkinson Study Group 1989, 1993, 1996). After follow-up (mean time of 14 ± 6 months), 38.6% (154/399) of selegiline-treated subjects reached the end-point or the time-to-disability requiring levodopa therapy, compared to 55.4% (222/401) subjects who did not receive selegiline. The projected median length of time to reach end-point was 9 months longer in the selegiline-treated group than in the groups treated with placebo or tocopherol alone. However, waning effects of selegiline after the first year coupled with slight, but significant improvement in motor performance after initiation of selegiline have been used as an argument in favor of a predominantly symptomatic, rather than neuroprotective effect. Data from other reports are also conflicting. Several studies suggest that it may exert some sort of protective effect, possibly by a mechanism other than MAO inhibition (Maruyama et al. 1997; Mytilineou et al. 1997, 1998). One study detected no deterioration after washout in *de novo* patients initially treated with selegiline (Palhagen et al. 1998), but most studies have concluded that while selegiline can delay the need for levodopa therapy, this does not translate into meaningful long-term therapeutic benefits (Brannan and Yahr 1995). Furthermore, selegiline does not appear to prevent the development of levodopa-induced motor fluctuations and dyskinesia (Parkinson Study Group 1996). Further research is needed to fully define the mechanism of action of selegiline in PD, and to clarify its role as a putative neuroprotective agent.

42.4 Symptomatic Therapy

Even though neurodegenerative diseases still have no cure, specific symptoms may be amenable to medical treatment (Table 42.3). While few randomized clinical trials of symptomatic therapy have been performed in individual neurodegenerative diseases, off-label use (*i.e.*, the prescription of an agent for a symptom or disease other than the one for which it is approved) is often helpful in improving quality of life for patients.

Table 42.3 Symptomatic therapy in neurodegenerative diseases

Symptom	Management	Dosage
<i>Psychiatric</i>		
Depression	Tricyclic antidepressants	20–100 mg QD
	Mirtazapine	15–30 mg QHS
	Selective serotonin uptake inhibitors (SSRI)	20–100 mg QHS
	Venlafaxine	37.5–75 mg BID-TID
	Bupropion	100 mg TID
Anxiety	SSRI antidepressants	20–100 mg QHS
	Buspirone	10 mg TID
	Mirtazapine	15–30 mg QHS
	Benzodiazepines	0.5–5 mg BID-TID
	Trazadone	50–100 mg BID-TID
Psychosis/ Agitation	Neuroleptics (Haloperidol)	0.5–3 mg QD
	Atypical Neuroleptics (Risperidone, Olanzapine, Quetiapine, Clozaril)	0.25–200 mg per day
	Benzodiazepines	0.5–30 mg per day
	SSRI medications	20–100 mg QD
Irritability/ Aggression	Divalproex sodium	250–2000 mg per day
	Carbamazepine	100–2600 mg per day
	SSRI antidepressants	20–100 mg QD
Pseudobulbar Affect	Tricyclic antidepressants	20–100 mg QHS
	Mirtazapine	15–30 mg QHS
	Venlafaxine	37.5–75 mg BID-TID
	Dextromethorphan/quinidine	30 mg/30 mg BID
	Lithium carbonate	300 mg QD-TID
	SSRI antidepressants	20–100 mg QD
<i>Sialorrhea</i>	Tricyclic antidepressants	20–100 mg QHS
	Atropine sulfate	0.4 mg Q 4–6 h
	Glycopyrrolate	1–2 mg TID
	Hycosamine sulfate	0.125–0.25 mg Q 4 h
	Diphenhydramine	25–50 mg TID
	Scopolamine transdermal patch	1.5 mg applied behind ear Q 72 h
<i>Motor</i>		
Spasticity	Baclofen	10–60 mg TID
	Tizanidine	2–8 mg QID
	Dantrolene	25–100 mg TID
	Benzodiazepines	2–10 mg TID
Cramps	Quinine sulfate	260–325 mg QD
	Vitamin E	400 IU TID
	Phenytoin	300 mg QHS
	Diazepam	2–10 mg TID
Tremor	Dopaminergic medication	300–3000 mg per day
	Amantadine	100–300 mg per day
	Beta blocker	10–30 mg BID-TID
	Primidone	50–250 mg QHS
	Clonazepam	0.5–2 mg QHS
Chorea	Neuroleptics	0.5–100 mg per day
	Atypical neuroleptics	0.5–160 mg per day
	Benzodiazepines	0.5–20 mg per day
	Amantadine	100–300 mg QD

(continued)

Symptom	Management	Dosage
<i>Autonomic</i>		
Orthostatic hypotension	Fludrocortisone	0.1–1 mg QD
	Midodrine	10 mg TID
<i>Gastrointestinal</i>		
Thick phlegm	Guaifenesin	200–400 Q 4 h
	Nebulized acetylcysteine	1 NEB Q 6–8 h
	Nebulized saline	1 NEB Q 4–6 hours
	Beta blocker	10–30 mg TID
Constipation	Increase fluid intake	N/A
	Increase fiber intake	N/A
	Docusate sodium	100 mg QD-BID
	Milk of magnesia	N/A
	Dulcolax	5–15 mg QD
	Lactulose	15–30 mL QD-BID
	Magnesium citrate	120–240 mL PRN
	Polyethylene glycol 3350	17 g QD
<i>Gentourinary</i>		
Urinary urgency	Oxybutynin	2.5–5 mg BID
	Tolterodine	1–2 mg BID
	Amitriptyline	25–75 mg QHS
	Oxytrol patch	3.9 mg QD
Erectile	Sildenafil citrate	50 mg PRN
Dysfunction	Vardenafil	
	Tadalafil	
	Avanafil	
<i>Sleep Disorders</i>		
REM sleep behavior disorder	Clonazepam	0.5–2 mg QHS
Insomnia	SSRI antidepressants	20–100 mg QD
	Tricyclic antidepressants	20–100 mg QHS
	Mirtazapine	15–30 mg QHS
	Zolpidem	5–10 mg QHS
	Eszopiclone	2–3 mg QHS
	Zaleplon	5–10 mg QHS
	Antihistamines	25–50 mg QHS
	Chloral hydrate	500–1000 mg QHS
	Benzodiazepine	2–5 mg QHS

42.4.1 Psychiatric Symptoms

Many patients with neurodegenerative diseases exhibit psychiatric symptoms, including depression and anxiety. Tricyclic antidepressants, such as amitriptyline and nortriptyline, may relieve depression. However, the anticholinergic effects of these medications can lead to confusion and must be used with caution in patients with cognitive compromise and in the elderly. Tricyclic antidepressants with low anticholinergic properties, such as desipramine or nortriptyline, can also be used. In PD, mirtazapine, which has noradrenergic and serotonergic effects, may relieve tremor in addition to reducing depression (Pact and Giduz 1999). While selective

serotonin reuptake inhibitor (SSRI) medications are helpful in the management of depression in all neurodegenerative disorders, they can exacerbate tremor, and sexual dysfunction is a common side effect.

Agitation, which may occur in patients with dementia, can be treated with anti-psychotic agents, mood stabilizing anti-convulsants, trazadone and anxiolytics (Doody et al. 2001). The atypical anti-psychotic medications are the treatment of choice for psychotic symptoms, such as hallucinations or delusions, particularly in those with parkinsonism when dopamine receptor blockage is contraindicated due to the potential to worsen motor symptoms. In these patients, clozapine, which may reduce tremor in addition to its anti-psychotic effects, is particularly effective. However, rare cases of agranulocytosis necessitate weekly blood counts and may limit its utility in PD. Quetiapine may be a next agent of choice due to the apparent fewer adverse motor effects compared to other medications in the class (Ondo et al. 2005). Irritability and aggression can be seen in HD, and less commonly in PD with dementia, AD, or ALS. SSRI medications and anticonvulsants such as valproic acid and carbamazepine can be effective in treating these symptoms.

In ALS, emotional lability or pseudobulbar affect can limit social interactions and quality of life. While no currently FDA approved therapies for pseudobulbar affect are available in ALS, SSRIs, tricyclic antidepressants, and dopaminergic therapy can be beneficial (Gordon and Mitsumoto 2007). One randomized, controlled trial showed that a combination of dextromethorphan and quinidine reduced emotional lability and improved quality of life in ALS (Brooks et al. 2004).

42.4.2 Sialorrhea

Drooling is an embarrassing symptom that is common in both ALS and PD. Anticholinergic medications are often helpful, but the benefits can be self-limiting and may require higher doses after initial improvement. Side effects include constipation, fatigue, and impotence. Urinary retention, blurred vision, tachycardia, orthostatic hypotension, dizziness and confusion may also occur. Drug selection depends on the severity and frequency of drooling. Hycosamine, which has a short half-life, is useful for sialorrhea that is associated with mealtimes or a particular time of the day. Transdermal scopolamine and oral glycopyrrolate provide a more continuous effect (Gordon and Mitsumoto 2007). Botulinum toxin injections have reduced sialorrhea in several small studies reported for both ALS and PD (Bhatia et al. 1999; Bushara 1997; Pal et al. 2000), but need to be used with caution in those with dysphagia. A more recent study showed that PD patients who received botulinum toxin injections into parotid and submandibular glands had significantly improved sialor-

rhea 1 month post-injection compared to placebo controls (Ondo et al. 2004). Additional clinical trials addressing botulinum toxin for the treatment of sialorrhea in ALS and PD patients are underway.

Thick phlegm can also be problematic in ALS. It is exacerbated by inadequate water intake, especially in patients with bulbar weakness, and by use of anticholinergic agents, which reduce serous secretions while sparing mucous secretions. Pharmacologic treatments that loosen phlegm include guaifenesin, nebulized acetylcysteine, nebulized saline, or beta adrenergic receptor blockers such as propranolol (Gordon and Mitsumoto 2007). Patients with a weak cough due to respiratory muscle involvement can use a cough-assist device or in-exsufflator to help clear secretions.

42.4.3 Motor Symptoms

In ALS, spasticity can be disabling. baclofen, a gamma amino butyric acid (GABA) analog, is the treatment of choice for spasticity, although the benefits are often modest. Patients may develop a sense of looseness or weakness, which can be minimized by slow dose titration. Other side effects include dizziness, fatigue and sedation. Intrathecal baclofen can be tried for those with severe or painful spasticity and inadequate response to oral treatment. Alternative medications to treat spasticity include dantrolene, tizanidine, and benzodiazepines. Dantrolene acts by blocking calcium release at the level of the sarcoplasmic reticulum and has the theoretical benefit of reducing excess neural excitation. At higher doses, liver function abnormalities may occur and require periodic monitoring. Tizanidine, an α_2 adrenergic agonist that has proven benefit for spasticity in multiple sclerosis, has similar side effects to baclofen. Benzodiazepines can be effective for spasticity, painful spasm and cramps. However, these medications can produce sedation and respiratory depression in high doses (Gordon and Mitsumoto 2007).

In PD, the most common motor symptoms are tremor, bradykinesia and rigidity. Beta blockers, primidone and clonazepam, can be used to treat postural tremor. Muscle relaxants are occasionally used for painful rigidity. Most of the typical motor symptoms, including rest tremor, respond to disease-specific therapy, at least early in the disease course. Some levodopa-induced motor complications, including chorea, dystonia, myoclonus and akathisia (Fahn 2000), can be reduced by prescribing lower more frequent doses of levodopa or by adding an anti-dyskinesia agent such as amantadine (Luquin et al. 1992).

Huntington's disease can cause chorea, bradykinesia, rigidity, and postural instability, among other motor symptoms. The chorea is often most noticeable and usually responds to treatment with dopamine receptor blocking or dopamine

depleting agents, but only at the expense of worsening bradykinesia. Atypical neuroleptics are also used to treat chorea and some behavioral symptoms. Other medications that are used to treat chorea include benzodiazepines and amantadine. Levodopa is not effective for bradykinesia due to HD because of its potential to worsen chorea and cognitive symptoms.

42.4.4 Autonomic Symptoms

Dysautonomia may produce orthostatic hypotension, sphincter abnormalities and sexual dysfunction. Orthostatic hypotension, particularly problematic in PD and Parkinson-plus syndromes such as multiple system atrophy (MSA), can be treated with salt, fludrocortisone, and midodrine (Jankovic et al. 1993). Fludrocortisone is a corticosteroid, but the exact mechanism on blood pressure is not fully understood. Side effects are similar to other glucocorticoids and mineralocorticoids. Midodrine stimulates $\alpha 1$ adrenergic receptors, but can cause significant hypertension in the supine position. Other common side effects include paresthesias, piloerection, dysuria, and pruritis.

42.4.5 Gastrointestinal Symptoms

Constipation is a common symptom in the neurodegenerative disorders. Inadequate fluid intake, dysphagia, dysautonomia, and immobility all contribute to constipation. Increasing fluid and fiber intake, along with increasing physical activity can help. Use of docusate sodium, milk of magnesia, dulcolax and senna may also improve symptoms. For severe constipation, lactulose, magnesium citrate and enemas can provide relief.

42.4.6 Genitourinary Symptoms

In neurodegenerative disorders, patients often experience urinary urgency, which is thought to be caused by spasm of the urinary sphincter or detrusor muscle. Oxybutynin (Ditropan) and tolterodine (Detrol) are effective for urinary urgency. For ALS patients and some PD patients, swallowing tablets may be difficult due to dysphagia. Extended release oxybutynin allows for infrequent daily dosing and oxytrol patches avoids the oral route of administration.

Sildenafil citrate (Viagra) has been found to be safe and effective for managing erectile dysfunction in PD, but can unmask orthostatic hypotension in patients with MSA (Hussain et al. 2001; Zesiewicz et al. 2000). Sildenafil is also effective for erectile dysfunction in other neurodegenerative disorders, but it should be used cautiously in those with cardiovascular disease.

42.4.7 Sleep Disorders

Sleeplessness in ALS has numerous causes. Respiratory insufficiency, difficulty repositioning in bed, anxiety and depression can all contribute to poor sleep. Treatment of depression with sedating antidepressants such as mirtazapine, tricyclic antidepressants, or trazadone can help promote sleep. Zolpidem, a non-benzodiazepine sleep aid, is effective and carries a low risk of respiratory depression. Other medications that can be helpful include antihistamines, chloral hydrate, and selective use of benzodiazepines (Gordon and Mitsumoto 2007). Non-invasive positive pressure ventilation can help relieve orthopnea in those with respiratory muscle weakness, and special equipment, such as a hospital bed, can reduce nighttime discomfort.

The sleep dysfunction in PD may also be multifactorial, but can also be due to an underlying sleep disorder. Parkinson's disease is commonly associated with rapid eye movement (REM) sleep disorder (Comella et al. 1998), which can be an initial manifestation of the disease. REM sleep disorder, best diagnosed with an overnight sleep study, can be treated with a nighttime benzodiazepine such as clonazepam.

In AD, non-pharmacological approaches to treating insomnia are undertaken first. Techniques include sleep restriction and keeping patients awake during the day as well as providing a cool comfortable quiet sleep environment at night. If pharmacologic management is necessary, sedating antidepressants or zolpidem may be helpful for promoting sleep in AD (Corey-Bloom and Galasko 1995). In those with nighttime confusion or sundowning, a low dose of a sedating atypical antipsychotic medication such as quetiapine can be helpful. Anticholinergic hypnotics should be avoided.

42.5 Current and Future Directions For Neuroprotection

42.5.1 Anti-inflammatory Agents

42.5.1.1 Nonsteroidal Anti-Inflammatory Drugs (NSAIDs)

Because inflammatory cells surround dying neurons in all neurodegenerative disorders, a recent approach to neuroprotection has been the use of anti-inflammatory agents. Nonsteroidal anti-inflammatory drugs (NSAIDs) reduce inflammation in part by inhibiting one or both of the cyclooxygenase (COX) enzyme isoforms. Inhibition of COX reduces prostaglandin synthesis leading to a general decrease in inflammation. While NSAIDs have demonstrated an ability to provide neuroprotection in animal models, human trials have been less rewarding.

Both celecoxib and rofecoxib, selective COX-2 inhibitors, had positive effects in the SOD1 mouse model of ALS (Drachman et al. 2002; Azari et al. 2005). However, a subsequent randomized controlled trial of celecoxib was reported not to impart a beneficial effect in 300 ALS patients (Cudkowicz et al. 2006). No differences in adverse effects between treated and control groups were detected, and the drug was well-tolerated at a dosage of 800 mg/day for 12 months. This trial had a high dropout rate, which may have impaired the ability of the investigators to detect a small impact of the drug if one existed.

Celecoxib and rofecoxib have also been studied in AD. Randomized double-blind, placebo-controlled trials failed to demonstrate a therapeutic benefit (Aisen et al. 2003). The rofecoxib trial used naproxen as a control; the results were consistent with other studies in which nonselective NSAIDs such as diclofenac, were ineffective in AD (Scharf et al. 1999). Other NSAIDs including ibuprofen, indomethacin, and sulindac sulfide have demonstrated potential efficacy in AD (in't Veld BA et al. 2001; Rogers et al. 1993), but trials failed to show positive effects as a result of NSAID therapy in mild to moderate AD (Pasqualetti et al. 2009; de Jong et al. 2008).

Pioglitazone is a member of the thiazolidinedione class of drugs that acts as an agonist for the nuclear peroxisome proliferator-activated receptor-gamma (PPAR- γ) and is indicated for type 2 diabetes to reduce insulin resistance. Preclinical and early clinical evidence suggested the neuroprotective properties of PPAR- γ agonists PD and neurodegenerative disorders (Nicolakakis et al. 2008; Chaturvedi and Beal 2008; Risner et al. 2006; Ristow 2004; Moreno et al. 2004). Although the neuroprotective mechanisms that PPAR- γ agonists afford have yet to be delineated, they have been shown to inhibit activation of microglia and astrocytes, and inhibit production of pro-inflammatory cytokines and nitric oxide (Storer et al. 2005). In a multi-center, double-blind, placebo-controlled, futility clinical trial of early PD patients, pioglitazone was administered for 44 weeks, but yielded no beneficial changes to UPDRS scores and suggested that pioglitazone failed to affect disease progression compared to placebo controls. (NINDS NET-PD FS-ZONE Investigators 2015).

42.5.2 Immunomodulation

42.5.2.1 Vaccination in ALS

Another approach is to harness the body's own immune system to regulate inflammation. In models of neurodegeneration caused by mechanical or biochemical injury, more neurons survive in the presence of a well-regulated evoked anti-self T cell-mediated response (Moalem et al. 1999). Vaccination can be used to induce nonpathogenic T cell

responses, including the activation of anti-inflammatory Th2 cells, which may migrate to lesion sites where they affect innate immune responses through the release of anti-inflammatory cytokines or by inducing the production of neurotrophic factors. Glatiramer acetate (GA, Copaxone, Cop-1), a random mix of four amino acids in a known molar ratio, was originally designed to mimic myelin basic protein and is now FDA approved for the treatment of multiple sclerosis (MS). Cop-1 can induce a protective T cell-mediated response without risk of causing an autoimmune disease. A study of acute and chronic motor neuron disease in animal models demonstrated that twice the number of motor neurons survived in Cop-1 vaccinated mice than control mice (Angelov et al. 2003). This study also showed that Cop-1 vaccination prolonged life span in SOD1 transgenic mice, although pathological findings were not reported. An initial trial in ALS patients revealed the modality to be safe in this disease (Gordon et al. 2006). ALS patients tolerated two different dosing frequencies in conjunction with riluzole, and patients mounted immune responses similar to those seen in MS patients. Larger trials of efficacy are planned.

42.5.2.2 Immunotherapy in AD

In AD, locally generated complement proteins are activated by extracellular amyloid deposits. Microglia aggregate at these deposition sites, and attack and destroy neuronal extensions. Research has focused on recruiting the immune system to remove beta amyloid (A β) protein deposits. Exogenous antibodies to A β protein selectively bind to senile plaques and cerebral amyloid in vivo, and the amyloid deposition can be ameliorated in A β precursor protein-transgenic mice by active immunization with the A β peptide (Walker et al. 1994). Other studies have confirmed that diverse anti-A β immunization schemes reduce cerebral A β burden and behavior deficits in AD transgenic mice (Dodel et al. 2003; Lemere et al. 2004).

A β immunization was initiated in humans after successful studies in rodents. Phase I studies were completed without major adverse events, and Phase II trials were initiated with AN 1792 (active immunization with A β 42 and QS-21 adjuvant). The trials were interrupted in 2002 because approximately 6% of those vaccinated developed aseptic meningitis (Orgogozo et al. 2003). Data from the incomplete trial suggest that AD patients who mounted a significant antibody response also showed signs of clinical benefit (Hock et al. 2003; Orgogozo et al. 2003). Autopsy findings from three patients who died approximately one year after the first immunization showed evidence of A β clearance (Ferrer et al. 2004; Masliah et al. 2005; Nicoll et al. 2003). However, two of the three brains also showed changes of encephalitis with infiltration of T lymphocytes, white matter lesions invaded by macrophages, and in one case, severe small vessel disease with multiple cortical hemorrhages (Ferrer et al. 2004; Nicoll et al. 2003).

The potential for A β immunization development as a disease modifying treatment in AD depends on whether serious side effects can be effectively controlled. Further studies are needed, especially in aged nonhuman primates, to clearly understand the adverse effects and how to allow for safe application of this therapy to humans (Orgogozo et al. 2003).

Granulocyte-colony stimulating factor (G-CSF) is a protein that induces the production of stem cells and granulocytes within the bone marrow and mobilizes bone marrow elements to the peripheral circulation (Thomas et al. 2002). However, this protocol alters the immune repertoire such that a more suppressive and alloprotective responsiveness is bestowed upon the immune system (Saraceni et al. 2015; Jun et al. 2004). In AD mice, administration of G-CSF diminishes A β deposition and reverses cognitive impairment (Sanchez-Ramos et al. 2009). In a pilot Phase I cross-over trial in 8 AD patients, G-CSF was administered over 5 days and was tolerated without serious events (NCT01617577). While no significant differences in cognitive ADAScog or paired associate learning (PAL) memory tests were detected at 14 week post-administration, total PAL tests of total PAL between G-CSF- and placebo-treated patients.

42.5.2.3 Immunotherapy in Prion Disease

Antibody based immunotherapy has also been evaluated in animal models of prion disease. In vitro assays have demonstrated that antibodies to the cellular prion protein (PrP^C) antagonize the deposition of disease-associated prion protein (PrP^{Sc}) (Sigurdsson et al. 2002). However, the induction of protective antiprion immune responses has been difficult in wild type animals because of tolerance to endogenous PrP^C. Some studies have shown the possibility to overcome this tolerance by inducing immune responses to bacterially expressed recombinant PrP (Heppner et al. 2001). However, developing antibodies that are capable of recognizing native cell surface PrP^C is more difficult (Heppner and Aguzzi 2004). One study demonstrated that anti-PrP antibodies cross-linked with PrP^C when directly injected into the brains of laboratory animals and subsequently provoked neurotoxicity (Solforosi et al. 2004); however, passive immunization studies have proven successful in animals (White et al. 2003). Anti-PrP^C antibodies represent a unique set of compounds that may prove beneficial in treating prion disorders, and could elicit protection through several avenues including reduction of available PrP^C substrates for conversion to PrP^{Sc}, stabilization of the PrP^C molecule to prevent conversion, and possibly prevention of the formation of PrP^{Sc} conversion intermediates (Rovis and Legname 2014).

42.5.2.4 Immunotherapy in Parkinson Disease

In the MPTP animal model of PD, glatiramer acetate (GA, Cop-1) has also been investigated. In that model, Cop-1-immune cells provided significantly more neuroprotection than placebo

(Benner et al. 2004). Mice received adoptive transfers of splenocytes from Cop-1 or ovalbumin immunized mice since MPTP-immunotoxicity precluded active immunization due to lymphotoxicity (Benner et al. 2004). In animals given splenocytes from Cop-1 immunized mice, T cells entered and accumulated in inflamed areas of the CNS. The T cells secreted interleukin-10, an inhibitory Th2 cytokine, which suppressed microglial activation, and also stimulated local expression of astrocyte-associated glial cell line derived neurotrophic factor. This immunization strategy minimized dopamine loss compared to control animals and resulted in significant protection of nigrostriatal neurons. CD4+, but not CD8+ T cells proved to provide neuroprotection in the MPTP model and suggested that regulatory T cells (Tregs) may play a crucial role in neuroprotection (Laurie et al. 2007). Indeed in a series of studies, adoptive transfer of as few as 3.5×10^6 Tregs from naïve animals delivered complete neuroprotection of nigral neurons and up to 90% protection of striatal termini, while concomitantly attenuated microglia-derived neuroinflammation in the MPTP model (Reynolds et al. 2007). Tregs also suppressed microglial activation and oxidative stress induced by stimulation with aggregated α -synuclein (Reynolds et al. 2009a, b). Immunization with nitrated- α -synuclein (N- α -syn) induces N- α -syn specific CD4+ effector T cells (Teffs) which exacerbate neuroinflammation and microglial activation, and increase neurodegeneration in the MPTP model. That Teffs are stimulated by N- α -syn, but not unmodified α -syn suggest the effectively breaking or evading immunological tolerance (Benner et al. 2008). Indeed N- α -syn-specific Th17 effectors were the principal neurotoxic effector cell type associated with a more neurotoxic phenotype. In the presence of these Th17 effectors, CD4+ Tregs counter N- α -syn-specific Teffs and protect dopaminergic neurons better than Tregs acting in the absence of Th17 effectors (Reynolds et al. 2010). These studies suggested that in PD, Teffs are no longer adequately controlled by Treg populations and could drive disease progression over a long period of time. Indeed, observational clinical studies showed that PD patients exhibited greater frequencies of T cells with effector phenotypes compared to age-matched caregivers (Saunders et al. 2012). Moreover, Teff frequencies correlated with increased severity of motor dysfunction as determined by UPDRS part III scores and were associated with diminished Treg function as determined by their ability to inhibit CD3/CD28-stimulated T cell proliferation. Two immune modulating agents, vasoactive intestinal peptide (VIP) and granulocyte-macrophage colony-stimulating factor (GM-CSF, CSF2) known to upregulate Treg function were neuroprotective in the MPTP model (Reynolds et al. 2010; Delgado and Ganea 2003; Kosloski et al. 2013). Using synthetic VIP receptor (VIPR) agonists altered to diminish degradation and increase half-lives, the Treg inducing and neuroprotective capacities were afforded by VIPR2

agonism compared to VIPR1 agonists or scrambled peptides (Olson et al. 2015). Moreover, treatment with VIPR2 agonist yielded CD4+ T cells that expressed a 45-fold increase in GM-CSF gene expression. Together, these studies resulted in the initiation of a double-blind, randomized, placebo-controlled Phase I clinical trial in PD patients treated daily with GM-CSF (sargramostim) at 6 µg/kg or placebo for 8 weeks and is estimated to be completed in 2016 (NCT01882010).

Immunophilin binding proteins are being developed as a potential immune based therapy in PD. Immunophilins, intracellular receptor proteins that bind to the immunosuppressive drugs cyclosporine A, FK506, and rapamycin, are 10–50 fold more abundant in the brain than in the immune system (Guo et al. 2001). The immunophilin ligands combine with immunophilin proteins to suppress the immune system by inhibiting the calcium activated phosphate calcineurin, and some promote nerve growth in vitro and in vivo. GPI 1046 (Guilford Pharmaceutical) and AMG-474-00 or NIL-A (Amgen) are immunophilin ligands that promote neuronal growth without demonstrating immunosuppressive effects (Gold et al. 1998; Steiner et al. 1997). These agents stimulate growth of nigrostriatal dopaminergic neurons spared after MPTP-induced damage to the substantia nigra. Human trials have been completed to determine whether these or other immunophilin ligands will be useful in slowing or reversing PD but results are unavailable.

42.5.3 Anti-apoptotic Therapies

One process associated with neurodegeneration is caspase enzyme-driven apoptosis, and drugs that inhibit the activation of caspase enzymes are being studied as a potential neuroprotective strategy.

42.5.3.1 Minocycline

Minocycline is a tetracycline antibiotic with anti-inflammatory and anti-apoptotic properties. It has been shown to delay disease onset and prolong survival in transgenic ALS mice (Kriz et al. 2002; Zhu et al. 2002). Two early randomized, placebo-controlled trials have been conducted in ALS. The first trial showed that minocycline can be used safely in combination with riluzole. The second trial showed that minocycline was tolerated at doses up to 400 mg/day (Gordon et al. 2004). However, upon completion of phase III, researchers reported that minocycline had a harmful effect on ALS patients (Gordon et al. 2007). The negative effects of minocycline on ALS patients, despite promising phase II results, are not clearly understood at this point.

Markers of apoptosis are also present in PD (Mogi et al. 2000; Tatton 2000). Administration of minocycline to 6-OHDA treated mice inhibits microglial activation, protects

dopaminergic neurons, and reduces markers of apoptosis (He et al. 2001). Minocycline has also been shown to prevent nigrostriatal dopaminergic neurodegeneration in MPTP mice models of PD (Du et al. 2001). Similarly, minocycline delays disease progression in animal models of HD, presumably by inhibiting caspase-1 and caspase-3 mRNA up-regulation and decreasing inducible nitric oxide synthase (iNOS) activity (Chen et al. 2000; Tikka et al. 2001a). In a Phase II NINDS-funded clinical trial of PD patients, treatment with minocycline over 18 months yielded no significant differences in the change of UPDRS scores or in the time to needed symptomatic therapy compared to placebo controls (NINDS NET-PD Investigators 2008).

42.5.3.2 TCH 346 (Omigapil)

TCH 346, which is structurally related to selegiline, binds to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in a mechanism that may prevent programmed cell death. It slows the degeneration of motor neurons in in vitro models of apoptosis (Carlile et al. 2000), but did not have a significant effect on survival in transgenic mice. Phase II/III trials in patients with ALS revealed no beneficial effects of TCH 346 on disease progression (Miller et al. 2007).

In PD, nuclear translocation of GAPDH in melanized nigral neurons has been interpreted as a marker for apoptosis (Dastoor and Dreyer 2001). After MPTP treatment in animal models, GAPDH is upregulated and undergoes nuclear translocation. TCH 346 has been shown to bind to GAPDH and rescue neurons from cell death in this model (Andringa and Cools 2000). TCH 346 also attenuates MPTP-induced toxicity in primates (Andringa et al. 2003). Clinical trials of TCH 346 in PD yielded similar results to trials in ALS. In a 12–18 month trial of 255 patients, no significant evidence of neuroprotection was seen at any of the doses evaluated (0.5, 2.5, or 10 mg) (Olanow et al. 2006).

42.5.3.3 Mitogen Activated Protein (MAP) Kinase Inhibitors

The cascade of apoptosis may be promoted by mitogen activated protein (MAP) kinase activation. CEP-1347, a small molecule MAP kinase inhibitor that crosses the blood brain barrier is protective in MPTP-treated mice (Saporito et al. 1999). A clinical trial showed good tolerability, no interference with L-DOPA pharmacokinetics, and no symptomatic effects of treatment over a 4-week period (Parkinson Study Group 2004). Phase II trials of CEP1347 were terminated during an interim analysis that demonstrated continuation of the trial would be futile (Parkinson Study Group PRECEPT Investigators 2007). Notably, the doses of CEP1347 used in the trial were selected from preclinical MPTP studies, but whether any of the doses actually inhibited MAP kinase activity in the human brain was not determined (Burke 2007).

42.5.4 Antioxidants

Another proposed mechanism of neuronal death is oxidative stress due to the accumulation of free radicals. Normal and abnormal cellular metabolic processes involving molecular oxygen produce free radicals, which can cause oxidative damage to lipids, proteins and nucleic acids (Andersen 2004).

42.5.4.1 Vitamin E

Alpha tocopherol (vitamin E), a naturally occurring antioxidant, delays disease onset in mutant human SOD1 transgenic mice (Gurney et al. 1996). One epidemiological study suggested that regular intake of vitamin E may reduce the risk of contracting ALS (Ascherio et al. 2005). A randomized, placebo-controlled trial of vitamin E at 500 mg twice daily dosages showed no significant difference in 12-month survival in patients with ALS (Desnuelle et al. 2001). This trial was probably underpowered due to insufficient sample size, and used a non-validated outcome measure, the Norris Scale. Vitamin E has also been studied in combination with other antioxidants. L-methionine, vitamin E, and selenium were studied in ALS in a double-blind placebo-controlled trial of the combinations taken three times a day (Stevic et al. 2001). After 12 months, the treatment group exhibited 80% survival compared to 50% survival in the control group, however, those findings that were not statistically significant (Orrell et al. 2005). Vitamin E was ineffective in slowing progression of PD in the DATATOP trial (Parkinson Study Group 1989).

42.5.4.2 Selegiline

Selegiline (or L-deprenyl) may have anti-oxidant and anti-apoptotic properties in addition to inhibiting MAO, and has been reported to increase SOD activity in the basal ganglia of rats (Knoll 1989). There have been several trials of selegiline 10 mg per day in patients with ALS: a randomized, placebo-controlled, double-blind trial (Lange et al. 1998), and a placebo-controlled crossover trial (Mitchell et al. 1995). Neither showed improvement in functional or subjective rating scales, but both trials were underpowered due to insufficient sample size and so may be considered inconclusive. In clinical trials with PD patients, treatment with selegiline was found to have beneficial effects during the first 12 months of treatment with delayed onset of disability that required L-DOPA therapy and improved motor function clinical scores (Parkinson Study Group 1989, 1993). The drug upregulates anti-oxidant enzymes such as SOD and catalase, affects pro-inflammatory mediators such as INF- γ , TNF- α , and IL-1 β , and increases trophic factors (Kitani et al. 2002).

42.5.4.3 N-acetylcysteine

N-acetylcysteine is a precursor of glutathione and a natural intracellular antioxidant. In a randomized, placebo-controlled trial in patients with ALS, 35 patients (65%) given 50 mg/kg of

N-acetylcysteine by daily subcutaneous infusion and 30 patients (54%) given placebo were still alive at 12 months, a difference that was not statistically significant (Louwerse et al. 1995). In a small clinical trial, patients with PD or Gaucher disease and healthy controls treated with N-acetylcysteine showed increased blood redox ratios and levels of brain glutathione by 7-T magnetic resonance spectroscopy (MRS) (Holmay et al. 2013).

42.5.4.4 Coenzyme Q10 (CoQ10)

CoQ10 is an essential cofactor of the mitochondrial electron transport chain, and may act as an antioxidant. It has been shown to have neuroprotective effects in models of ALS, PD and HD (Beal 2002). An open label escalation trial in ALS was completed in 2004 with dosages up to 3000 mg per day well-tolerated (Ferrante et al. 2005). An NINDS-funded randomized controlled phase II trial of CoQ10 showed insufficient improvement to justify a larger phase III trial (Kaufmann et al. 2009).

A phase II trial has also been completed in PD (Shults et al. 2002). Over a period of 16 months, CoQ10 was safe and well tolerated at doses up to 1200 mg/day. Trends toward less disability occurred in treated patients and the benefits were greater in those receiving the highest dosage. Phase III trials aimed at examining whether CoQ10 could be used to slow disease progression in early PD verified that safe and well-tolerated, however no evidence of clinical benefit was detected (Parkinson Study Group QEI et al. 2014).

42.5.4.5 AEOL 10150

AEOL 10150 (manganese (III) meso-tetrakis (di-N-ethylimidazole) porphyrin) is a compound that can catalytically decompose biological oxidants such as peroxynitrite via its ability to cycle between MN (III) and MN (IV) states. Several investigators have reported the presence of nitrotyrosine in affected tissue in motor neuron disease; however, no cause and effect relation between protein nitration and neuronal death has been established (Crow et al. 2005). AEOL 10150 prolonged survival in the SOD1 ALS model when administered at disease onset (Zoccolella et al. 2009). Phase I trials of AEOL 10150 showed that a single injection was safe and well-tolerated in 25 patients with ALS (Zoccolella et al. 2009). Using the SOD1 mouse model of ALS, researchers have described AEOL 10150 as one of the most promising drugs for preventative and therapeutic trials in patients with ALS (Benatar 2007), however no current clinical trials using AEOL 10150 are in progress.

42.5.5 Trophic Factors

42.5.5.1 Insulin-Like Growth Factor (IGF-1)

Nerve growth factors promote neuronal sprouting in vitro, and reduce nerve cell death in vivo. Viral delivery of IGF-1 was shown to prolong survival and delay the onset of disease

in the ALS mouse model (Kaspar et al. 2003). Human trials of viral vector delivery are planned, but the safety of the modality must be shown before the FDA will allow trials to proceed. In two randomized controlled trials of IGF-1 in ALs, one showed slowed progression of functional impairment using an non-validated outcome measure (Lai et al. 1997), the other showed no significant change using the same outcome tests (Borasio et al. 1998). In a phase III trial of ALS patients, twice daily subcutaneous injections of IGF-1 for 2 years were found to provide no significant effect on muscle effect, clinical score, or tracheostomy-free survival (Sorenson et al. 2008). In a small clinical trial of ALS patients, high dose of IGF-1 (3 µg/kg) given intrathecally every 2 weeks for 40 weeks slowed decline of motor functions for some clinical signs, and afforded a modest, but significant beneficial effect without serious adverse effects (Nagano et al. 2005).

In animal studies, IGF-1. N-terminal tripeptide of IGF-1 was peripherally administered to 6-OHDA mouse models of PD and results indicated that administration after the onset of nigrostriatal dopamine depletion improved long-term parkinsonian motor deficits; however IGF-1 administration did not prevent the loss of tyrosine hydroxylase in the substantia nigra pars compacta or the striatum (Krishnamurthi et al. 2004). In human studies of drug-naïve PD patients, increased IGF-1 serum concentrations were associated with better clinical scores of movement and cognitive function, while poorer scores were associated with lower serum IGF-1 levels (Pellecchia et al. 2014; Picillo et al. 2013). Human trials of IGF-1 have yet to be conducted in PD.

42.5.5.2 Vascular Endothelial Growth Factor (VEGF)

Vascular endothelial growth factor (VEGF) is an angiogenic factor, shown important in tumor biology and recently has been implicated in neurodegeneration and therapies (Jin et al. 2000). Moreover, increasing evidence points to the involvement of other members of the VEGF family including, VEGF-B, VEGF-C, VEGF-D, and placental growth factor-2 (PLGF2) on a variety of neuronal cell types (Carmeliet and Ruiz de Almodovar 2013). Researchers have shown that reduced VEGF in persons carry a higher risk of ALS, and targeting the VEGF promoter region in transgenic mice causes an ALS-like syndrome (Storkebaum et al. 2005). Also, intramuscular delivery of VEGF to mSOD1 mice delays onset and prolongs survival. In a clinical study of AD patients, elevated levels of VEGF in CSF was found to be associated with positive clinical outcomes (Hohman et al. 2015). In the 6-OHDA mouse model of PD, VEGF promoted neuroprotection by activating glial proliferation and promoting angiogenesis (Yasuhara et al. 2004). Human trials will likely be initiated in the future, once optimal dosing and route of administration have been established.

42.5.5.3 Brain Derived Growth Factor (BDNF)

Brain derived neurotrophic factor (BDNF) has been shown to have a positive effect in preclinical models of neurodegeneration and psychiatric disorders (Nagahara and Tuszynski 2011). It prolonged survival, protected against glutamate neurotoxicity, and slowed disease progression in the wobbler mouse, an early model of ALS. In an early study with ALS patients, systemic administration of BDNF failed to show benefit for survival, but patients with early respiratory impairment showed significant benefit (BDNF Study Group 1999). Attempts to increase motor neuron exposure by intrathecal administration of BDNF showed no improvement in autonomic or motor function (Ochs et al. 2000; Beck et al. 2005). Regardless of delivery route, the failure of BDNF to reach the appropriate motor neuron targets is a consistent issue (Nagahara and Tuszynski 2011; Ankeny et al. 2001; Pardridge et al. 1998).

BDNF was also evaluated in the rat 6-OHDA model of PD, and was compared to glial cell line derived neurotrophic factor (GDNF). Results of the study showed that GDNF was more effective than BDNF in the model, but that there were no significant benefits from either of these neurotrophic factors (Sun et al. 2005).

In animal models of AD, BDNF has been shown to reverse age-associated changes in neurons, reduce neuronal death, and improve learning and memory, but did not change amyloid plaque density (Blurton-Jones et al. 2009; Nagahara et al. 2009).

42.5.5.4 Ciliary Neurotrophic Factor (CNTF)

Ciliary neurotrophic factor (CNTF) was shown to protect wobbler mice, to be required for preservation of adult motor neurons in knockout mice, and to reduce immunoreactivity in the spinal cord of ALS patients. This drug advanced as far as Phase III clinical trials, however, no significant difference in survival was observed between CNTF and placebo groups (ALS CNTF Treatment Study Group 1996; Hurko and Walsh 2000).

CNTF has also been investigated in mouse models of PD. Although CNTF had potent neurotrophic effects for injured adult rat dopaminergic substantia nigra neurons, it did not prevent the disappearance of the transmitter synthesizing enzyme tyrosine hydroxylase (Hagg and Varon 1993).

42.5.5.5 Glial Derived Neurotrophic Factor (GDNF)

GDNF has been a particularly promising approach to neuroprotection in ALS and PD (Gash et al. 1998; Lapchak 1998). GDNF enhances survival of the midbrain dopaminergic neurons in vitro and rescues degenerating neurons in vivo (Tseng et al. 1997). Intracerebroventricular (ICV) administration of GDNF in monkeys ameliorated parkinsonism and produced 20% enlargement of nigral neurons accompanied by increased fiber density (Gash et al. 1998). A GDNF-levodopa

combination reportedly reduced levodopa side effects in experimental monkeys (Miyoshi et al. 1997). A pilot trial administering GDNF ICV to humans with moderately advanced PD was abandoned because of lack of efficacy and the frequent occurrence of nausea, anorexia, tingling, hallucinations and depression (Nutt et al. 2003). Autopsy of one case showed no evidence of significant trophic effects on nigrostriatal neurons (Kordower et al. 1999). In another Phase 1 trial of PD patients, GDNF was administered into the putamen for 1–2 years with no serious side effects and improvement of motor scores and daily living activities (Gill et al. 2003; Patel et al. 2005). In a randomized controlled clinical trial, recombinant human GDNF (liatermin) was administered by intraputamenal infusion (Lang et al. 2006). Treatment resulted in increased ^{18}F -DOPA uptake, but did not improve motor scores, and was terminated due to safety concerns in preclinical trials, device-related serious events, and production of neutralizing antibodies to GDNF in several patients. Subsequent MRI analyses yielded no significant cerebellar differences between pre- and post-treatment examination of the patients (Chebrolu et al. 2006).

GDNF shows protective capability in axotomized motor neurons (Gimenez et al. 2011; Buj-Bello et al. 1995) and in preclinical models of ALS (Park et al. 2009; Suzuki et al. 2007; Li et al. 2007; Mohajeri et al. 1999). Human pluripotent stem cells engineered to produce GDNF and acquired under cGMP protocols have been used in preclinical models with the intent to use in ALS patients in the future (Gowing et al. 2014; Shelley et al. 2014).

Laboratory studies are now testing lentoviral and AAV vectors to deliver GDNF to the striatum of monkeys with MPTP-induced parkinsonism. One study demonstrated reversal of functional deficits, extensive and long term GDNF expression, augmentation of dopaminergic function, and prevention of nigrostriatal degeneration (Kordower et al. 2000; Kells et al. 2012; Bartus et al. 2011; Richardson et al. 2011). A Phase I clinical trial (NCT01621581) in PD patients testing the safety and efficacy of surgically implanted AAV2-GDNF is currently underway. The new delivery system provides a hopeful therapeutic strategy for PD, and has also been beneficial in the SOD1 model of ALS (Lu et al. 2003; Wang et al. 2002).

42.5.6 Antiglutamatergic Agents

42.5.6.1 Gabapentin

Gabapentin, an antiepileptic agent, may reduce glutamate release and thereby, reduce excitotoxicity. Gabapentin showed a small but significant effect on survival in SOD1 transgenic mice (Gurney et al. 1996). An early phase clinical trial in ALS showed a non-significant slower rate of decline in arm strength due to gabapentin (Miller et al. 1996a). A phase III study using a higher dose revealed no change in strength and a more rapid

rate of decline of the forced vital capacity (Miller et al. 2001), exemplifying the need to better define dose in early phase trials before preceding to definitive trials.

42.5.6.2 Topiramate

Topiramate reduces glutamate release from neurons and antagonizes activation of the alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor, a glutamatergic excitatory amino acid receptor. A double-blind, placebo-controlled trial conducted in 296 ALS patients over 12 months showed a faster rate of decline in arm strength and no effect on survival from topiramate (Cudkowicz et al. 2003). This trial had a large dropout rate and proceeded to a phase III trial without first exploring the effects of topiramate in the SOD model.

Topiramate was recently studied in the marmoset model of PD (Silverdale et al. 2005). Overactive AMPA receptor-mediated transmission may be involved in the pathogenesis of levodopa-induced dyskinesias. Topiramate significantly reduced levodopa-induced dyskinesias without affecting the antiparkinsonian action of levodopa. Further studies are needed to better elucidate the potential benefits of topiramate in PD.

42.5.6.3 Lamotrigine

Lamotrigine is another glutamate release inhibitor that inhibits voltage-sensitive sodium channels and stabilizes neuronal membranes, thus has been studied as a candidate neuroprotecting agent in neurodegenerative diseases. In ALS, 300 mg of lamotrigine per day had no effect when compared to placebo in a small number of patients (Ryberg et al. 2003). Lamotrigine also failed to show symptomatic benefit in patients with PD (Shinotoh et al. 1997), but neither study had adequate power to truly assess efficacy (Braga et al. 2002).

42.5.6.4 Ceftriaxone

Ceftriaxone was selected from the recent NIH-supported high throughput screening initiative in ALS. Approximately 1050 FDA approved drugs, for which the safety and toxicity are already known, were screened using in vitro assay systems (cell survival, cytochrome C release, protein aggregates, etc.). Positive results or “hits” were then tested in the transgenic model. Ceftriaxone was shown to increase glutamate transporter activity and prolong motor neuron survival. Ceftriaxone is a third generation cephalosporin that also protects against SOD toxicity, radiation induced neurodegeneration in in vitro models (Tikka et al. 2001b), and has the ability to cross the blood brain barrier. Phase I/II clinical trials in ALS patients testing the pharmacokinetics, tolerability, and safety of ceftriaxone showed the drug was well-tolerated at optimal dosing levels (Berry et al. 2013; Zhao et al. 2014). A Phase III trial (NCT00349622) was continued using two doses of ceftriaxone or placebo administered over

12 months and has been completed, however statistical analyses and publication of primary and secondary outcomes are pending.

42.5.6.5 Talampanel

Talampanel, an AMPA antagonist with antilglutamatergic activity, is also being evaluated in neurodegeneration. Talampanel has been shown to be neuroprotective after traumatic brain injury in rats (Belayev et al. 2001), and AMPA receptor antagonists have been shown to prolong survival in the transgenic mouse model of ALS (Van Damme et al. 2005). Phase II clinical trial of talampanel in ALS patients was well-tolerated, but did not show significant results in primary outcome measures; however, the rate of decline was slowed in isometric arm strength, timed hand movements and scores in ALS Functional Rating Scale (ALSFRS) (Pascuzzi et al. 2010). An extended clinical trial (NCT00982150) was terminated. Several Phase II trials of talampanel for PD have been completed, but results are unavailable.

42.5.6.6 Remacemide

Remacemide as an anticonvulsant and NMDA antagonist has been studied in PD and some models. It enhanced the effects of levodopa in parkinsonian rats and monkeys (Greenamyre et al. 1994). A randomized, controlled trial of remacemide in 279 patients with PD and motor fluctuations showed trends towards improvement of "on" time, but no evidence of neuroprotection (Shoulson et al. 2001). In a small HD trial, remacemide tended to alleviate chorea, but failed to slow functional decline (Huntington Study Group 2001). Later examination of those data suggested that after adjusting for age, longer CAG repeats were associated with greater clinical progression and may confound the results in clinical trials (Ravina et al. 2008).

42.5.7 Stem Cell Transplant Therapy

Advances in stem cell therapy have received a great deal of press in recent years. The science provides hope for potential therapeutic interventions in neurodegenerative diseases, but it is in its early stages and the mechanisms that control stem cell proliferation, differentiation into specific cells and optimal functional recovery are needed. With public expectations, patients will likely search for non-orthodox and unproven sources of treatment for which the internet provides a plethora of pages extolling the promise of stem cells, and various forms of stem cell therapy in a variety of countries. The administration of stem cells in an uncontrolled manner and without long-term follow-up by medical protocols that permit rational scientific conclusions remain an issue with some commercial ventures. Moreover, ethical concerns have clouded the collection and use of human fetal

tissue as sources of stem cells for transplantation; however the use of other sources as stem cells and tightly controlled programming protocols provide substantial advances in the possible use of neuronal replacement therapy (Lindvall 2015; Castorina et al. 2015; Haidet-Phillips and Maragakis 2015; Diaz et al. 2015; Tong et al. 2015).

42.5.7.1 Stem Cell and Gene Therapy and Parkinson Disease

The loss of specific dopaminergic neurons in PD makes the prospect of replacing missing or damaged cells a potential therapy. Recent double-blind, placebo-controlled trials of primary human embryonic dopaminergic tissue used functional neuroimaging and neuropathological investigations to demonstrate integration of transplanted dopaminergic neurons in host striatum (Freed et al. 2001; Olanow et al. 1996). However, only subpopulations of PD patients displayed significant benefits, and some patients developed uncontrollable side effects due to excess dopamine production (Freed et al. 2001; Olanow et al. 2003). Postmortem analysis at 18 months post-transplantation, showed robust engraftment of the tissues and seemingly no adverse effects of transplantation on the tissues (Kordower et al. 2008). Later autopsies of 8 patients demonstrated long-term survival of the grafts; however, long term benefits were limited in scope. Postmortem analysis showed that host factors of PD also affected the neurons from transplanted tissues as well as the transplant recipients (Li et al. 2008; Kordower et al. 2008; Mendez et al. 2008). Three patients showed typical PD neuropathology with α -synuclein inclusions from long-term transplants, but no Lewy pathology was evident from patients that succumbed up to 4 years after transplantation. Increased Lewy pathology was noted with increasing age of the grafts. Additionally, Lewy pathology was noted to be associated more with those patients with long-term transplants that had received tissue fragments rather than cell suspensions.

Retinal pigmented epithelia (RPE) are dopaminergic support cells in the neural retina. RPE cells on gelatin beads, also called Spheramine, produce levodopa (Watts et al. 2003). An open label clinical trial of transplantation of Spheramine was conducted in 6 patients with PD and implantation therapy was deemed safe and well tolerated at 6 months post-implantation (Bakay et al. 2004). However a larger double-blind, placebo-controlled trial in advanced PD showed no anti-parkinsonian benefits compared to control surgery (Gross et al. 2011). In another clinical trial, human RPE cells from 10–20 week postmortem tissues were cultured and unilaterally transplanted into the postcommissural putamen of 12 PD patients (Yin et al. 2012). The surgery was well-tolerated and 11/12 patients showed improved UPDRS scores at 3 months which peaked at 12 months and declined over the next 24 months. PET analysis detected trends of increased DA release during the first 6 months.

The activity of both GABA efferents to the subthalamic nucleus and its targets within the basal ganglia are affected in PD. Glutamic acid decarboxylase (GAD) is the rate-limiting enzyme for GABA production and thus gene therapy consisting of GAD gene insertion may serve as an alternative PD treatment strategy (LeWitt et al. 2011). In patients who received the bilateral delivery of AAV2-GAD, there was a greater decrease in their UPDRS scores correlating with an improvement in motor control compared to patients who received sham surgery. The efficacy of GAD gene therapy supports its further investigation as a PD therapy and also shows promise for gene therapy in other neurodegenerative and neurological disorders (LeWitt et al. 2011).

42.5.7.2 Stem Cell Therapy and Huntington Disease

In animal models of HD, transplanted striatal cells survive, grow, and establish afferent and efferent connections (Freeman et al. 2000). One open label human trial indicated that grafts may have restored function (Hauser et al. 2002). Recipients showed cognitive and motor improvements that were associated with reductions in striatal and cortical hypometabolism. The success of grafting may be sensitive to the age of the donor and to the degree of neuronal loss in the patient.

In HD rodent models, mesenchymal and adipose derived stem cells have been shown to improve HD pathology through the secretion of neurotrophic factors and reduction in proinflammatory cytokines. Further studies will be required prior to the use of stem cell transplants in clinical trials, however studies-to-date suggest that stem cell therapy has great potential to effectively treat HD (Maucksch et al. 2013).

42.5.7.3 Stem Cell Therapy and Amyotrophic Lateral Sclerosis

Stem cell therapy poses a greater challenge in ALS than in other neurodegenerative diseases because of the length of motor axons and growing evidence that neurodegeneration in ALS may be mediated by neuronal and glial influences. Late stage fetal cortical neurons have been shown to replace apoptotic neurons when grafted into adult mouse neocortex, to receive afferents from host brain, and to project to the contralateral hemisphere (Fricker-Gates et al. 2002). Fetal motor neurons grafted to the adult rat spinal cord lacking motor neurons migrate to the ventral horn and make functional connections with skeletal muscle (Clowry et al. 1991). Whether these neurons are integrated into neuronal circuits is unclear. It is also unknown if similar neuronal replacement could work in the brains of individuals with ALS. A series of Phase 1 clinical trials using bone marrow-derived mesenchymal stem cells (MSCs) were injected into the spinal cord of ALS patients and followed for various lengths time (Mazzini et al. 2003, 2006, 2008, 2010, 2012). The trials demonstrated that

intraspinal injection of MSCs was generally safe, and beneficial trends included slowing the rate of decline of the forced vital capacity and of the ALS-FRS score. However, neither graft survival nor transplant growth could be confirmed. Several other trials have reported that MSC transplantation in ALS patients is well-tolerated (Feldman et al. 2014; Garcia Santos et al. 2013; Riley et al. 2012; Blanquer et al. 2012; Glass et al. 2012; Karussis et al. 2010; Deda et al. 2009; Oh et al. 2015), but only a few studies yielded significantly positive outcomes for patients (Karussis et al. 2010; Oh et al. 2015). Using a spinal cord-derived neural stem cell line, NSI-566 (Glass et al. 2012; Riley et al. 2012) implanted into spinal cords of ALS patients, a Phase II trial showed the maximum dose of 16 million cells was well-tolerated and at 9 months post implant, 47 % (7/15) patients responded to the treatment as measured by non-declining slope of ALSFRS scores, increased grip strength, and retention of lung function.

42.5.7.4 Stem Cell Therapy and Alzheimer's Disease

Preclinical studies in animal models of AD have shown that stem cells, including embryonic stem cells, iPSCs, MSCs, and adipose-derived stem cells (ADSCs) have the capacity to model familial AD (if derived from AD patients); improve memory, cognitive, and locomotor functions; remove A β plaques from hippocampal tissues; reduce A β deposits by induction of M2-like microglia; support and enhance autophagy; and improve acetylcholine levels (Salem et al. 2014; Shin et al. 2014; Ma et al. 2013; Yang et al. 2013; Park et al. 2013; Darlington et al. 2013; Yagi et al. 2011; Lee et al. 2009; Tang et al. 2008; Amemori et al. 2015). Several clinical trials using stem cells in AD patients have been initiated or completed, but results have not yet been posted (NCT01297218, NCT01547689, NCT02054208, and NCT02600130).

42.6 Others

42.6.1 Amyotrophic Lateral Sclerosis

42.6.1.1 Calcium Channel Blockers

Calcium channel blockers antagonize excitatory amino acid receptor activation. A randomized, double-blind, placebo controlled, crossover study of nimodipine was conducted in patients with ALS (Miller et al. 1996b). There was no significant difference in the rate of decline of pulmonary function or limb strength with treatment compared to placebo and the authors concluded that nimodipine was ineffective in slowing the progress of ALS.

The efficacy of verapamil, a calcium channel blocker, was studied in a clinical trial in which the treatment of phase was compared to a natural history phase (Miller et al. 1996c).

During months 1–3, patients were not given drug and the natural history of their progression was measured. Following the month 3 visit, verapamil was started at 40 mg and titrated to a daily dose of 240 mg per day. The decline in forced vital capacity and limb strength were not significantly different between the treatment phase and natural history phase.

42.6.1.2 Creatine

A key pathological feature in the animal model of ALS is abnormality of the mitochondria (Wong et al. 1995). Creatine, which acts as a mitochondrial energy buffer and may also have anti-oxidant properties, provided a dose dependent improvement in survival in the transgenic SOD1-G93A mouse (Klivenyi et al. 1999). A subsequent randomized placebo-controlled trial using 5 g per day showed no significant benefit in survival (Groeneveld et al. 2003). This trial broke new ground in several ways. First, the investigators used a sequential analysis plan, so that the trial was stopped once the null hypothesis could no longer be rejected, saving 18 months over a fixed duration trial. Second, the dropout rate was nearly zero, because the investigators made home visits when necessary to obtain data from patients no longer able to travel to the centers. A second double-blind, placebo-controlled trial of 5 g per days also did not show a benefit (Shefner et al. 2004), but this trial likely had inadequate power due to small numbers of patients enrolled. Neither trial may have been dosed adequately.

42.6.1.3 Sodium Phenylbutyrate

Transcriptional dysregulation may contribute to the pathogenesis of neurodegeneration (Gonzalez de Aguilar et al. 2000). Sodium phenylbutyrate, a histone deacetylase inhibitor (HDAC inhibitor), regulates transcription and has provided improvement in survival, body weight and motor performance in the SOD mouse model (Ryu et al. 2005). The safety and tolerability of sodium phenylbutyrate has been evaluated in a trial funded by the Veterans Administration and the Muscular Dystrophy Association and shown that the lowest dose was therapeutically efficient in improving histone acetylation levels (Cudkowicz et al. 2009).

42.6.1.4 SOD-1 Clearing Agents

Motor neurons may have a high threshold to activation of the heat shock protein pathway, which is involved in protein repair. Arimoclomol, an inducer of heat shock proteins, delays disease progression and improves survival in the murine model (Kieran et al. 2004). In safety trials, arimoclomol was shown to be well tolerated and safe at up to 300 mg/day in ALS patients (Cudkowicz et al. 2008). Additional phase II/III clinical trials are underway.

An FDA-approved drug for malaria treatment, pyrimethamine (Daraprim) is showing promise in ALS treatment. Pyrimethamine reduces SOD1 levels in cultured cells, mice,

and ALS patients (Pandya et al. 2013; Lange et al. 2013). Another SOD-1 clearing agent, edaravone, was shown to effectively slow symptom progression in mSOD1-G93A mice but further characterization of edaravone is necessary (Pandya et al. 2013).

42.6.1.5 Ribonucleic Acid Interference

Mutant gene expression leading to neurodegeneration might be reduced by gene silencing techniques. RNA interference (RNAi) molecules have been generated that target the SOD1 gene and reduce its expression. RNAi has prolonged survival in the SOD1 model (Ralph et al. 2005; Raoul et al. 2005). The technology will only apply to the 2–5% of patients whose ALS is due to mutations in the gene encoding SOD1, and so obtaining adequate numbers of patients to conduct clinical trials may be difficult.

42.6.1.6 Olesoxime

mSOD1 aggregation in the mitochondria is linked with spinal cord-specific dysfunction of mitochondria and may play a role in the pathogenesis of ALS. Neuroprotection could therefore be achieved using compounds that target mitochondria (Pandya et al. 2013). Olesoxime (TRO-19622 or mitotarget) has been shown to be protective in in vivo and in vitro models of ALS. In mSOD1-G93A mice, olesoxime increased lifespan by delaying disease onset rather than slowing disease progression (Pandya et al. 2013; Bordet et al. 2007).

42.6.1.7 Combination Therapy

Because of the complexity of the mechanisms underlying neurodegeneration, testing combinations of agents that target different processes could theoretically be more beneficial than individual agents alone. The combinations of minocycline and creatine, and celecoxib and creatine showed additive effects in the SOD1 model (Klivenyi et al. 2004; Zhang et al. 2003). Phase II/III clinical trial have been completed and results are pending (Gordon et al. 2004).

42.6.2 Alzheimer Disease

42.6.2.1 Statins

Statins, used predominantly in the treatment of hypercholesterolemia, act by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which regulates the synthesis of cholesterol. Statins are also agonists of peroxisome proliferator activated receptors (PPARs), which are part of the nuclear receptor superfamily and when activated, can suppress transcription of pro-inflammatory genes (Chinetti et al. 2000). In vitro and in vivo studies have shown that a decrease in serum cholesterol inhibits the production of beta amyloid and plaque (Fassbender et al. 2001; Simons

et al. 1998). A randomized, controlled trial evaluating the efficacy of simvastatin on disease progression in patients with AD provided conflicting results. Overall, the plasma levels of beta amyloid protein remained relatively unchanged after 6 months of treatment, but those with better cognitive function demonstrated a greater reduction in beta amyloid protein levels than patients with more severe dementia (Simons et al. 2002). The mechanism of how lowering cholesterol affects beta amyloid production is still unclear; studies have demonstrated that statins do not alter beta amyloid morphology (Hoglund et al. 2004). It may be that the neuroprotective properties of statins are due to antioxidant and anti-inflammatory effects.

42.6.2.2 Secretase Inhibitors

Beta amyloid peptide is cleaved from the beta amyloid precursor protein by the beta secretase (or beta amyloid cleaving enzyme, BACE) and gamma secretase enzymes (Cummings 2004). Experimental studies in animal models have shown that secretase inhibition lowers the amount of beta amyloid protein in the brain and can reduce the formation of beta amyloid protein aggregates (Dewachter and Van Leuven 2002). While BACE and gamma secretase are viable therapeutic targets, they have thus far been refractory to drug development. Gamma secretase inhibitors diminish beta amyloid protein load in the brain, but also cleave other proteins essential for signaling in cellular proliferation. Gamma secretase is essential for embryogenesis and gamma secretase knockout mice are not viable (Dewachter and Van Leuven 2002). Until these limitations can be overcome, the potential for gamma secretase inhibitors for AD therapy is restricted.

BACE is another therapeutic target. One study showed that raising BACE expression increased plaque load in beta amyloid precursor protein-transgenic mice (Willem et al. 2004). However, the development of pharmacological inhibitors of BACE has been challenging due to its large catalytic site, which does not bind small molecules effectively.

42.6.3 Huntington Disease

42.6.3.1 Sodium Phenylbutyrate

Impaired gene transcription is thought to be a key event in the cascade that leads to neurodegeneration in HD. Mutant huntington can bind to histone acetyltransferase, reducing histone acetylation and ultimately suppressing gene transcription. HDAC inhibitors act to relax the DNA conformation and facilitate transcription by keeping DNA acetylated. HDAC inhibitors may also be effective in preventing sequestration of certain transcription factors by mutant huntington during the process of aggregation (Ferrante et al. 2003). Sodium phenylbutyrate was tested in the R6/2 mouse model

of HD and was shown to significantly increase survival, improve motor function and minimize brain atrophy (Gardian et al. 2005). An initial dose-finding study of sodium phenylbutyrate in humans was conducted and found to be safe and well-tolerated but no further clinical trials were completed (Hogarth et al. 2007).

42.6.3.2 Ethyl-EPA

Ethyl-EPA (ethyl-eicopentaenoate) is a semisynthetic, highly purified derivative of the n-3-fatty acid EPA. Ethyl-EPA acts to preserve mitochondrial function by targeting peroxisome proliferator activated receptors. Ethyl-EPA reduces loss of neuronal function by inhibiting caspase activation and apoptosis, and by reducing mitochondrial damage in models of neurodegeneration (Van Raamsdonk et al. 2005). A randomized, double-blind, placebo-controlled trial of ethyl-EPA was conducted in patients with HD. This study showed that there was no benefit in the intent to treat cohort of patients with HD. However, exploratory analysis revealed that a significantly higher number of patients in the cohort treated with ethyl-EPA showed stable or improved motor function (Puri et al. 2005). Phase III clinical trials of ethyl-EPA on HD patients revealed no benefit in total motor score change, function, cognition, or global impression compared to control patients (Huntington Study Group TREND-HD Investigators 2008).

42.6.4 Parkinson Disease

42.6.4.1 Creatine

Administration of MPTP produces parkinsonism in experimental animals by a mechanism involving impaired energy production. MPTP is converted to 1-methyl-4-phenylpyridinium (MPP+), which blocks complex I of the electron transport chain. Supplementation with creatine, a substrate for creatine kinase, may increase phosphocreatine and cyclophosphocreatine levels, buffer against ATP depletion and exert neuroprotective effects. Oral supplementation of creatine produced significant protection against MPTP-induced dopamine depletion in mice (Matthews et al. 1999). Based on these results, a randomized, double-blind, futility clinical trial of creatine was conducted in 2006 (NINDS NET-PD Investigators 2006). The study found that creatine and separately, minocycline, could not be rejected as futile and that they should be considered for phase III clinical trials to determine whether they alter the long-term progression of Parkinson disease. The trial was terminated early after showing treatment with creatine for at least 5 years did not improve clinical outcomes. Thus the findings did not support the use of creatine in patients with Parkinson's disease (Kieburtz et al. 2015).

42.7 Review Questions/Problems

- Which one of these medications for Alzheimer's disease is NOT a cholinesterase inhibitor?
 - galantamine
 - rivastigmine
 - tacrine
 - memantine*
- Amantadine is a useful medication in the treatment of dyskinesias in Parkinson Disease. What side effect limits its use in the elderly?
 - diarrhea
 - sialorrhea
 - dyskinesias
 - neuropsychiatric and cognitive effects*
- Which is the only FDA approved medication for amyotrophic lateral sclerosis?
 - memantine
 - lamotrigine
 - riluzole*
 - coenzyme Q10
- Memantine is approved for use in which disease?
 - moderate to severe Huntington disease
 - early stages of Huntington disease
 - moderate to severe Alzheimer disease*
 - early stages of Alzheimer disease
- In ALS patients, sialorrhea can be a major symptom as bulbar weakness worsens. Which of these medications is ideal for a patient with swallowing difficulty?
 - amitriptyline
 - scopolamine*
 - diphenhydramine
 - glycopyrrolate
- Which one of these medications are used to treat chorea in Huntington disease?
 - risperidone*
 - levodopa
 - fluoxetine
 - bupropion
- Proposed mechanisms in neurodegenerative diseases include all of the following EXCEPT:
 - oxidative stress
 - apoptosis
 - inflammation
 - excess GABA*
- Which of these medications currently being investigated in neurodegenerative diseases have anti-apoptotic properties?
 - remacemide
 - minocycline*
 - insulin growth factor
 - ceftriaxone

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Abstract

Substantial progress has been made over the past 25 years in terms of understanding the underlying pathophysiology, developing diagnostic and monitoring tools, and, most importantly, in the treatment of Multiple Sclerosis (MS) and its symptoms. Clinical trials have contributed greatly to this progress, as both successes and failures have helped understand that MS is far more complex than was initially appreciated. The spectrum of clinical phenotypes was thought to be an expression of varying severity of the disease rather than of different pathophysiological processes. Generally, treatment was reserved for later stages of the disease, when accrual of disability was well underway.

Keywords

Experimental allergic encephalomyelitis • Gadolinium • Glatiramer acetate • IFNBeta-1a • IFNBeta-1b • Mitoxantrone • Natalizumab • Neuromyelitis optica • Sclerosis

43.1 Introduction

Multiple Sclerosis (MS) is the most common disease of the central nervous system (CNS) to cause disability in young adults. It is an immune-mediated inflammatory disease of the brain and spinal cord that typically presents in the second or third decade of life and affects women about two times more often than men (Noseworthy et al. 2000). The most common clinical presentation, representing approximately 85–90 % of cases, is of relapsing-remitting disease (RRMS) characterized by episodes of discrete neurological symptoms that resolve fully or with some residual deficit. The very first clinical episode is termed clinically isolated syndrome (CIS). Over time, the frequency of relapses decreases and, in the majority

of patients, slowly progressive accumulation of disability ensues; this stage of the illness is termed secondary progressive MS (SPMS). Approximately 10–15 % of people with MS present with slowly progressive disease from the onset (primary progressive MS or PPMS) (Miller and Leary 2007). Additionally, a progressive-relapsing (PRMS) course, described as progressive disease from onset with superimposed acute relapses, has inconsistently been referred to as a distinct presentation in the literature. These four phenotypes, based on description of clinical course alone, were first formally proposed in 1996 (Lublin and Reingold 1996) and have represented the basis for categorization of MS for research and clinical management purposes.

At the cellular level, a prominent role of T cells in the immune mediated injury in MS has long been recognized. It is now clear that in addition to T cells, B cells are also important players. A role of innate immunity is increasingly appreciated with macrophage and microglia acting as antigen presenting cells, contributing to oligodendrocyte injury and creating a chronic, diffusely inflammatory environment (Moore et al. 2015). Histopathological studies have shown that distinct immune-mediated pathways may lead to acute demyelination in individual patients (Lassmann et al. 2001)

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and that mechanisms driving immune-mediated injury may change over time, with shifts between predominantly adaptive immune responses to innate immunity over the natural course of the disease (van Horssen et al. 2012) and possibly compartmentalization of the immune response in later stages of the disease (Lassmann et al. 2012).

Pathologic changes are appreciated throughout the white matter such that MS can no longer be considered simply a focal or multifocal disease; and lesions are not restricted to white matter, but occur throughout the CNS parenchyma, including cerebral cortex and deep gray matter (Dutta and Trapp 2014).

Moreover, it is now clear that in parallel to immune-mediated injury, a neurodegenerative process is already evident in the earliest stages of the disease with extensive neuroaxonal degeneration (Trapp et al. 1999). Neurodegenerative features increase over the course of the disease, predominating in later phases, and are now recognized to be the primary mechanism leading to accrual of irreversible disability over time. The relationship between inflammation and neurodegeneration remains unclear, however at least in the earliest stages of the disease it is thought that axonal damage is a direct result of the acute inflammatory lesion.

As a result of an improved understanding of the disease, increasingly more potent and targeted immunotherapies have been developed together with a paradigm shift toward treating MS in its earliest stages in an attempt to arrest or slow the process before irreversible injury occurs. To this end, MRI has been instrumental, permitting to satisfy diagnostic criteria of dissemination in time and space (denoting multifocal, recurring bouts of inflammation; McDonald diagnostic criteria) already at the time of first clinical presentation (CIS) (Polman et al. 2011). Recently, the classification of clinical phenotypes of MS originally proposed in 1996 (Lublin and Reingold 1996) was revised (Lublin et al. 2014). The new schema, which takes into account both inflammatory and neurodegenerative contributions, is more complex but more accurately reflects current understanding of underlying biology of the disease. Grouping of patient cohorts based on biologically meaningful differences is expected to enhance the ability of clinical trials in MS to further advance our understanding of this disease.

Immune abnormalities in cerebrospinal fluid (CSF) have been extensively studied in multiple sclerosis. The presence of oligoclonal bands in CSF are often used as evidence of polyclonal B cell activation and as a supportive criteria for diagnosis of Primary progressive MS (Polman et al. 2011). It is noted that while FDA-approved therapies are able to suppress relapses and contrast enhancing lesions by MRI, most do not have any effect on the oligoclonal bands, likely due to the lack of any effect on long-lived plasma cells. CSF cytokine abnormalities and markers of neuronal injury may

on the other hand have potential role as biomarkers for monitoring disease progression and effects of treatment, (Komori et al. 2015) but to date have not entered clinical practice.

43.2 FDA-Approved Disease Modifying Therapies (DMT)

The armamentarium available for treatment of MS has grown rapidly over the past two decades. The first medications to be FDA-approved were interferon β -1b in 1993, followed in 1996 by glatiramer acetate. Since that time, a total of 10 additional drug products, as well as a generic formulation of glatiramer acetate, have been approved (Fig. 43.1; Tables 43.1 and 43.2). The use of MRI in monitoring clinical trials in MS was transformative. The ability to quantify contrast enhancing lesions and accumulation of new lesions was far superior to the clinical scales and lead to the identification of drugs that could modify the course of RRMS. All the approved medications target and suppress the active inflammation that underlies clinical relapses and contrast enhancement detected by MRI. None of these medications have proven effective in arresting the more gradual accumulation of disability occurring in progressive forms of MS.

43.3 Trials in Progressive MS

Indeed, the failures of these medications in progressive forms of MS have been instrumental in revealing the distinct nature of the inflammatory and neurodegenerative processes in this disease. This was first recognized in the collective experiences of trials of interferon β , in which it quickly became clear that patients beginning treatment later in the course of their illness did not gain the same benefits as those who began treatment early. While trials in SPMS consistently showed reductions in relapse rate and accumulation of new MRI lesions, time to disability progression (assessed by EDSS) was not impacted (Secondary Progressive Efficacy Clinical Trial of Recombinant Interferon-Beta-1a in 2001; Cohen et al. 2002; Andersen et al. 2004; Kappos et al. 2004; Panitch et al. 2004; Bates 2011).

Similar results have been replicated even with the most effective anti-inflammatory agents available. Coles et al. (2006) reported a cohort of 36 patients with SPMS treated with alemtuzumab, who were subsequently followed longitudinally for 14 years. At study entry, mean disease duration was 11 years; EDSS was 6.5; and patients had an average of 0.7 relapses per year. Alemtuzumab led to reduction in annualized relapse rate (ARR) to 0.02 and a 90 % decrease in new lesion formation by MRI. Despite this, the EDSS continued to worsen, on average by 0.2 points per year with associated brain atrophy by MRI. At a follow up 7 years later, continued

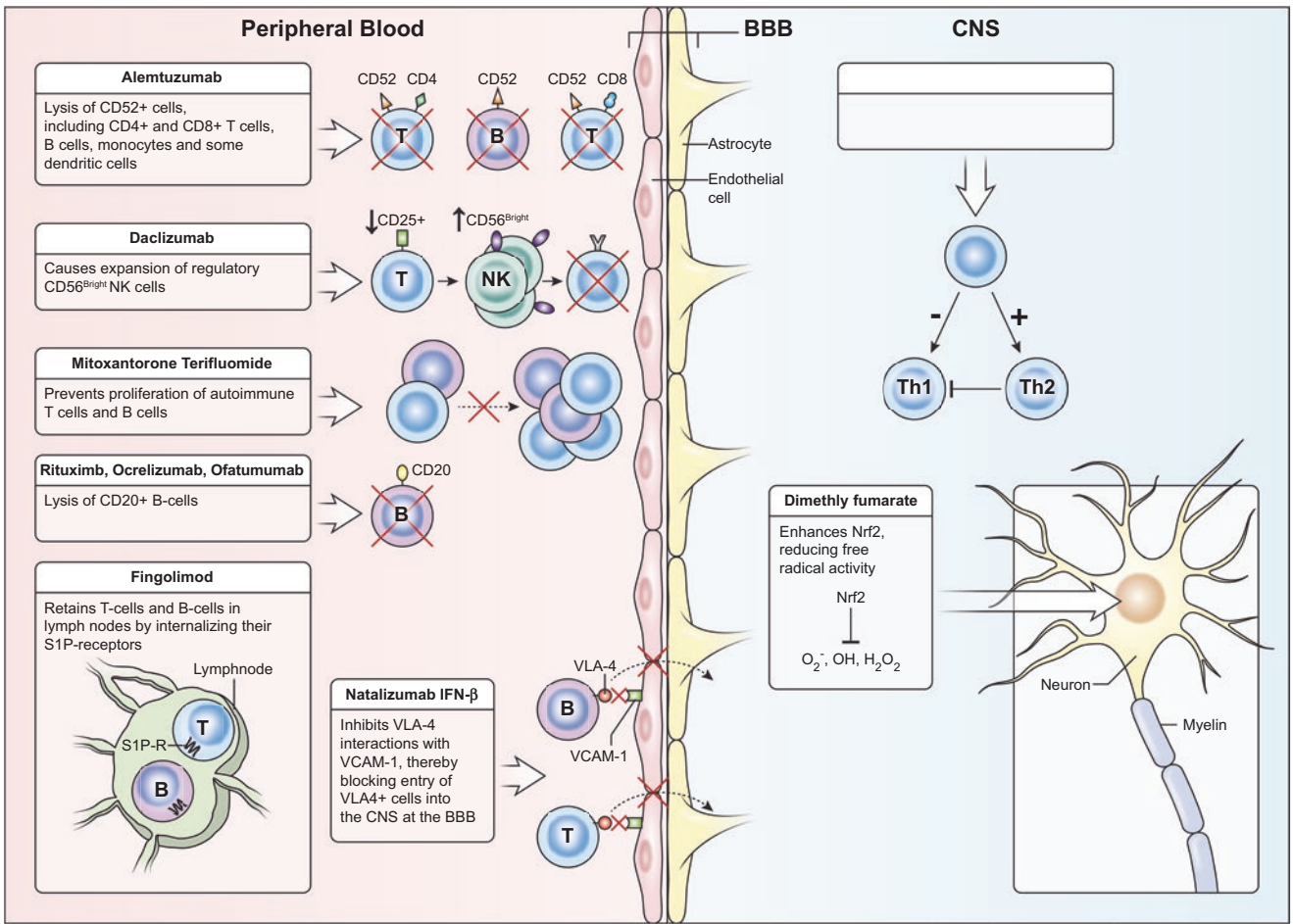


Fig. 43.1 Mechanisms of Action of approved disease modifying therapies for MS

Table 43.1 FDA approved disease modifying therapies for MS

	IFN beta 1-b		IFN 1-a		Glatiramer acetate		Pegylated IFN
Brand	Betaseron	Extavia	Avonex	Rebif	Copaxone		Plegridy
Year Approved	1993	2009	1996	2002	1996	2014	2014
Dosing	QOD		Weekly	3× week	QD	3× week	QOW
Reduced RR (%)	33		18	30	29	34	36
MRI(%)	83		50	88	35	45	86
Adverse reactions	Flu-like symptoms. Injections site reactions. Liver toxicity. Depression. Allergic reactions. Leukopenia. Anemia. Seizures				Injection site reactions. Immediate post-injection reaction		Flu-like symptoms. Injection site reactions. Depression. Allergic reactions. Liver toxicity. Anemia. Seizures. Congestive heart failure
Monitoring	Hepatic panel and CBC baseline, month 3, month 6 then yearly				None		As for other IFN formulations

brain atrophy had occurred despite stable T2 lesion load, again noted at follow-up 14 years post treatment, by when median EDSS had increased to 7.5 (Hill-Cawthorne et al. 2012). Subjects with the highest T2 lesion load at baseline were noted to have progressed the most during the observation period (Coles et al. 2006). This study suggested that while progression may occur through non-inflammatory mechanisms, accrual of disability may somehow depend upon earlier inflammatory injury (Jones and Coles 2014).

Early trials of hematopoietic stem cell transplantation (HSCT) were conducted in patients with progressive forms of MS, often in patients with significant disability. These studies too showed that despite aggressive immunosuppression, disability continued to progress, consistent with non-inflammatory factors underlying progressive neurodegeneration. Long-term follow-up showed that patients who did the best were those who had active CNS inflammation before HSCT (Fassas and Mancardi 2008).

Table 43.2 FDA approved disease modifying therapies for MS

	Mitoxatrone	Natalizumab	Fingolimod	Teriflunomide	Dimethyl fumarate	Alemtuzumab
Brand	Novantrone	Tysabri	Gilenya	Aubagio	Tecfidera	Lemtrada
Pivotal trials		AFFIRM, SENTINEL	FREEDOMS I, FREEDOMS II, TRANSFORMS	TEMPO, TOWER	DEFINE, CONFIRM	CARE-MS I, CARE-MS II
Year approved	2000	2004, 2006	2010	2012	2013	Under review
Dosing	Every 3 months	Monthly IV	Daily PO	Daily PO	BID PO	Annual course IV
Reduction RR (%)	67	68	54	31	44–50	55 (naïve) 49 (treated)
MRI (%)	84	92	82	57	74–90	>90
Adverse reactions	Blue-green discoloration of urine and sclera. Myelosuppression. Hepatotoxicity. Congestive heart failure	Headache. Gastrointestinal distress. Hypersensitivity reactions. Hepatotoxicity	Headache. Diarrhea. Hepatotoxicity. Bradycardia with first dose. Macular edema	Hair thinning. Diarrhea. Nausea. Hepatotoxicity. Renal toxicity. Peripheral neuropathy. Leukopenia. Hypertension	Flushing. Gastrointestinal distress. Hepatotoxicity. Lymphopenia. Hypersensitivity	Rash. Headache. Fever. Thyroid dysfunction. Infusion reactions. HSV reactivation
Recommended monitoring	CBC and LFT baseline and prior to each dose	CBC and LFT baseline and Q6 months	CBC baseline and every 6 months	UPT	CBC and LFT baseline and every 6 months	Baseline and monthly CBC (platelets). Serum creatinine and UA at baseline and then monthly
	Echocardiogram baseline and prior to each dose; yearly following discontinuation	JCV baseline and every 6 months	Baseline VZV titer	LFT baseline monthly in 6 months — 1st year, then Q6 month		Baseline and Q3 months TFT
	UPT before each dose	In JCVAb + subjects, MRI brain Q 3–6 months	Ophtho exam baseline and month 3 (continued in patients with DM or uveitis)	Baseline TB test		Monitor lab work for 48 months after last dose
			Baseline ECG; 6 h monitoring with first dose			Baseline and yearly skin exams

The best candidates for HSCT have since been determined to be young patients with short disease duration, in RRMS phase (Krasulova et al. 2010) and with active inflammation by MRI (Fassas et al. 2011).

Thus, clinical trial experience has strengthened the notion that progressive neurodegeneration in MS is not directly driven by active inflammation. It is however important to note that while progressive forms of MS have significantly less prominent inflammatory infiltrates compared to RRMS, histopathology clearly shows that adaptive and innate immune mechanisms do persist (Bruck et al. 2002; Kutzelnigg and Lassmann 2014). Whether neurodegeneration is truly an independent process or whether lack of efficacy of anti-inflammatory strategies might be due to qualitative differences in this smoldering inflammation or to its compartmentalization behind an intact barrier, is not yet fully clear.

43.4 Clinically Isolated Syndrome and Radiologically Isolated Syndrome

A duality of MS pathophysiology is now widely accepted and has reshaped management and research in this disease. It has led to the notion of a window of therapeutic opportunity for immunotherapies early in MS; to the development of diagnostic criteria permitting earlier and earlier diagnosis (Polman et al. 2011); and to a focus on CIS clinical trials. Several clinical trials have demonstrated the benefit of initiating DMT after the very first clinical manifestations of MS (Jacobs et al. 2000; Comi et al. 2001, 2009; Kappos et al. 2009) leading to delay in conversion to clinically definite MS, as defined by occurrence of relapse. As a result, in the United States, first-line injectables are approved for use in this setting (Sellner et al. 2010).

How early to initiate treatment remains a dilemma. As MRI has become more commonplace, the incidental identification of patients meeting radiological criteria for MS, despite no clinical symptoms, has been described with prevalence estimated to be between 0.06 and 0.7 % of the general population. Cohorts of these patients have been followed longitudinally and it has been seen that approximately one-third go on to develop neurological symptoms over a follow-up period of 2–5 years (Granberg et al. 2013).

How to manage these patients clinically has not yet been studied in the controlled trial setting and different approaches have been proposed ranging from doing nothing unless the patient becomes symptomatic; intermittently following the patient clinically and radiologically; to the initiation of off-label treatment. Since in the individual patient the clinical relevance of these MRI findings long term is uncertain, and due to the potential impact of DMT both in terms of risk and cost, this latter approach cannot be advocated until studies demonstrate benefit.

43.5 Treatment Strategies: Escalation vs. Induction

The currently available DMTs differ in terms of mechanism of action of immunomodulation, efficacy, side effects and risk profile. Additionally, factors such as route and frequency of administration, general tolerability as well as type of monitoring required are important factors for patient acceptability and adherence. Very few head-to-head comparison studies have been carried out and unfortunately, there is still little data on which to construct a rational framework for optimal therapeutic management.

Most common treatment is initiated with a moderately effective, low risk agent following which patients are monitored for evidence of clinical and radiological disease activity. Often patients can remain stable long-term on such a regimen. A decision to switch therapy is based on determination of treatment failure. There are no specific criteria to define treatment failure, but generally this can be due to insufficient suppression of disease activity or due to inability of the patient to comply with the treatment regimen.

Adherence to DMTs has been reported to be about 60 %, (Treadaway et al. 2009) which is comparable to self-administration of treatments in other chronic illnesses (Brandes et al. 2013). This is often due to unrealistic expectations of treatment effects, such as expectation of resolution of established neurological deficits, or because patients are not adequately educated about possible adverse reactions of therapy. To improve adherence, identification of goals of therapy as well as education and assistance with management of side effects are of great importance. Generally switching therapy before 6 months due to poor tolerability should be discouraged as this improves over time.

Switching therapy in this paradigm can occur as a treatment “escalation,” when a therapy with greater efficacy is initiated, or as a “lateral switch” to a drug with comparable efficacy but different mechanism of action. One such treatment algorithm is provided in Fig. 43.2.

An alternative treatment strategy is that of aggressive induction therapy to suppress all inflammatory activity and possibly “reset” the immune repertoire, followed by long-term maintenance therapy. At this time, there are no studies supporting this strategy as effective for improving long-term outcomes. With the wider use of powerful immunosuppressive strategies, such as alemtuzumab and HSCT, the value of such a treatment strategy will be evaluated.

In the future, development of pathway-specific biomarkers may lead to a new era of personalized medicine with the ability to identify optimal treatment strategies based on biological patterns.

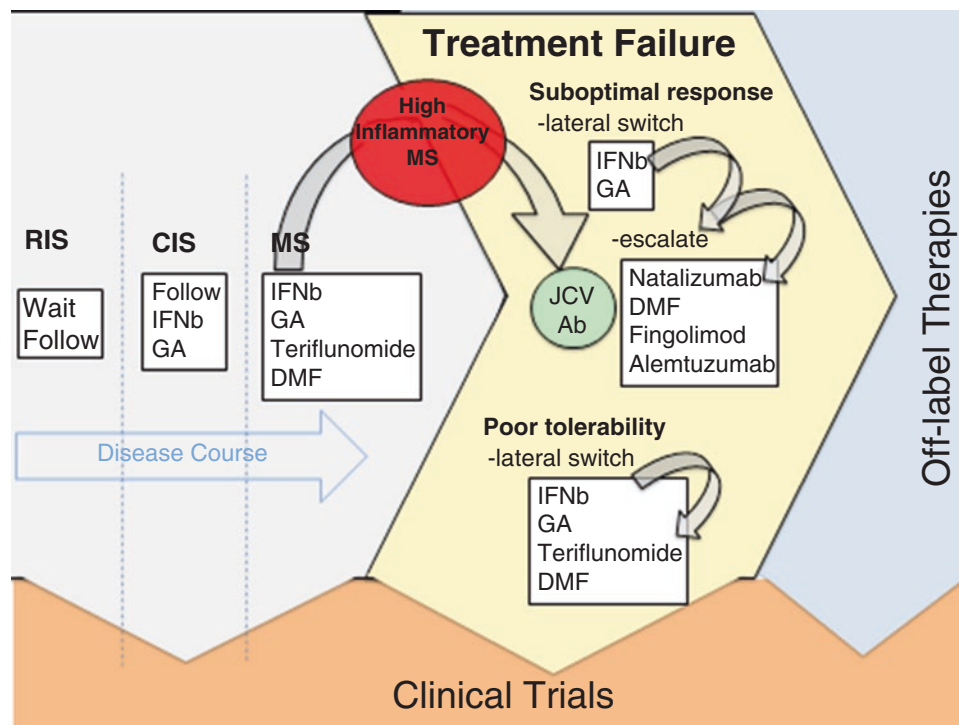


Fig. 43.2 Treatment Algorithm. RIS: current evidence does not support initiation of therapy but rather to wait and/or actively follow patients closely for development of symptoms. CIS: evidence suggests that initiating DMT can delay conversion to clinically definite MS; first line injectable therapies can be pursued or patients can be followed clinically off therapy. MS: first-line therapy should be initiated, typically with medications having moderate efficacy and good safety profile. For patients with high inflammatory disease burden, consideration can be given to directly initiating therapies with greater efficacy albeit

greater risk profile. JCV Ab status can guide decision. Treatment failure should prompt change in therapy. If this is due to suboptimal treatment response, either lateral switch to another first line agent or treatment escalation to class of drug with greater efficacy albeit greater risk profile can be pursued. If this is due to poor tolerability, it is reasonable to try lateral switch first. JCV Ab status and patient preference can help guide decision. At any stage of disease course, participation in Clinical trials is an option. Off label therapies should be reserved for clinical trial setting and/or for patients who have failed approved treatments

43.6 The DMTS

43.6.1 First-Line Injectable Therapies

Interferon- β (1b, 1a, peginterferon β -1a)

First formulation, interferon β -1b SQ QOD approved in 1993.

43.6.1.1 Pregnancy Category C

43.6.1.2 Background

The failure of interferon gamma ($\text{IFN}\gamma$) as a therapeutic (Panitch et al. 1987) and the observation that it provoked exacerbations led to the investigation $\text{IFN}\beta$ for the treatment of MS as it was a known inhibitor of $\text{IFN}\gamma$. Interferons are naturally occurring proteins that mediate antiviral, anti-proliferative and immunomodulatory cellular response to various stimuli; exactly which of these effects are relevant for the efficacy of this class of medications in MS is not entirely clear.

43.6.1.3 Mechanism of Action

Interferons have pleiotropic biological effects. Immunomodulatory effects believed to be important for therapeutic efficacy include up-regulation of anti-inflammatory cytokines (IL-4, IL-10, IL-27) and down-regulation of pro-inflammatory cytokines (IL-2, IL-12, $\text{IFN}\gamma$, $\text{TNF}\alpha$); inhibition of T cell adhesion and migration across the blood-brain barrier; induction of T-regulatory cells; and reduction in T cell activation (Dhib-Jalbut and Marks 2010).

43.6.1.4 Formulations

Several formulations are available including $\text{IFN}\beta$ 1a administered by intramuscular injection; $\text{IFN}\beta$ 1a administered by subcutaneous injection; $\text{IFN}\beta$ 1b administered by subcutaneous injection; and peginterferon β -1a, which is interferon β -1a conjugated to a methoxy-polyethylene glycol (PEG molecule). This latter modification renders the drug more stable, permitting a less frequent dosing schedule of every 2 weeks.

43.6.1.5 Efficacy

Interferon β_1b subcutaneous, interferon β_1a subcutaneous and interferon β_1a intramuscular preparations have been shown to decrease the relative risk of annualized relapse rate by 34 % (The IFNB Multiple Sclerosis Study Group 1993), 33 % (Ebers 1998) and 18 % (Jacobs et al. 1996) respectively as compared to placebo, while reduction of gadolinium enhancing lesions was 83 %, 88 %, and 50 % respectively. These studies suggest a dose response with this class of drugs.

The phase III ADVANCE study (Calabresi et al. 2014) demonstrated comparable efficacy of pegylated interferon to unconjugated formulations, with a reduction in adjusted annualized relapse rate of 36 % as compared to placebo and an 86 % reduction in gadolinium enhancing lesions. The major advantage of the pegylated drug is the less frequent dosing, thereby improving tolerability, as well as possibly a lower incidence of neutralizing antibodies (Hu et al. 2012; Craddock and Markovic-Plese 2015).

It is worth noting that IFNB formulations are not effective for neuromyelitis optica (Tanaka et al. 2009) and may even lead to exacerbation of disease (Shimizu et al. 2010; Uzawa et al. 2010). Such paradoxical reactions should prompt reconsideration of the MS diagnosis.

43.6.1.6 Side Effects

The most frequent side effects, shared by all the interferon formulations, include flu-like symptoms, which occur in up to 75 % of patients (Moses and Brandes 2008).

Flu-like side effects, including fever, chills, myalgias, arthralgias and headache, usually begin within a few hours of administration and improve within 24 h. Typically these side effects lessen over time, with most patients noting improvement within weeks—3 months of initiation of therapy. It has been noted that low body weight and female gender are associated with greater risk of these side effects (Moses and Brandes 2008).

Strategies to mitigate flu-like side effects include beginning dosing with reduced dose (one-quarter to half dose) and titrating up slowly every 2–4 weeks. Premedication with nonsteroidal analgesics, to be taken at the time of injection and then as needed over the subsequent 24 h, is helpful. Timing of dose administration to best suit a patient's lifestyle, (i.e.—dosing in the evening such that symptoms occur during sleep or if sleep is disrupted, dosing earlier in day).

For patients experiencing significant flu-like symptoms, low dose prednisone (30 mg PO for the first 2 weeks, tapered over 2 weeks) or alternatively 10 mg given at time of injection and then every 4–6 h for the subsequent 12 h can be considered. Pentoxifylline 800 mg given twice daily as an anti-inflammatory drug that blocks cytokine production has also been shown to be of benefit and could be considered if other strategies do not work (Brandes et al. 2009).

Some degree of injection site reaction can occur in as many as 86–92 % of patients. These are most common with subcutaneous administration and vary greatly in terms of severity, with skin necrosis representing the most severe manifestation. This latter has been reported in up to 3–4 % of patients receiving subcutaneous injections (Moses and Brandes 2008).

Injection technique is critical for mitigating these reactions. Keeping the site clean; rotating sites and avoiding sites that develop worse reactions; icing skin or using a topical anesthetic cream before injecting; warming the syringe or vial in one's hand prior to injecting can reduce pain; and use of autoinjectors can all be helpful strategies. Typically injection site reactions lessen over the first 56 months of treatment.

Laboratory abnormalities, including hepatic enzyme elevations and lowered white blood cell counts occur in almost 40 % of patients. The majority of patients with mild elevations ($<2.5 \times \text{ULN}$) will return to normal range despite continued treatment with IFN. Greater elevations ($2.5\text{--}5 \times \text{ULN}$) should prompt lowering or suspension of dosing and close monitoring; once values return to normal range, re-challenge can be considered. Greater than $5 \times \text{ULN}$ should prompt discontinuation of treatment and diagnostic work-up. All patients should be counseled to limit exposure to common hepatotoxins, including alcohol and acetaminophen. IFN-induced leukopenia does not typically improve with dose reduction and usually fails re-challenge.

Transient, subclinical thyroid dysfunction and autoimmunity has been reported in post-marketing surveillance.

A higher incidence of depression, already more common in MS than the general population, is associated with IFN treatment (40 % in IFN-treated patients compared to 21 % in GA-treated patients). Mood should be monitored and treated as appropriate (Patten et al. 2003).

Headache is reported with IFN treatment, either together with flu-like symptoms or independently—and can be managed with triptans as needed.

43.6.1.7 Monitoring

It is recommended that white blood cell counts and hepatic enzymes at baseline and again 1, 3, and 6 months after initiating treatment, with yearly checks thereafter as long as values are stable.

43.6.1.8 Special Considerations: Neutralizing Antibodies (NAbs)

Up to 45 % of patients receiving treatment with preparations of interferon beta will develop NABs. These typically develop within 2 years, most often in the first 4–6 months, of initiating treatment. In some patients this may be associated with treatment failure, as defined by persistent clinical and/or MRI disease activity. IFNB1b preparations are more immunogenic

than IFN β 1a preparations, with intramuscular IFN β 1a having the lowest incidence of NABs. This latter may be due both to formulation, dosing and to route of administration. Reported frequencies of neutralizing antibodies are 2–6 % for IFN β 1a given intramuscularly; 12–28 % for IFN β 1a given subcutaneously and 28–47 % for IFN β 1b when given subcutaneously (Bertolotto et al. 2014). Increased likelihood of development of NABs may be related to the presence of antigen presenting cells such as the Langerhans cells in the skin. Of note, NABs cross react with other formulations; therefore switching to a different formulation is not beneficial.

Concomitant autoimmune diseases:

IFN β is ineffective and can even worsen diseases where the Th17 immune response is prominent such as neuromyelitis optica, systemic lupus erythematosus, rheumatoid arthritis and ulcerative colitis (Axtell et al. 2011). Hence patients should be screened for other autoimmune diseases and IFN β should be avoided if other autoimmune diseases are concomitantly present.

43.6.2 Glatiramer Acetate

Approved in 1996

43.6.2.1 Pregnancy Category B

43.6.2.2 Background

The discovery of glatiramer acetate was fortuitous. In the production of synthetic polypeptides with amino-acid composition similar to MBP with the intent of creating encephalitogenic mixtures for generating EAE, some of the copolymers were found to protect mice from disease. Cop 1, randomly composed of L-alanine, L-lysine, L-glutamic acid and L-tyrosine in a molar ratio of 4.2:3.4: 1.4:1.0 was the most effective in ameliorating EAE and was ultimately developed as a disease modifying agent (Teitelbaum et al. 1971).

43.6.2.3 Mechanism of Action

The therapeutic effects of GA are complex and not completely understood.

Most studies indicate the principle mechanism of GA is to shift the peripheral T cell response from a predominant pro-inflammatory Th1 to anti-inflammatory Th2 pattern. Additionally, GA-induced CD4 and CD8 regulatory immune cells are thought to enter the CNS and mediate *in situ* anti-inflammatory cytokine shifts and bystander immunomodulation (Arnon and Aharoni 2004; Aharoni 2014).

43.6.2.4 Formulations and Dosing Regimens

In 2014, a modified dosing regimen of 40 mg three times weekly was approved providing an alternative to the previous 20 mg daily SQ regimen. A generic formulation was approved in 2015.

43.6.2.5 Efficacy

Glatiramer acetate SQ daily led to a 29 % reduction in relapse rate compared to placebo (Johnson et al. 1995). The alternative thrice weekly dosing regimen was found to be at least as effective, with a reduction in relapse rate of 34 % (Khan et al. 2013). A reduction in gadolinium enhancing lesions of 35 % (Comi et al. 2001) and 44.8 % (Khan et al. 2013) respectively.

43.6.2.6 Side Effects

Glatiramer acetate is generally well tolerated with injection site reactions (in up to 73 % of patients) being the most common side effect. This latter can be associated with development of lipatrophy. Strategies for mitigating injection site reactions are analogous to those employed for IFN β formulations.

Another adverse reaction, unique to GA, is an immediate post-injection reaction, characterized by flushing, chest pain, palpitations and anxiety that can last a few minutes or more than an hour, and then spontaneously resolves. This reaction was reported to have occurred in 15 % of subjects at least once during a 2–3 year period (Johnson et al. 1995). While this reaction can be dramatic and sometimes mistaken for angina, it very rarely re-occurs and does not require discontinuation of the medication or adjustment of dosage. The exact etiology of the chest pain is unclear but does not appear to be of cardiac origin.

Asymptomatic lymphadenopathy is also associated with GA treatment and can occur in up to 30 %.

43.6.2.7 Monitoring

None

43.6.3 Oral Therapies

Fingolimod

Approved in 2010

43.6.3.1 Pregnancy Category C

43.6.3.2 Background and Mechanism of Action

Fingolimod was the first oral therapy to be FDA-approved for the treatment of MS. Its development was the result of rational drug design targeting the S1P receptor on lymphocytes, thus preventing their egress from secondary lymph organs and reducing the number of circulating lymphocytes. The drug affects naïve and central memory T cells, while sparing effector memory T cells (Chun and Hartung 2010); this may explain the relatively low incidence of opportunistic infections.

43.6.3.3 Efficacy

In registration trials, fingolimod demonstrated approximately 50 % relative reduction in ARR compared to placebo and reduction of gadolinium contrast enhancing lesions by about 82 % (Kappos et al. 2010; Calabresi et al. 2014).

43.6.3.4 Side Effects

The most common side effect reported is liver enzyme elevation in approximately 10 % of treated patients and leading to discontinuation of treatment in 3.8 %; upon discontinuation of treatment values returned within normal range in the majority of patients within a few months.

Reduction in lymphocyte counts by 73 % compared to baseline is observed within 1 month of treatment initiation, typically remaining stable thereafter. Up to 18 % of subjects develop more profound lymphopenia, with absolute lymphocyte counts below 200 (Kappos et al. 2014a).

Transient bradycardia and atrio-ventricular block following first dose administration and resolving by 1 month after treatment initiation is reported to occur in 0.5 % of subjects. Due to this effect, use of this drug is contraindicated in patients with CAD, heart failure, Mobitz type II 2nd degree or 3rd degree AV blocks; prolonged QT interval or patients being treated with Class Ia or Class III antiarrhythmic drugs (Wingerchuk and Carter 2014).

Macular edema was reported in 0.4 % of patients included in the registration trials. In the majority this developed within the first 6 months of treatment and resolved with discontinuation of treatment, and patients with history of uveitis were at increased risk for developing this adverse reaction.

Blood pressure elevations occur in approximately one third of subjects, with systolic over 160 mmHg and/or diastolic above 100 mmHg in about 9 %.

There appears to be a specific predisposition to herpes infections in patients treated with fingolimod, with overall incidence of about 11 %. To date, 6 serious infections have been reported, including 3 fatal cases, including disseminated zoster and herpes virus type-1 encephalitis (Kappos et al. 2014a); these were reported both at the higher dose (1.25 mg) explored in the registration trials as well as the lower (0.5 mg) FDA-approved dose.

PML has been reported to date in 2 patients treated with fingolimod (for 8 months and 4 years, respectively) in whom no other risk factor or drug exposure was recognized (FDA safety communications, Novartis safety communications).

43.6.3.5 Monitoring

EKG at baseline; 6 h cardiac monitoring with first dose administration. Ophtho exam at baseline and 3 months; people with DM or history of uveitis, require continued yearly monitoring. CBC at baseline and every 6 months.

43.6.4 Teriflunomide

Approved in 2012

43.6.4.1 Pregnancy Category X

43.6.4.2 Background

Teriflunomide is the principal active metabolite of leflunomide, which was approved for treatment of rheumatoid arthritis in 1998.

43.6.4.3 Mechanism of Action

Teriflunomide has a cytostatic effect on rapidly dividing T and B lymphocytes mediated by its inhibition of *de novo* pyrimidine synthesis.

43.6.4.4 Efficacy

Compared to the other DMTs, teriflunomide has relatively modest efficacy with a 31 % relative risk reduction in annualized relapse rate compared to placebo (O'Connor et al. 2011); 36 % reduction in TOWER (Confavreux et al. 2014) and a reduction of gadolinium enhancing lesions by 80 % (O'Connor et al. 2011).

43.6.4.5 Side Effects

Elevated liver enzymes occurs in about 12–14 % of patients, with elevations greater than 3×ULN in about 6 %. Current monitoring recommendations as well as black box warning for hepatotoxicity are based on the experience with leflunomide. Self-limited, mild to moderate diarrhea and/or nausea are reported in approximately 15 % of patients. Hair loss or thinning occurs in approximately 10–13 % of patients; this typically improves within 6 months and is reported to lead to treatment discontinuation in less than 1.5 % of patients. Leucopenia with decrease of about 15 % occurring in the first 6 weeks of treatment. Cases of tuberculosis were observed during clinical studies with teriflunomide, prompting recommendation perform tuberculin skin test prior to initiation of therapy. Increased blood pressure with frank hypertension in approximately 3–4 %. Peripheral neuropathy is a rare adverse reaction, occurring in about 1–2 % of patients. Based on animal studies, there is risk of teratogenicity and fetal death.

43.6.4.6 Special Considerations: Pregnancy

Teriflunomide is the only immunomodulatory drug used to treat MS shown to have a teratogenic effect. Moreover, since there is potential risk of male-mediated embryo-fetal toxicity, and therefore reliable contraception should be used by both females and males being treated with teriflunomide.

The half life of teriflunomide is 18–19 days and it can take months or up to 2 years to completely eliminate the drug (Wingerchuk and Carter 2014). For females or males desiring pregnancy, current recommendations are to discontinue treatment and undergo procedure for accelerated elimination with cholestyramine or activated charcoal; these regimens lead to 98 % decrease in teriflunomide plasma concentrations

at the end of 11 days. Following this procedure, verification that plasma concentrations are less than 0.02 mg/L is advised as this is expected to have minimal risk (package insert).

43.6.4.7 Monitoring

Complete blood cell count at baseline and at 6 months.

Liver enzymes monthly for the first 6 months—1 year.

TB screen and UPT before treatment initiation.

43.6.5 Dimethyl Fumarate

Approved in 2013

43.6.5.1 Pregnancy Category C

43.6.5.2 Background

Dimethyl fumarate (DMF) is a small molecule derived from fumaric acid. It was initially proposed as a therapeutic for psoriasis in the 1950s based on the notion that this disease was due to abnormal citric acid cycle. While this rationale did not prove to be correct, a combination of ester derivatives of fumaric acid including DMF did ultimately prove effective for the treatment of psoriasis and in 1994 this treatment strategy was approved for use in Europe (Bomprezzi 2015).

43.6.5.3 Mechanism of Action

While the precise mechanism of immunomodulation is incompletely understood, it appears that DMF promotes pleiotropic anti-inflammatory and cytoprotective activities mediated by activation of the NRF2 pathway.

43.6.5.4 Efficacy

The phase III trials DEFINE (Gold et al. 2012) and CONFIRM (Fox et al. 2012) demonstrated a relative reduction of the annualized relapse rate of 50 and 44 % compared to placebo.

43.6.5.5 Side Effects

The most common side effects experienced with this agent include flushing (40 %), gastrointestinal distress (diarrhea, abdominal pain; approximately 20 %), lymphopenia, and elevated liver enzymes (2 %).

Flushing and gastrointestinal symptoms typically decreases over time and can be mitigated by taking doses with food. Flushing can be further managed with ASA or anti-histamine, while gastrointestinal symptoms can be managed with H2 blockers and proton-pump inhibitors.

Lymphopenia is well described in patients treated with fumaric acid derivatives. The experience with DMF in MS is that mean lymphocyte counts drop 30 % during first year of treatment and then plateau. Severe, persistent lymphopenia has developed in about 2 % of patients; this was usually evident

already in the first year of treatment and persisted if treatment continued. Counts increased, although not back to baseline, within ~4 weeks after discontinuation (Tecfidera package insert December 2014).

PML has been reported in patients treated with fumaric acid derivatives for the treatment of psoriasis who experienced prolonged, severe lymphopenia with absolute lymph counts less than 500/mL. It is important to note that this has also been reported in patients in absence of severe lymphopenia (Nieuwkamp et al. 2015; Mrowietz and Reich 2013). As of April 2015, a single patient with MS and treated with DMF has been reported to have developed PML; similar to the previous experience in psoriasis, this patient had presented severe, persistent lymphocytopenia for 3.5 years (Rosenkranz et al. 2015).

43.6.5.6 Monitoring

It is recommended to monitor blood work, including CBC and hepatic panel, at baseline and then every 6 months. Treatment should be discontinued in people with absolute lymph counts below 500.

43.6.6 Intravenous Infusions

Mitoxantrone

Approved in 2000 for the treatment of worsening relapsing remitting, progressive-relapsing, secondary progressive MS.

43.6.6.1 Pregnancy Category D

43.6.6.2 Background

Mitoxantrone is a synthetic anthracenedione agent originally developed for treatment of cancer.

43.6.6.3 Mechanism of Action

It interacts with topoisomerase-2 and interferes with DNA synthesis and repair; as such, it is a potent immunosuppressant due to its effects on rapidly proliferating immune cells including macrophage, T cells and B cells. Mitoxantrone is the only disease modifying therapy approved for the treatment of secondary progressive multiple sclerosis.

43.6.6.4 Efficacy

Mitoxantrone led to a 67 % reduction in annualized relapse rate compared to placebo (Hartung et al. 2002) and an 84 % reduction in gadolinium enhancing lesions compared to methylprednisolone (Edan et al. 1997).

43.6.6.5 Side Effects

Treatment with mitoxantrone is associated with risk of irreversible congestive cardiomyopathy. Cumulative dose is the primary risk factor for cardiotoxicity. Cardiac evaluation

must be performed prior to initiation of treatment and before administration of each dose. Significant decrease of left ventricular ejection fraction or in patients with LVEF <50 % are contraindications for use. Lifetime cumulative dose must be limited to 140 mg/m² due to increased risk of cardiomyopathy above these thresholds (Marriott et al. 2010; Rivera et al. 2013). Cardiotoxicity may develop years after stopping treatment; it is thus recommended that patients undergo yearly quantitative LVEF evaluation to monitor for this delayed toxicity.

Risk of leukemia has been variably reported; taken together the incidence is estimated to be approximately 0.81 %. This too can be a delayed complication, occurring even years after discontinuing dosing (Marriott et al. 2010). Persistent amenorrhea is estimated at approximately 22 % (Rivera et al. 2013).

Additional side effects include severe local tissue damage with extravasation, temporary blue-green discoloration of urine and sclera, and myelosuppression. Mitoxantrone should not be administered in patients with ANC less than 1500 cells/mm³ and due to hepatic clearance, should not be administered in patients with abnormal liver function.

43.6.6.6 Monitoring

Evaluation of LVEF at baseline, prior to each dose and yearly after stopping treatment. CBC and LFT every 3 months prior to dosing.

43.6.7 Natalizumab

Approved 2003; suspended 2005; re-introduced 2006.

43.6.7.1 Pregnancy Category C

43.6.7.2 Background

Rational drug design targeting α 4-integrin, cell-adhesion molecule that mediates homing of immune cells to the central nervous system (Drews 2006).

43.6.7.3 Mechanism of Action

Natalizumab blocks α 4-integrin on lymphocytes, thus reducing trafficking of lymphocytes into the CNS.

43.6.7.4 Clinical Efficacy

The pivotal trials of natalizumab demonstrated a 68 % reduction of relapse rate compared to placebo, with a 92 % reduction of gadolinium enhancing lesions and 83 % reduction of new or enlarging T2 lesions (Polman et al. 2006).

43.6.7.5 Side Effects

The most common side effects include headache, infusion reactions and hepatic toxicity.

A rarer but potentially fatal side effect of natalizumab that has greatly influenced the use of this drug is progressive multifocal leucoencephalopathy (PML). Natalizumab was first approved in 2003. Following the development of PML in two subjects enrolled in the Phase III SENTINEL trial and a subject who had been treated with natalizumab for Crohn's disease, the drug was withdrawn from the market. In 2006, it was reintroduced in the United States under a restricted distribution program called the TOUCH Prescribing Program.

Risk stratification for PML of patients being treated with natalizumab can guide management. Several risk factors for the development of PML have been identified, including latent JCV infection, duration of treatment with natalizumab and history of previous treatment with immunosuppressive therapies. Current risk stratification, based on long-term follow-up of patients enrolled in clinical trials as well as post-marketing surveillance, takes into account these three variables.

JC virus infection status can be measured by serum anti-JCV Ab, a surrogate marker of JCV exposure. The risk of PML in subjects who are JCV Ab negative and who have never received prior immunosuppressive therapy is negligible and estimated to be 1:10,000.

In subjects who are JCV Ab positive, there is an overall 10-fold increase in risk for development of PML (1:1000). In subjects with greater than 2 years treatment duration, this increases to 4.6 in patients without prior immunosuppressive therapy and to 11.1 per 1000 for those with history of prior immunosuppressive therapy.

Recently it has been proposed that determination of JCV Ab titer, measured as the JCV Ab Index (determined based on reference quality control standards), can help further stratify JCV Ab positive patients. Titers below 1.5 were shown to be associated with risk of PML of 0.1 per 1000 during the first 2 years of therapy. For patients treated longer than 24 months, this risk reached 1.3 per 1000. For patients with index >1.5, the risk of PML was 1 per 1000 during the first 2 years of treatment, and reached 8.5 per 1000 for longer duration (Plavina et al. 2014). For patients who had received prior immunosuppressive therapy, the index did not predict risk. Based on these findings, current recommendations are that before initiating treatment with natalizumab, patients should undergo JCV Ab testing with index determination. Current understanding of risk of development of PML based on infection status and duration of treatment is summarized in Fig. 43.3.

Although longitudinal measurements of JCV Ab have demonstrated these values to be stable over an 18 month period in the majority of individuals (Plavina et al. 2014), the risk of exposure to JCV is recognized to be continuous over time with a seroconversion rate of about 1–2 % per year (Wingerchuk and Carter 2014).

Therefore JCV status needs to be monitored every 6 months in order to reassess risk. Close clinical and MRI

Fig. 43.3 JCV infection status and risk for development of PML

JCV Ab	Prior IS	JCV Ab Index	0-24 months	>24 months
+	-	<0.9	0.1:1,000	0.1:1,000
		0.9 - 1.5	0.1:1,000	1.3:1,000
		>1.5	1:1,000	8.6:1,000
-	+		1.6:1,000	11.1:1,000
			0.1:1,000	

monitoring, especially after the first 2 years of treatment, must be performed. New onset of clinical symptoms—especially if represented by symptoms such as aphasia, seizures, apraxia or visual symptoms which are highly suspicious of PML—must prompt discontinuation of treatment and diagnostic work up. Of note, since diagnosis of PML can occur following discontinuation of natalizumab, it is recommended that close monitoring continue for 6 months following discontinuation of treatment.

The typical clinical presentation of PML is of subacutely progressive neurological symptoms in the setting of immunosuppressed state. In such a patient, MRI can be helpful in identifying lesions suggestive of PML, characterized by T2W hyperintense white matter lesions at the gray-white junction or in the brainstem or cerebellum, with or without contrast-enhancement. The diagnosis is confirmed based on JCV PCR in CSF. In subjects with typical clinical presentation and characteristic MRI but negative CSF JCV PCR, in whom alternative diagnoses have been discarded, brain biopsy for histology and in situ hybridization can confirm PML. Plasma exchange has been shown to accelerate the removal of natalizumab from circulation (Khatri et al. 2009); therefore this is the recommended course of action should a diagnosis of PML be confirmed. Of note, following rapid removal of natalizumab, PML-related immune reconstitution inflammatory syndrome can develop, which in itself can be potentially life-threatening (Carruthers and Berger 2014).

43.6.7.6 Monitoring

Anti-JC virus antibody before initiation of treatment and then every 6 months thereafter.

CBC and hepatic enzymes and baseline and then every 6 months. For JCV Ab+ subjects, MRI of the brain every 3–6 months for PML surveillance; in such patients, it is recommended to limit treatment to 24 months.

43.6.8 Alemtuzumab

Approved 2014

43.6.8.1 Pregnancy Category C

43.6.8.2 Background

Alemtuzumab originated as the rat monoclonal directed against human CD52 known as Campath-1 (derived from the Pathology department of Cambridge University). It was subsequently developed as a T cell depleting agent as part of conditioning regimen in HSCT and as a therapeutic for lymphoproliferative disease (Hale et al. 1998).

43.6.8.3 Mechanism of Action

CD52 is a cell surface glycoprotein present on all T and B cells, monocytes and eosinophils; targeting this receptor leads to rapid and long-lasting depletion of peripheral lymphocytes. The rate of reconstitution of the immune cell compartments depends on the cell type, with B cells recovering quickly, while CD4 and CD8 T cell lymphopenia lasts 35 and 20 months respectively. It is generally believed that lymphopenia alone cannot account for the efficacy of this drug in suppressing inflammatory activity. Supported by the observation that reconstituting immune compartments are represented by

mature naïve cells dominating over memory cells, some resetting of the immune repertoire may contribute to the long-lasting benefits (Craddock and Markovic-Plese 2015).

43.6.8.4 Efficacy

The two pivotal trials showed that compared to IFN β 1a, there was a 55% and 49% reduction in ARR in CARE-MS I and CARE-MS II respectively (Cohen et al. 2012; Coles et al. 2012).

43.6.8.5 Side Effects

Almetuzumab causes infusion reactions in approximately 92% of patients; these can be serious in 3%. It is recommended to premedicate with 1000 mg methylprednisolone or equivalent prior to infusion and for the first 3 days of each treatment course. Premedication with antihistamine and/or antipyretics can also be considered. Infusion reactions can be delayed, and subjects must be observed for at least 2 h following each infusion.

An increased risk of malignancies, most commonly including thyroid cancer (0.3%), melanoma (0.3%), is recognized.

Increased risk of HSV infection, most commonly localized manifestations, usually in the first month after therapy; it is recommended to administer anti-viral prophylaxis to be continued for 2 months following treatment or until CD4 lymphocyte counts are $\geq 200/\mu\text{L}$. Test for VZV antibodies and consider vaccination for Ab negative subjects prior to initiating treatment.

Secondary autoimmunity is the most common serious side effect, most commonly manifesting as autoimmune thyroid disease (34%). Idiopathic autoimmune thrombocytopenic purpura (ITP) is reported in about 2%; and glomerular nephropathies occurred in 0.3%. All secondary autoimmune syndromes have been reported to develop even years after the last dose, and therefore patients and physicians should remain vigilant. The increased incidence of autoimmune diseases may be due to the effect of the drug on regulatory T cells; alternatively it has been attributed to homeostatic expansion of residual self-reactive clones (Zwang and Turka 2014).

Lymphopenia is a direct pharmacodynamics consequence of treatment with alemtuzumab, with absolute lymphocyte counts reaching a nadir at about 1 month post-infusion. Counts reach lower limits of normal in 40% of patients by 6 months, and in 80% by about 12 months.

UPT should be checked prior to dosing and effective contraception is recommended for 4 months following a course of treatment.

43.6.8.6 Special Considerations

Oral prophylaxis for HSV is recommended for 28 days upon initiation of treatment. Vaccination schedules must be completed 6 weeks prior to dosing. No live vaccines should be administered following dosing.

43.6.8.7 Monitoring

All monitoring to be continued until 48 months following last dose. CBC at baseline and then monthly thereafter. Serum creatinine at baseline and monthly thereafter. Urinalysis at baseline and monthly thereafter. TFTs at baseline and every 3 months thereafter. Yearly skin exams to monitor for melanoma.

43.7 Immunosuppressive Therapies in MS

Before the advent of FDA approved immunomodulatory therapies immunosuppressive therapies were the mainstay of treatment of MS, and continue to be used in patients with aggressive or refractory course of disease. A summary of evidence supporting use of these agents follows.

43.7.1 Cyclophosphamide

Cyclophosphamide is an alkylating agent that binds to DNA and disrupts cell replication. It targets rapidly proliferating cells, including immune cells, acting as a general immunosuppressant. Several studies have been conducted with the first being an open label study in 32 patients with chronic progressive MS (Hommes 1975), which showed benefit especially in patients with shorter disease duration. Subsequent studies have shown that cyclophosphamide is effective as add-on rescue therapy to IFN β 1a leading to a 45% reduction in relapse rate and 82% reduction in contrast enhancing lesions as compared to IFN β 1a alone (Smith et al. 2005). Similar efficacy was reported in an open label studies (Gonsette et al. 1977; Reggio et al. 2005).

More recently, renewed attention has been given to its use in treatment of refractory pediatric MS and one retrospective study in 17 children has suggested (Makhani et al. 2009) that this was safe and effective.

43.7.2 Methotrexate

Oral immunosuppressant inhibits dihydrofolate reductase, essential for purine and thymidylate synthesis and cell proliferation and growth. A few studies have suggested a trend toward clinical benefit in chronic progressive MS (Goodkin et al. 1995) and in RRMS (Currier et al. 1993). On the other hand, no benefit was found in a trial of methotrexate as add-on therapy to IFN β 1a (Cohen et al. 2009). Generally, systematic reviews have thus concluded there is a limited role of methotrexate for the treatment of MS (Gray et al. 2004). More recently a retrospective case series was reported suggesting benefit of intrathecal methotrexate in SPMS (Sadiq et al. 2010), however this has not been replicated.

43.7.3 Azathioprine

Steroid sparing purine analog that targets rapidly proliferating cells through inhibition of purine synthesis. A large RCT was done on 354 patients and showed a trend toward beneficial effect on some clinical outcome measures. Since then, several RCT studies have been performed which suggest modest relative risk reduction in relapse rate (20 %). More recently, a non-inferiority trial suggests that it may be at least as effective in reducing relapses and new brain lesions and interferon β formulations (Massacesi et al. 2014).

43.7.4 Mycophenolate Mofetil

Inhibitor of inosine 5'-monophosphate dehydrogenase type II, resulting in interruption of purine biosynthesis within activated T and B lymphocytes and macrophages. Limited data exists, with the largest RCT investigating MMF as monotherapy was conducted in 35 RRMS with trend toward benefit in the primary MRI outcomes (Frohman et al. 2010). A recent retrospective study in 344 patients treated with MMF suggested significant reduction in ARR by 68 % (Michel et al. 2014).

43.7.5 Cyclosporine

Selective and reversible inhibitory effect on T cells via block of calcineurin and thus downstream transcription of IL-2 and related cytokines.

A few clinical trials of cyclosporine in MS were pursued in the late 1980s/early 1990s. One study showed a 50 % reduction in relapse rate and delay to first relapse in RRMS subset. The authors concluded however that the adverse event profile, and specifically nephrotoxicity, outweighed benefits (Rudge et al. 1989).

In one of the first sizable (547 patients with progressive MS) double-blind, placebo controlled studies in MS, cyclosporine was found to satisfy one of its three efficacy criteria (delayed time to becoming wheelchair bound), failing however to show an effect on time to sustained progression or time to dependency in ADL. In this study, adverse reactions contributed to a 44 % drop out rate (The Multiple Sclerosis Study Group and Wolinsky 1990).

43.8 Emerging Experimental Therapeutic Strategies

43.8.1 Daclizumab

Daclizumab is a monoclonal antibody directed against the alpha chain of the high affinity IL-2 receptor. A newer formulation, daclizumab high-yield process, differs in glyco-

sylation and likely results in decreased antibody-dependent cellular toxicity.

The rationale for exploring daclizumab in MS was the proposed blockade of CD25+ T cells, based on the concept that MS is a CD4 mediated disease. Indeed a decrease by about 25 % of this T cell population is observed, however this does not fully account for therapeutic effects seen. Interestingly, patients treated with daclizumab were found to have a seven to eightfold expansion of immune regulatory CD56^{bright}NK cells in the blood and CSF (Bielekova et al. 2006). This expansion appears to correlate with clinical response. Other mechanisms have been described including blockade of cross-presentation of IL-2 by dendritic cells to T cells (Wuest et al. 2011) and a reduction of lymphoid inducer cells (Perry et al. 2012).

Two phase II double-blind RCT in RRMS, the CHOICE study using daclizumab as add-on therapy to IFN (Wynn et al. 2010) and the SELECT study who received daclizumab high-yield process as monotherapy.

These studies showed a 95 % reduction in relapse rate and 78 % reduction in contrast enhancing lesions compared to placebo; (Gold et al. 2013) and a 70 % reduction in CEL and 43 % decrease in relapse rate in the high dose group compared to IFN β (Wynn et al. 2010). The Phase III study DECIDE was completed in 2014.

43.8.2 Anti-B Cell Strategies: Rituximab, Ocrelizumab, Ofatumumab

CSF oligoclonal bands have been recognized as one of the most consistent findings in MS patients since the 1970s (Laterre et al. 1970; Link and Muller 1971). Traditionally however CNS immunoglobulins, including OCB, have been considered an epiphenomena and a significant role of B cells to CNS injury in MS was not fully recognized until more recently. In addition to producing antibodies, B cells act as antigen-presenting cells and contribute to the cytokine milieu. Antibody deposition is seen in acute demyelinating lesions (Breij et al. 2008) and more recently, ectopic lymphoid follicles containing B cells have been recognized in the meninges of patients with chronic MS and are associated with greater disease severity (Serafini et al. 2004).

Targeting B cells as a strategy for the treatment of MS was initially undertaken based on the observation in EAE models that B-cell deficient mice leads to characteristic lack of demyelination (Svensson et al. 2002). This was interpreted to suggest that demyelination required the presence of pathogenic T cells as well as presence of autoantibodies.

Rituximab is a chimeric monoclonal antibody, first approved for treatment of non-Hodgkin's lymphoma in 1997. In a phase II study in RRMS, (Hauser et al. 2008) rituximab led to a rapid and significant reduction of CEL and ARR. The rapid response implied this effect was not due to

reduction of autoantibodies but more likely to be a direct effect on the B cells themselves.

Ocrelizumab is a recombinant humanized monoclonal antibody, expected to be better tolerated than rituximab. In a phase 2 study, the number of CEL was reduced by 89 % and 96 % with reduction in ARR by 80 % and 73 % respectively in the high and low dose arms, as compared to control arms (Kappos et al. 2011). Phase III trials are underway.

Ofatumumab is a humanized antibody that binds to a different region of CD20. Its slower rate of dissociation provides for improved complement-dependent cytotoxicity (Sorensen et al. 2014). Ofatumumab was explored in a phase 2 study where it was found to suppress gadolinium enhancing lesions by more than 99 % as compared to placebo.

43.8.3 Vitamin D

Vitamin D deficiency as an explanation of the geographic distribution of MS has generated much interest. The Nurse's Health Study and the Nurses' Health Study II (Munger et al. 2004) showed that those taking supplements with Vit D (>400 IU/day) had less chance of developing MS. This protective role of vitamin D was supported by a retrospective study looking at vitamin D levels in previously stored blood samples (Munger et al. 2006).

A pilot study in 15 patients with RRMS treated with calcitriol, with primary outcome of safety, suggested a decreased MRI and clinical activity compared to baseline pre-treatment (Wingerchuk et al. 2005). Similarly, a phase I/II RCT in 50 MS also showed trend in reduction in ARR (Burton et al. 2010) while another phase II RCT demonstrated no benefit on relapse rate (Shaygannejad et al. 2012). Further studies to define a possible role in protecting or reducing risk of relapse or MRI activity are underway (Bhargava et al. 2014).

43.8.4 Estriol

Pregnancy, especially in the last trimester, is associated with a significant reduction in relapse rate in patients with MS and estriol is considered the sex hormone most likely mediating these effects. A small phase II study with crossover design of estriol showed a decrease in the number of CEL by 82 % (Sicotte et al. 2002). In 2014, a Phase II RCT of estriol as add-on to glatiramer acetate was completed.

43.8.5 Laquinimod

Laquinimod is a derivative of linomide, an anti-inflammatory agent shown to be effective for MS but found to have cardiopulmonary toxicity that precluded further development as a

therapeutic. Laquinimod is thought to exert its anti-inflammatory effects via suppression of the NF-kappa signaling pathway. Two phase III trials have been completed to date. ALLEGRO demonstrated a modest reduction in annualized relapse rate compared to placebo (Comi et al. 2012) while BRAVO did not show a significant reduction in relapse rate (Vollmer et al. 2014). A third phase III study exploring a higher dosing is underway.

43.8.6 Hematopoietic Stem Cell Transplantation

Autologous hematopoietic stem cell transplantation (HSCT) has been pursued for the treatment of autoimmune disease as a way to repair, rather than suppress, the immune system (Atkins and Freedman 2013). Efficacy of this approach is attributed not simply to the profound immunosuppression used in the conditioning regimen, but to the subsequent immune reconstitution with a renewed self-tolerant, T cell repertoire. New thymic outputs with reduced numbers of proinflammatory effector T cells and increased numbers of regulatory cells are also thought to contribute to the resetting of the immune system (Muraro 2015).

More than 800 cases of MS have been treated with HSCT in recent years (Mancardi et al. 2015); a wide variety of protocols have been used, which together with differences in patient cohorts and outcome assessments, leads to difficulty in comparing results. Recently two larger prospective studies have been reported that begin to better define a viable future role for HSCT in the treatment of MS.

HALT-MS is an on-going, open label phase II study of high-dose immunosuppressive therapy and HSCT with a planned 5-year follow-up after transplantation. Twenty-five patients with RRMS were enrolled. The 3-year interim analysis was completed and event free survival (defined as death or evidence of MS disease activity or progression of disability) was reported to be 82.8 % at 2 years and 78.4 % at 3 years following HSCT (Nash et al. 2015). This is a striking result when compared to the AFFIRM study in which only 37 % of patients treated with natalizumab had no radiologic or clinical activity after 2 years (Havrdova et al. 2009) or CARE-MSII in which only 32 % of patients treated with alemtuzumab had no radiologic or clinical activity after 2 years (Coles et al. 2012).

Mancardi et al. (2015) reported the results of the first RCT comparing intermediate intensity conditioning (BEAM) followed by HSCT to BEAM followed by treatment with mitoxantrone (ASTIMS). This led to complete suppression of gadolinium enhancing lesions compared to 56 % of subjects in the control arm, and a reduction in relapse rate by about 30 % as compared to mitoxantrone, sustained at 4 years despite only symptomatic therapy.

For both these studies, reported AEs were expected and reversible, occurring early after high dose immunochemotherapy with no early treatment-related mortality.

The possibility of pursuing less intense non-myeloablative conditioning regimen holds appeal for anticipated reduced morbidity compared to myeloablative regimens. A large case series of 121 patients treated with non-myeloablative conditioning regimen demonstrated that 50 % and 64 % of patients had sustained improvement in EDSS scores at 2 and 4 years respectively, relapse free survival of 89 % at 2 years and 87 % at 4 years as well as significant reduction in gadolinium enhancing lesions sustained at 5 years post transplant (Burt et al. 2015). A phase III study in RRMS with evidence of active inflammation using non-myeloablative conditioning regimen is underway (NCT00273364).

43.9 Clinical Trial Failures

Clinical trials can fail for a variety of reasons, including flawed trial design or inadequate dosing or route of administration, but also as a result of fundamental gaps in our understanding of the pathophysiology of a disease, despite apparently rational considerations. As with any experiment, trial failures represent opportunities to revise and correct our disease model. This is especially true when unexpected worsening of disease occurs as a result of interventions.

Unexpected adverse reactions and/or unexpectedly severe adverse reactions can sometimes lead to an interventional strategy being abandoned. One such example is that of cladribine. This is an adenosine deaminase-resistant purine nucleoside, demonstrated promising efficacy in 2 phase III studies, with a relative risk of ARR reduction of 54.5 % and 57.6 % and reduction in gadolinium-enhancing lesions by 87.9 % and 85.7 % at high and low dose respectively compared to placebo (Cook et al. 2011) and delay in conversion to CDMS (Leist et al. 2014). Its concerning safety profile, including risk of malignancy, myelosuppression, opportunistic infections arrested further development of this drug as a therapeutic.

43.10 Drugs That Made MS Worse

43.10.1 IFN γ

In 1987 an open label study of 2 doses of interferon γ was undertaken based on previous benefit seen in studies using interferon α and β (Jacobs et al. 1981, 1986; Knobler et al. 1984). Seven out of 18 subjects developed clinical exacerbations, which was significantly greater than expected based on pretreatment rate. This was attributed to a direct effect of increasing HLA class II expression on immune cells. This study marked the beginning of an effort to identify therapeutics that could block IFN γ , including IFN α and β .

43.10.2 PDE Inhibitors

Rolipram is a selective cyclic nucleotide phosphodiesterase inhibitor that targets PDE-4, the predominant isozyme present in inflammatory cells. Inhibition of PDE-4 leads to an increase of intracellular cAMP levels and can thereby modulate immune functions such as T cell activation and cytokine secretion. In vitro and in EAE models, rolipram reduced production of TNF α by activated T cells. PDE-4 inhibitors were effective in several models of EAE (Sommer et al. 1995, 1997).

An open-label Phase I-II study was terminated early after enrolling only 8 patients, due to poor tolerability and increased number of contrast enhancing lesions in 5/8 patients (225 % increase, $p=0.052$). No clinical deterioration was associated with this MRI activity and pharmacologic activity of rolipram was as predicted with inhibition of Th1 and Th17 cell function (Bielekova et al. 2009). The authors concluded that factors other than simplistic Th1/Th2 balance, including possibly effects on regulatory cell populations, might need to be considered.

Ibudilast is a non-selective PDE-inhibitor, shown to shift in vitro (Yoshimura et al. 1998) and in vivo (Feng et al. 2004) cytokine profile from Th1 to Th2, and specifically to reduce TNF- α production by immune cells including microglia. A Phase II RCT in 297 patients showed no benefit on rate of newly active lesions or on relapses (Barkhof et al. 2010). PDE inhibitors are no longer being pursued as anti-inflammatory agents in MS, but still hold interest as early data has suggested a possible neuroprotective role, yet to be confirmed (Fox 2010).

43.10.3 TNF Inhibitors

TNF is an important pro-inflammatory cytokine and there is evidence that it contributes to tissue injury in MS: TNF is found in active demyelinating lesions; has a direct toxic effect against oligodendrocytes in vitro; patients with MS have elevated TNF levels in serum and CSF and TNF production is increased prior to and during clinical exacerbations. TNF inhibitors have been very effective in the treatment of chronic inflammatory diseases, including RA and spondyloarthropathies (Kaltsonoudis et al. 2014).

Based on this rationale, the TNF inhibitor infliximab was studied in a small open label phase I study of 2 patients with rapidly progressive MS. Both patients developed an increase in number of gadolinium enhancing lesions by MRI associated with laboratory biomarkers indicating increase of active inflammation (IgG Index and CSF pleocytosis) (van Oosten et al. 1998).

Subsequently a phase II placebo controlled study in 168 patients with lenercept, a soluble TNF receptor fusion protein, was pursued. This study was terminated due to an unex-

pected 68 % increase in relapse rate in the lenercept treatment arms compared to the placebo arm. This effect was evident already in the first month of treatment, and was associated with a dose-dependent decrease in time to first exacerbation was seen (1999).

A paradoxical role of TNF in MS is further supported by an unexpectedly high occurrence of CNS demyelinating disease has been described in anti-TNF-treated individuals, with a prevalence of about 0.05 and 0.2 % (Ramos-Casals et al. 2010). The relatively low rate of this adverse outcome has been interpreted as an unmasking of underlying predisposition to demyelinating disease rather than a direct causal effect.

More recently, an MS-associated splice variant of TNFR1 was described; this mutated protein is secreted rather membrane bound and is capable of neutralizing circulating TNF, thereby acting as an endogenous TNF inhibitor (Gregory et al. 2012). Additional effects of the mutated protein leading to enhanced production of TNF-driven pro-inflammatory activity has also been described and may further contribute to MS disease susceptibility (Ottoboni et al. 2013).

TNF has thus emerged as a pleiotropic factor that acts in a complex network, with pro-inflammatory and anti-inflammatory effects. The net effect of modulation of this cytokine likely depends on the environment in which it is found and possibly genetic susceptibility.

43.10.3.1 APL

Altered peptide ligands are short peptides with amino-acid substitutions in key T-cell contact positions that can block specific T cell responses to the native antigen by displacing it or by inducing regulatory T cell populations (Bielekova and Martin 2001).

Two phase II studies were conducted using identical APL MBP₈₃₋₉₉ (Kappos et al. 2000; Bielekova and Martin 2001). Both studies were terminated early due to poor tolerance of the intervention, mainly related to hypersensitivity reactions. While neither trial demonstrated statistically significant difference between treated and untreated patients (Kappos et al. 2000) or baseline vs. treatment (Bielekova and Martin 2001), Bielekova *et al.* reported a higher than expected incidence of relapses, seen in 3 out of the 8 enrolled subjects, associated increase in gadolinium enhancing lesions. In 2 of these 3 patients, the exacerbations were directly linked to APL-induced 1000-fold expansion of MBP₈₃₋₉₉-specific T cells in peripheral blood and CSF. This was taken to support the encephalitogenic potential of MBP-specific T cells in MS.

43.10.4 Atacicept

Atacicept is a recombinant fusion protein, made of the extracellular ligand-binding portion of the human TACI receptor linked to a recombinant Fc domain of human immunoglobulin

G. It binds and blocks the activity of two B cell growth factors: B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL). Both factors are essential for B cell differentiation, maturation and survival. Atacicept targets mature B cells (antigen-driven B cell responses) and plasma cells and late stages of B-cell development while sparing B cell progenitors and memory cells.

ATAMS (Kappos et al. 2014b) a 36-week placebo-controlled phase II study of atacicept in RRMS was terminated early due to more than doubling of relapse rate in all doses of study drug explored and associated dose-dependent decrease in time to first relapse. Over the subsequent 60-week safety follow-up, relapse rate returned to pre-treatment baseline in parallel with B cells counts and Ig concentrations returning toward pre-dose values, suggesting biological relevance of these pharmacological effects. The concurrent phase II study of atacicept in optic neuritis (ATON) was terminated early based on the ATAMS results. Although difficult to draw conclusions since very few patients completed the study, a higher proportion of patients converting to clinically definite MS was observed in the atacicept arm compared to placebo (35.3 % vs. 17.6 %) (Sergott et al. 2015).

Different explanations have been proposed for these unexpected effects, including the decrease in immunoglobulin levels precluding non-specific occupation of Fc receptors on antigen-presenting cells and/or disruption of the balance between effector and regulatory B cells (Luhder and Gold 2014).

This study, similarly to both the lenercept and daclizumab experiences, demonstrates that the net effect of specific interventions in this complex network of checks and balances can lead to unpredictable outcomes. In order to benefit and advance understanding of immune-physiology and pathophysiology, to be gained both from negative and positive studies alike, clinical trials must include measures of biological activity of study interventions.

43.11 Drugs That Cause CNS Demyelination

43.11.1 Fludarabine

Fludarabine is a purine nucleoside (Warrell and Berman 1986) used to treat myeloproliferative malignancies and as immunosuppression prior to BMT.

At doses higher than 100 mg/m² fludarabine has been described to cause a delayed, progressive demyelinating encephalopathy in approximately 36 % of subjects within 6–8 weeks of treatment; this has limited the use of this otherwise well-tolerated chemotherapeutic agent (Spriggs et al. 1986; Chun et al. 1991). This effect is dose-dependent, such that at lower doses (<25 mg/m²) neurotoxicity occurred in 0.2 % (Chun et al. 1986).

Clinically patients most often present with visual deficits. Neuropathology of autopsy cases has shown leukoencephalopathy with predominant involvement of occipital and parietal lobes, with vacuolization, demyelination, PAS-positive macrophage infiltration and axonal loss (Ding et al. 2008).

More recently ocular toxicity, specifically characterized by retinal ganglion and bipolar cell loss, optic nerve atrophy and macrophage infiltration has been described (Ding et al. 2008; Bishop et al. 2010); ocular toxicity is not be limited to high dose therapy.

It has been suggested neurological complications caused by fludarabine may be due to specific, direct toxic effect on susceptible neural progenitor cells and non-dividing myelinating oligodendrocytes in regions with greatest metabolic activity. (Ding et al. 2008; Hafner et al. 2014).

43.12 Opportunistic Infections in Patients with MS Due to Immunomodulatory Therapy

43.12.1 Progressive Multifocal Leukoencephalopathy

43.12.1.1 Therapies Implicated

PML is caused by a ubiquitous polyomavirus, JC virus. Immunomodulatory therapies associated with an increased risk of PML include natalizumab, rituximab, and efalizumab (Nath and Berger 2012). Mycophenolate mofetil also carries a black box warning for PML. Any broadly immunosuppressive therapy may be associated with PML, but separating the contribution of the immune abnormality of the underlying disease from the therapy may be difficult.

43.12.1.2 Treatment

The only unequivocal improvement in outcome attends restoration of immune function. Therefore, removing the offending agent is critically important aim of therapy is to restore immune function (Fox 2011).

43.12.1.3 Withdrawal of Immunomodulatory Therapy

Discontinuation of the drug may be sufficient for drugs with a short half life.

Removal of the drug by plasmapheresis (PLEX) may be needed. To date, PLEX has only been demonstrated to effectively remove natalizumab and the effects on the immune system of some monoclonal antibody therapies, such as, rituximab, do not lend themselves to rapid reversal. Serum natalizumab concentrations are reduced by a mean of 92 % from baseline to 1 week after three PLEX sessions ($p < 0.001$). Although average alpha4-integrin saturation was not reduced after PLEX because it was tightly bound to the lymphocytes,

it was reduced to less than 50 % when natalizumab concentrations were below 1 ug/mL. PBMC trans migratory capacity increased 2.2-fold after PLEX ($p < 0.006$) suggesting partial functional recovery (Khatri et al. 2009).

Immune restoration is the cornerstone of treatment of PML; however, the inflammatory response associated with it may result in IRIS. This has been observed in the majority of patients with treated for natalizumab associated PML and may be fatal. Early treatment with high dose steroids (1 g methyl prednisone/day for 5 days) is necessary followed by a slow oral taper over 2 months as evidenced by analysis of retrospective studies (Tan et al. 2011).

43.12.1.4 Antiviral Therapies

Although several agents have been demonstrated to suppress JC virus replication in vivo, such as, cytosine arabinoside, camptothecin, mefloquine (Brickelmaier et al. 2009) and, in some but not all studies, cidofovir (Andrei et al. 1997; Hou and Major 1998). A carefully designed clinical trial of cytosine arabinoside for HIV-associated PML showed no benefit (Hall et al. 1998). Observational trials have failed to show any benefit of cidofovir in HIV-associated PML (Gasnault et al. 2001; Marra et al. 2002). Similarly, a trial of mefloquine that used CSF JC virus copy numbers as its primary endpoint failed to show an effect of the drug.

43.12.2 Herpes Simplex Virus-1 (HSV-1) Encephalitis

43.12.2.1 Therapies Implicated

There have been isolated cases reported with natalizumab and fingolimod.

43.12.2.2 Treatment

National guidelines for treatment of viral encephalitis were established for the United Kingdom (Solomon et al. 2012). This consensus statement recommended that all patients undergo neuroimaging studies prior to cerebrospinal fluid evaluation. The etiological diagnosis is best made by polymerase chain reaction for the viral genome. While they suggested that there was no role for a brain biopsy in the initial assessment of patients with suspected HSV encephalitis; it may be useful in patients with suspected HSV encephalitis who are CSF PCR negative and deteriorating despite acyclovir to confirm the diagnosis or to identify alternative disorders.

43.12.2.3 Acyclovir

Standard dosage: 10 mg/kg IV (over 1 h) given every 8 h; MAX 20 mg/kg every 8 h. Duration of treatment in the original randomized trials of aciclovir for HSV encephalitis was 10 days. However, clinical relapse after 10 days treatment

are known to occur (Dennett et al. 1996). As a consequence most clinicians now use at least 14–21 days intravenous treatment in confirmed cases. Some advocate repeating a CSF examination at 14–21 days, and continuing treatment until the CSF is negative of virus by PCR (Solomon et al. 2012). A prolonged duration of therapy may be more important in the immunosuppressed patients.

43.12.3 Varicella Zoster Virus Infection

43.12.3.1 Therapies Implicated

In immunosuppressed patients, single or multidematomal eruptions of shingles may occur. Unusual presentations may include a CNS or retinal vasculitis with infarcts in the absence of a rash. Rarely, it may cause acute encephalitis without a vasculitis or a rash (De Broucker et al. 2012). In United States, three drugs have been approved for treatment which includes acyclovir, valacyclovir and famciclovir. In Europe, brivudin is also approved for treatment. All CNS complications should be treated with IV acyclovir while shingles can be treated with oral valacyclovir or famciclovir.

43.12.3.2 Famciclovir

Standard dosage: Shingles: 500 mg orally every 8 h for 7–10 days. Dosage needs to be adjusted in patients with renal insufficiency.

43.12.3.3 Special Consideration

Famciclovir is a prodrug that gets metabolized to penciclovir which is the active form of the drug.

43.13 Review Questions

Your brother had some “double” vision problems lately and has felt very tired. Your family physician sent him to a neurologist who scheduled several tests. On follow up, the neurologist says that your brother has optic neuritis that may potentially be associated with multiple sclerosis. The neurologist also recommends that your brother consider beginning an interferon β therapy.

1. What clinical data would support initiating IFN β therapy even before a definitive diagnosis of MS?
2. What clinical information, in association with verification of the clinical symptoms of optic neuritis and fatigue would be sufficient to verify that the diagnosis is definitive MS using the revised MacDonald criteria?
3. Your brother has received information on the two FDA approved IFN β -1a products. What are the differences in dose, dosing schedule, potential for side effects and efficacy for the two products?

4. Over the next year, your brother has 2 more episodes that include problems with walking and numbness in his right hand. Are any other criteria necessary for a definitive diagnosis of MS by the revised McDonald criteria?
5. What would you expect an MRI to show if your brother truly has MS?
6. Your brother has been on IFN β -1a IM therapy for 9 months and is still complaining of flu like symptoms and muscular aches and pain following each injection. What other therapeutic option(s) does he have?
7. How has the diagnosis of MS changed your brother's life expectancy?
8. Your brother has just had a major relapse that has left him unable to walk. What is the standard therapy for this type of acute relapse and what is the postulated mechanism of action?
9. If the previous scenario had been your sister rather than your brother, would the prognosis have been different?
10. Your brother continues to progress rapidly with no apparent remission even on standard immunotherapy for RR-MS. What therapy is FDA approved for rapidly progressing MS?

43.14 Answers

1. The CHAMPS study showed that patients who were started on Avonex with your brother's symptoms had a 1% risk of meeting criteria for definitive MS in 18 months compared to a 16% risk for those who did not begin therapy.
2. The presence of lesions on a brain MRI with and without gadolinium (Gd) to evaluate for the presence of recent and evolving plaques CSF and EMG data are often also used to verify MS.
3. The two FDA approved IFN β -1a products are Avonex® (Biogen-Idex) which is the lowest total weekly dose available at 33mcg IFN β -1a to be injected intramuscularly (IM) once a week and Rebif® (Serono) which is available in doses of 22 mcg and 44 mcg for subcutaneous (SQ) injection three times weekly. Both reduce MS relapses significantly. Both have similar side effect profiles. The lower dose may cause fewer side effects but there is also some evidence that the lower the dose the less effective in reducing relapses.
4. If an MRI confirms the presence of active lesions, and your brother has clinical verification of at least 2 episodes separated by time in at least two different sites. MacDonald criteria are met.
5. There should be evidence of lesions within the brain or spinal cord as a result of the recent exacerbations. Since they are relatively recent, one would expect Gd to infil-

trate the brain parenchyma through breaches in the cerebrovascular endothelium.

6. There are several options that should be discussed with his neurologist. He could first try pre-medicating with an over the counter NSAID before his weekly injection. Ibuprofen and naproxen are effective in alleviating some flu like symptoms in some patient. If that is not effective, there is also evidence that oral corticosteroids taken in conjunction with the IFN β injection will decrease the inflammatory side effects. The other possibility is to change to a non IFN β therapy. Glatiramer acetate (Copaxone[®]) has a similar efficacy profile without the flu like side effects.
7. Prior to approval of the current therapies, life expectancy from MS was only decreased by 2–3 years. Since the longest any approved therapy has been available is approximately 10 years, there is no additional information available. In general with all things considered, his expectancy should not be significantly different that yours.
8. The standard of therapy is a Methyl prednisolone (Solu-Medrol[™]) with IV treatment for 3–5 days at 500–1000 mg/day. There is no evidence that steroids reduce the relapse rate but they are effective in treating the acute inflammation.
9. Men often have a more aggressive and progressive MS than women. With frequent clinical and MRI monitoring to determine if the current therapy is adequate, his prognosis should be better than if he were diagnosed 10 years ago.
10. The only FDA approved therapy is mitoxantrone (Novantron[®]) with a recommended dosage for treatment of multiple sclerosis at 5–12 mg/mL IV every 3 months. Acute side effects of mitoxantrone include nausea and alopecia. Because of cumulative cardiotoxicity, the drug can be used for only 2–3 years (or for a cumulative dose of 120–140 mg per m²).

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Abstract

HIV-associated neurocognitive disorders follows viral invasion of the central nervous system and can lead to impairments in memory, problem solving, behavior and decision making capabilities. Disease is divided into asymptomatic neurocognitive impairment, mild neurocognitive disorder and HIV-associated dementia. In an advent of combination antiretroviral therapy disease severity has been attenuated with mild dysfunction becoming the prominent feature. Also operative has been the emergence of confounders and comorbid conditions that may cause cognitive impairments and behavioral deficits such as depression, liver disease, drug toxicities and nutrition. This chapter serves to review the virologic and immune disease parameters, disease diagnosis, antiretroviral and adjunctive therapeutic considerations to combat disease.

Keywords

Antiretroviral therapies • HIV-associated neurocognitive disorders • HIV-replication • Neuroimaging

44.1 Introduction: HIV-Associated Neurocognitive Disorders (HAND)

According to recent estimates, over 35 million people are currently living with HIV-1 worldwide (WHO 2014). Estimates for the lifetime prevalence for neurologic consequences of HIV involving the central nervous system (CNS) and peripheral nervous system (PNS) are between 40 and 70 % of HIV-infected individuals (McArthur et al. 2005; Simioni et al. 2010). With ART treatment, the rates of opportunistic infections of the CNS and PNS have been reduced

(Mamidi et al. 2002; Habata et al. 1999; Roulet 1999), however the prevalence of neurological disorders directly caused by HIV have increased (Antinori et al. 2007). HIV infection can directly cause peripheral neuropathy, vacuolar myelopathies, and HAND even in immune-competent hosts (Childs et al. 1999; McArthur et al. 2003, 2005; Antinori et al. 2007).

HIV-Associated Neurocognitive Disorders (HAND) encompass a spectrum of cognitive impairment with sub-syndromes of varying severity. These include asymptomatic neurocognitive impairment (ANI), mild neurocognitive disorder (MND), and HIV-associated dementia (HAD), which are characterized by cognitive, motor, and behavioral abnormalities (Kaul et al. 2005) and are classified by patient neuropsychological test performance and neurological and behavioral functioning (Table 44.1) (Antinori et al. 2007). HIV-associated asymptomatic neurocognitive impairment (ANI) is the least severe form of HAND and includes individuals with only mild cognitive impairment in neuropsychological testing and without functional impairment in daily activities. In HIV-associated MND, individuals have a higher level of cognitive impairment, which interferes with

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Table 44.1 Diagnostic criteria of HAND

HAND diagnosis	Functional status	Neuropsychological testing criteria
Asymptomatic neurocognitive impairment (ANI)	Mild cognitive impairment with no effect on ADLs	Below 1 SD in ≥ 2 of 5–7 domains
Minor neurocognitive deficit (MND)	Mild cognitive impairment with impact on ADLs	Below 1 SD in ≥ 2 of 5–7 domains
HIV-associated dementia (HAD)	Marked cognitive impairment with marked impact on ADLs	Below 2 SD in ≥ 2 of 5–7 domains

ADLs activities of daily living, SD standard deviation

daily functioning, as determined by self-reporting or by observation of others. HAD, the most devastating form of HAND has been considered a late complication of HIV infection, often in the setting of uncontrolled viremia and severely depleted CD4+ T-cell counts. In HAD, cognitive impairment is associated with marked interference with day-to-day functioning and HAD is considered a significant independent risk factor for death due to AIDS (Liner et al. 2008).

44.1.1 Role of HIV Replication in HAND

HIV enters the CNS compartment early in the course of infection and yet not all HIV+ individuals go on to develop HAND. Understanding how HIV replicates in the CNS compartment, the role of ART in suppressing the CNS reservoir and the circumstances that promote neurocognitive deficits are necessary for developing effective therapeutics. Recent studies confirm early entry (within weeks of systemic infection) of HIV into the CNS, and these studies have also confirmed a predominance of T lymphocyte-tropic R5 HIV strains in the CSF of newly-infected individuals, with a later increase in the prevalence of macrophage-tropic R5 variants (Sturdevant et al. 2015). These findings suggest infiltration of HIV-infected T lymphocytes into the CNS early after initial infection and throughout the course of later infection, with adaptation of virus to replication in brain macrophages in at least some individuals (Sturdevant et al. 2015). Predicting which infected individuals are at high risk for developing HAND based upon early entry events remains uncertain, but certainly worthy of intensive study.

HIV infection in the CNS can be clinically silent throughout the infection course, as is evident by the number of HIV+ patients lacking neurocognitive deficits. However, even those who are virally suppressed by ART are at risk for developing HAND, and this risk is most strongly linked to the individual's historic CD4 T lymphocyte nadir (Ellis et al. 2011). A recent study examined the prevalence of neurocognitive deficits in patients with undetectable serum HIV RNA over a median time of 48 months. Within this cohort, 27% had cognitive complaints and within this group, HAND was diagnosed in 84% (ANI 24%, MND 52% and HAD 8%). Of the 73% of patients without cognitive complaints, an additional 64% were found to have HAND on formal neuropsychological

testing (ANI 60%, MND 4% and HAD 0%) (Simioni et al. 2010). Furthermore, data suggest that neurological injury can progress even while patients are on virally suppressive ART. A recent review summarizes studies that demonstrate significantly elevated CSF neurofilament light-chain (NFL, a specific biomarker of neuronal injury) not only in ART-naïve individuals but also in individuals on suppressive ART, in comparison with age-matched HIV-negative controls (Jessen Krut et al. 2014). Additional evidence indicates that neuronal damage affected prior to initiation of ART is not fully reversible (Tozzi et al. 2007). In a cohort of individuals with AIDS, 21% of those who had undetectable blood and CSF HIV RNA progressed to HAD (Sevigny et al. 2007). And initiation of neuronal damage and clinically evident HAND can occur before AIDS criteria are met, as is evident by a prospective study that identified HAND in 8–34% of aviremic patients who had no comorbidities and with a CD4 nadir of greater than 200 cells/ μ L (Cysique et al. 2006). As such, HAND cannot be mediated simply by uncontrolled HIV replication in the CNS compartment and the neuropathogenesis of this syndrome must take into account accumulating neuronal damage even in the absence of clinically detectable HIV replication. These new insights underscore the need for adjunctive neuroprotective therapies, as there are no current therapies for improving neurologic outcomes other than ART, which targets only viral replication (Sacktor et al. 2002; Antinori et al. 2007; Brew 2004).

44.2 The Role of Neuroimaging in the Diagnosis of HAND

Apart from neuropsychological testing, which assesses current HAND symptoms and signs; there is limited evidence to support predictive usefulness of either serum or CNS biomarkers in detecting the risk for development of HAND. The most promising biomarker, which has only been validated for CSF, is neurofilament light-chain (NFL). This is a marker for axonal injury but it is not specific to HIV-induced injury (Gisslen et al. 2007, 2009). Neuroimaging has been investigated for the diagnosis and staging of HAND, and for monitoring the CNS response to therapy. Additionally, neuroimaging has been utilized in assessing for other causes of neurocognitive dysfunction in HIV+ patients, including

CNS lymphoma, PML, cerebral toxoplasmosis and other opportunistic infections that have distinct abnormalities.

Throughout the course of HAND, cerebral atrophy is greater than predicted by age, although cerebral atrophy is not a specific to HIV infection alone, as it can occur with associated nutritional deficiency or drug and alcohol abuse. However, characteristic magnetic resonance imaging (MRI) signal changes due to HIV are often observed both within subcortical (basal ganglia) and cortical (frontal grey/white matter and posterior grey matter) compared to HIV-controls (Aylward et al. 1995). A newer MRI study has revealed loss of structural integrity and micro-edema in the global white and cortical gray matter, as well as in the thalamus and basal ganglia in MND patients compared to controls (Granziera et al. 2013). Multiple regression analysis showed a significant interaction of subcortical nuclei changes and executive indices in HIV+ patients with MND, suggesting that multi-contrast MRI at high field strength may be able to differentiate ART-treated HIV+ patients with mild neurocognitive deficits from those without (Granziera et al. 2013).

Functional MRI (fMRI) is used to examine the hemodynamic response related to neural activity, and impairment of normal recruitment of brain regions suggests brain dysfunction. Using this technique, it has been shown that HIV+ individuals have greater parietal activation for a simple attention task and greater frontal and parietal activation during more complex tasks (Chang et al. 2001). This is believed to represent evidence of modulation of neural circuits during HIV infection and greater use of brain reserve in order to meet cognitive demand. A recent meta-analysis of fMRI studies examining the fronto-striatal-parietal network revealed functional differences in the left inferior frontal gyrus and caudate nucleus and this dysfunction was qualitatively related to neurocognitive impairment (Plessis et al. 2014). Additionally, baseline functional connections may be compromised in HAND, in a similar way to those seen in normal aging (Thomas et al. 2013). With future studies, fMRI may be able to better characterize the networks involved in the cognitive decline during HIV infection and could then be utilized to monitor progression and efficacy of therapeutics.

MR Spectroscopy (MRS) adds additional information to traditional MRI by determining the relative concentrations of target brain metabolites. *N*-acetyl-aspartate (neuronal marker), choline (marker of cellular proliferation and inflammation), creatinine (brain metabolism and reference marker) and myoinositol (marker of gliosis) are the most commonly measured. Early after seroconversion, MRS metabolites are altered (Lentz et al. 2011) with neuroimaging alterations correlating with inflammation and neuronal injury (Valcour et al. 2012; Sailasuta et al. 2012). HIV+ patients with chronic infection have reduced levels of *N*-acetyl-aspartate (NAA) and increased choline and myoinositol (Harezlak et al. 2014; Paul et al. 2008; Yiannoutsos et al. 2008). However, many of

these same alterations are seen with aging, making it a more relevant examination for younger HIV-infected individuals.

Fluorodeoxyglucose-positive emission topography (FDG-PET) imaging is used to measure metabolic activity in neurons. Studies have identified two unique metabolic signatures in HIV infection. First, there is evidence of a hypermetabolic state, especially prominent in the striatum, which appears to be specific for HIV infection and CNS involvement, even in the setting of normal neuropsychological performance testing. This has been seen as evidence of abnormal functional connectivity within subcortical areas in the setting of HIV infection (von Giesen et al. 2000). Secondly, in chronic infection, there is generalized hypometabolism in cortical and subcortical regions, which correlate with, age, extent of cerebral atrophy and neurocognitive status (Rottenberg et al. 1996). A transition from a hypermetabolic state to a hypometabolic one in subcortical regions was associated with the development of functional deficits and progression to HAD (von Giesen et al. 2000). Even in the ART era there are subtle FDG-PET changes that have been observed, with at least half of virologically suppressed HIV+ individuals on ART having evidence of hypometabolism in the mesial frontal lobes (Andersen et al. 2006). Interestingly, a significant age effect has been demonstrated in frontal brain regions suggesting a synergistic interaction between aging and HIV (Towgood et al. 2013). As neuroimaging techniques continue to advance, it is hoped that they can be used to diagnose HAND, characterize its severity and evaluate the efficacy of novel therapeutic candidates in longitudinal studies.

44.3 HAND in the Era of Antiretroviral Therapy (ART)

The largest and most comprehensive cross-sectional study examining the prevalence of cognitive impairment in HIV+ infection is the CNS HIV Antiretroviral Therapy Effects Research (CHARTER) cohort study. Cognitive impairment was found in 814 (52%) of 1555 HIV seropositive patients attending treatment centers in the United States and reflects all causes of impaired cognitive function in HIV+ individuals (Heaton et al. 2010). While the most severe form of cognitive impairment, HAD, was rare, the milder categories of ANI and MND remained common even in those receiving ART and with minimal comorbidities.

44.3.1 The Effectiveness of ART in HAND

Since the introduction of ART, there has been a profound reduction in the incidence of HAD from approximately 20% in the pre-ART era to a current prevalence of between 2 and 5% (Heaton et al. 2010). This reduction due to ART represents

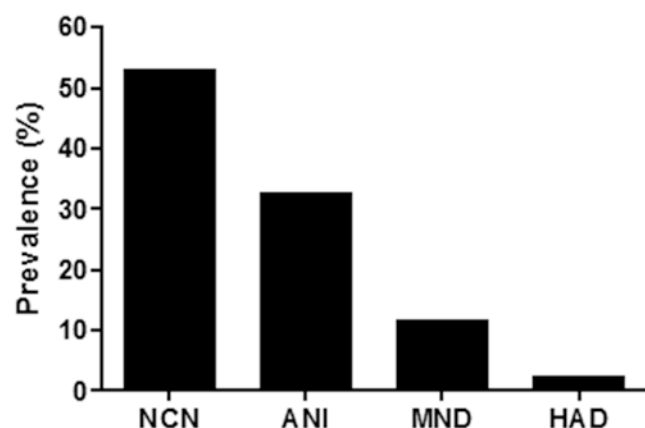


Fig. 44.1 Prevalence of HIV-associated neurocognitive disorders in the USA after introduction of ART in patients without confounding comorbidities (CHARTER study, $n=1316$) (Heaton et al. 2010). *NCN* neurocognitive normal, *ANI* asymptomatic neurocognitive impairment, *MND* mild neurocognitive impairment, *HAD* HIV-associated dementia

the only consistent therapeutic treatment success for HAND patients to date. However, the prevalence of MND and ANI has increased in the ART era (reviewed in Nightingale et al. (2014)). While estimates of the prevalence of HAND vary, it is evident that HIV-associated neurocognitive impairment does persist, even in those with virologic suppression on ART. In the CHARTER cohort study, prevalence estimates were 33% for ANI, 12% for MND, and 2% for HAD (Heaton et al. 2010) (Fig. 44.1).

Failure of consistent suppression of viral replication with ART could contribute to HAND persistence. With ART treatment, many patients have shown durable and complete suppression of HIV replication as determined by plasma viral RNA quantification, although a surprisingly high overall rate of HIV ‘blipping’ (defined as spontaneous expression of viral RNA copies (plasma, CSF)) in individuals on ART with sustained antecedent undetectable VLs with return to undetectable levels (Grennan et al. 2012) of up to 25% has been reported. This suggests that ART efficacy in maintaining consistent viral suppression has been overestimated, and that viral ‘blipping’ in ART-treated patients might represent a risk factor for HAND (Chen et al. 2014).

An additional concern for patients who are now being maintained on ART for decades is the possibility of ART neurotoxicity. Long-term treatment with ART has been demonstrated to have a range of systemic side effects including hepatic steatosis, peripheral neuropathy, cardiomyopathy, pancreatitis, ototoxicity, retinal lesions and lipodystrophy. Complete suppression of HIV replication in the CNS reservoir has been limited, in part due to the poor blood–brain barrier penetrance of ART. As new compounds, regimens and methods are developed to increase the penetrance of antiretroviral compounds, consideration will have to be given to the possibility of direct neurotoxicity by these compounds.

There is known to be substantial variation in the toxicity of various NRTIs in different tissue types and thus far, relatively little is known about the possibility of antiretroviral toxicity within the CNS compartment. In vitro work has demonstrated that several of the first line antiretroviral agents cause direct neurotoxicity when used at concentrations seen in the CSF of patients on ART (Robertson et al. 2012). Further work is needed to determine if ART causes a similar effect in the CNS of patients on long-term ART and if and how boosting CNS penetrance of these antiretrovirals alters neuronal function and cognition.

44.3.2 ART Treatment Strategies

While ART has significantly decreased the incidence of HAD, presumably by lowering systemic and CNS viral loads, more recent efforts have focused on improving the penetration of ART past the blood brain barrier. In theory, improved penetration or retention of bioavailable ART drugs in the CNS would slow HAND progression by decreasing CNS viral replication and reducing concomitant release of neurotoxins from infected and activated macrophages/microglia and immune activated astrocytes (Letendre et al. 2008; Ellis et al. 2007; Spitznberger et al. 2007). However, clinical trials have been unable to reduce the incidence or progression of HAND in patients treated with ART regimens with high CNS penetrance efficacy (CPE) (Reviewed in Nightingale et al. (2014)). Additionally, there is some evidence to suggest that increasing ART penetrance into the CNS may be detrimental. A 2009 study by Marra *et al.* suggested that ART regimens with strong CNS penetration and reduced CSF viral loads were associated with poorer neurocognitive outcomes (Marra et al. 2009). While further clinical studies are needed, this study highlights the importance of considering drug toxicity when promoting ART in the CNS. More recently, it has been proposed that choosing ART regimens based upon their efficacy in reducing HIV replication in monocyte and macrophage lineages may be more efficacious in targeting HAND pathogenesis (Shikuma et al. 2012). Effective therapies for HAND will likely require combination therapy that not only targets viral replication in the relevant cell populations in the CNS, but also addresses the indirect pathways known to contribute to HIV-associated neurodegeneration.

44.3.3 Viral ‘Blipping’ in ART-Treated Patients

Further research is underway to examine the relationship between CSF viral replication in systemically controlled HIV+ patients maintained on ART and its potential link to HAND. The term “viral blipping” has been applied to the observation that HIV RNA has been intermittently detected

at low-levels when assayed in the serum and/or CSF of HIV+ patients maintained on therapeutic ART. Up to 25 % of ART treated individuals may show non-sustained viral blipping in the plasma and it has been estimated that 10–30 % of patients who have clinical suppression of HIV replication by serum measurements have detectable CSF HIV RNA (Grennan et al. 2012). This suggests that ART, while extremely effective, cannot currently eliminate all viral replication and that the CNS can be a privileged reservoir that should be considered independently of the systemic compartment (Eden et al. 2010).

The role of viral blipping remains undetermined, as does its potential link to neuroinflammation pathways and development of HAND. A recent study has proposed that plasma HIV blipping contributes to immune activation, as measured by increased T cell activation. HIV+ patients on ART were characterized as blippers (having a one time viral load between 50 and 1000 copies/mL following at least 180 days of undetectable VL) or non-blippers. Patients with plasma viral blips showed increased T-cell activation compared to those without blips (Zoufaly et al. 2014). While this study was assaying peripheral markers of immune activation, it suggests that a similar mechanism may be occurring in the CNS within HIV-infected monocytes/macrophages and that this may contribute to neurocognitive deficits and disease progression. In a small study of 11 patients by Canestri et al., CSF samples of HIV-suppressed individuals with neurologic symptoms were collected and monitored for viral blipping. Of these 11 patients, 10 had evidence of pleocytosis in their CSF, suggesting an association between CSF blipping and inflammation in the CSF (Canestri et al. 2010). Additionally, ART resistance mutations were found in seven of eight CSF specimens supporting the theory that blipping is associated with increased duration of ART and the emergence of ART resistance (Eden et al. 2010; Pozniak et al. 2009). As these patients did not undergo neuropsychological testing, the link between viral blipping in the CNS, neuroinflammation and presence of HAND remains speculative.

The presence of these viral blips have been controversial with suggestion that they could promote changes in immune responses for better control of HIV infection while an opposing hypothesis states that they predict an increased risk of virological failure. In a retrospective analysis of an ART interruption study, it was found that individuals with plasma blips (termed episodes of intermittent viremia) above 200 copies/mL had lower CD4+ T cell counts and higher activation markers while receiving ART. After ART interruption, these individuals had a significantly higher viral rebound despite having a greater specific immune response against HIV. Of note, most of these differences disappeared when smaller blips of 20–200 copies/mL were included (Castro et al. 2013). Taken together, this study provides evidence that blipping can drive immune activation and also that the

magnitude of the blip can determine future virological failure. Given the prevalence of viral blipping and ongoing evidence of neuroinflammation in HIV+ individuals on ART, it is unlikely that ART will ever be a monotherapy for HAND, and as such, development of adjunctive therapeutics will be necessary to mitigate cognitive sequelae of HIV infection.

44.4 Proposed Adjunctive Therapies in HAND

Initially, known therapeutics for other neurodegenerative and neuropsychiatric disorders were trialed in HAND. Specifically, medications with FDA approval for use in Alzheimer's and Parkinson's disease have been examined. Mood stabilizers, antidepressant medications and antioxidants have also been investigated.

44.4.1 Therapies Applied to Other Neurodegenerative Diseases and HAND

Noted clinical similarities between the neurodegeneration seen as a consequence of HIV infection and other neurodegenerative diseases such as Alzheimer's and Parkinson's has prompted investigation into the currently approved neuroprotective therapies in the treatment of HAND (Table 44.2). Memantine (Namenda) is approved in the treatment for Alzheimer's disease and acts as a non-competitive NMDA receptor antagonist. In vitro and in vivo animal studies have shown that memantine inhibits gp120 and Tat-induced neurotoxicity (Nath et al. 2000a; Anderson et al. 2004; Toggas et al. 1996) and in a SIV-infected macaque model, increases levels of brain-derived neurotrophic factor (BDNF) and conserves dopamine function (Meisner et al. 2008). A short term clinical trial in HAND patients demonstrated that memantine improved neuronal metabolism, indicative of neuroprotection, but did not cause significant neurocognitive improvement (Schifitto et al. 2007a). A longer-term follow up study in this patient cohort failed to reveal a clinically demonstrable neurological benefit (Zhao et al. 2010).

Selegiline (Deprenyl), a monoamine oxidase B (MAO-B) inhibitor used in the treatment of early-stage Parkinson's disease, is proposed to act as a neuroprotectant by reducing the antioxidant burden of the cell (Magyar and Szende 2004). However, recent studies have shown neither a reduction in biomarkers of oxidative stress nor evidence of cognitive improvement with short-term (24 weeks) transdermal selegiline (Schifitto et al. 2007b, 2009a). Rivastigmine, an acetylcholinesterase inhibitor approved for Alzheimer's and Parkinson's disease, was examined in a 20 week, placebo controlled crossover study in aviremic HIV patients with HAND. While there was no change in the primary outcome

Table 44.2 Proposed therapeutics for HAND

Generic (Brand) name	Molecular target	Proposed effects	References
Memantine (Namenda)	NMDA receptor antagonist	Increases BDNF levels, conserves dopamine function, inhibits gp120 and Tat-induced neurotoxicity, improves neuronal metabolism, short-term treatment provided no neurocognitive improvement	Meisner et al. (2008), Nath et al. (2000b), Toggas et al. (1996), Anderson et al. (2004), Schifitto et al. (2007a) and Zhao et al. (2010)
Selegiline (Deprenyl)	MAO-B Inhibitor	Proposed to reduce antioxidant burden of cell, may improve psychomotor speed, no clinical evidence of cognitive improvement over 24 week treatment	Sacktor et al. (2000), Magyar and Szende (2004), Schifitto et al. (2009a) and Schifitto et al. (2007b)
Rivastigmine (Exelon)	Acetylcholinesterase inhibitor	Proposed to increase acetylcholine levels in the CNS. Improved processing speed and possible effect on executive functioning after 20 week treatment	Simioni et al. (2013)
Sodium valproate (Depakote) and lithium	GSK-3 β inhibitor	Reduces neurotoxicity, improved neuropsychological performance	Ances et al. (2008), Tong et al. (2001), Dou et al. (2003), Everall et al. (2002), Schifitto et al. (2006), Letendre et al. (2006) and Schifitto et al. (2009b)
SSRIs: Citalopram (Celexa), Paroxetine (Paxil)	Serotonin transporter	May decrease HIV viral levels in CSF, improved adherence to ART	Ances et al. (2008) and Letendre et al. (2007)
Minocycline	5-lipoxygenase and others	Decreases CCL2 levels in CSF, suppresses HIV replication and inhibits secretion of TNF- α , IFN- γ and IL-2 by lymphocytes	Copeland and Brooks (2010), Colovic and Caccia (2003), Si et al. (2004), Zink et al. (2005) and Szeto et al. (2010)

of cognition, processing speed improved and there was a possible, albeit non-significant effect, on executive functioning (Simioni et al. 2013). While memantine, selegiline and rivastigmine have shown some limited effectiveness, they are clearly not suitable monotherapies for HAND. In addition to using such drugs prior to neurocognitive decline, effective neuroprotective therapies will likely have to be used in combination with ART and over the duration of viral infection to provide maximal clinical benefit.

44.4.2 Therapies Applied to Neuropsychiatric Disorders and HAND

In addition to therapies for neurodegenerative diseases, compounds in clinical use for neuropsychiatric disorders are also under investigation in HAND (Table 44.2) (Ances et al. 2008). Sodium valproate (VPA) and lithium are approved for treatment of bipolar disorder and related mood disorders, and both inhibit glycogen synthase kinase-3 β and provide neuroprotection against HIV-induced toxicity in vitro and in mouse models of HIV encephalitis (Tong et al. 2001; Dou et al. 2003; Everall et al. 2002). Several small pilot studies have demonstrated improved neuropsychological performance in HAND patients following short-term VPA or lithium therapy (Schifitto et al. 2006, 2009b; Letendre et al. 2006). Additional studies using selective serotonin reuptake inhibitors (SSRIs), including citalopram and paroxetine are also under consideration as adjunctive therapies for HAND (Ances et al. 2008; Letendre et al. 2007). SSRIs were predicted

to directly decrease levels of HIV replication, although clinical trials have not yet found a significant improvement when an SSRI is used as an adjunctive therapy (Benton et al. 2010; Horberg et al. 2008). However, using an SSRI or other antidepressant medication in order to treat clinical depression in HIV+ individuals increases ART compliance and thereby improves virologic control, immune status, and would be predicted to improve cognitive functioning (Horberg et al. 2008).

44.4.3 Other Tested Therapies for HAND

Other adjunctive therapies for HAND are focused on targeting inflammation cascades that contribute to neurotoxicity. Minocycline is a broad-spectrum tetracycline antimicrobial that is capable of efficiently crossing the blood–brain barrier and suppressing HIV replication in microglia, macrophages and lymphocytes; the cell types mediating the neuropathogenesis of HAND (Colovic and Caccia 2003; Si et al. 2004; Zink et al. 2005; Szeto et al. 2010). In addition, minocycline has anti-inflammatory and antioxidative properties and can inhibit the secretion of the inflammatory cytokines including TNF- α , IFN- γ , and IL-2 by lymphocytes (Szeto et al. 2010). Experimental studies using SIV-infected macaques demonstrated that minocycline decreased CSF levels of CCL2, a marker of CNS inflammation, and decreased the severity of encephalitis (Zink et al. 2005). However, a 24-week clinical trial in which 73 ART-naïve, HIV+ patients were treated with minocycline demonstrated no improvement in cognitive functioning (Nakasujja et al. 2013). However, analysis of

CSF from a subset of patients demonstrated a significant reduction in lipid markers of oxidative stress (ceramides) in those who were treated, suggesting that a longer minocycline treatment period should be considered (Sacktor et al. 2014).

44.5 Current Therapeutic Considerations

Several new antiretroviral therapies, including integrase inhibitors and virus entry inhibitors, are now available clinically and are being investigated as therapeutics in HAND. Other active therapeutic considerations include inflammation, the immune response and oxidative stress.

44.5.1 Modified ART Regimens: Integrase Inhibitors

There are several novel ART-based therapeutic regimens and treatment strategies that are under current investigation for HAND. Raltegravir (Isentress), an integrase inhibitor, is being investigated as a potential neuroprotectant when used as part of intensification therapy in individuals with suppressed viral loads on ART. Theoretically, raltegravir when added to traditional ART regimens would reduce infection of healthy cells from chronic and latently infected cells replicating virus at low levels. In a small clinical trial (NCT00672932), raltegravir was added to home ART regimens for a 12-week period of time and CSF markers of immune activation and inflammation (neopterin) and blood CD8+ T cell activation (co-expression of surface CD38 and HLA-DR) was assessed. There was no significant difference observed in these domains after 12-weeks of intensification therapy with raltegravir (Dahl et al. 2011). A clinical trial was terminated due to inability to successfully recruit patients (NCT01448486) and as such, no clinical data linking raltegravir intensification therapy with neurocognitive performance is available.

44.5.2 Modified ART Regimens: Chemokine Receptor Blockade

The monocyte-derived brain macrophage and infiltrating T lymphocytes have each been proposed as potential HIV brain reservoirs, and as such each could be considered a critical target for novel therapeutics in HAND (Sturdevant et al. 2015). Accordingly, maraviroc (Selzentry) a CCR5 receptor antagonist and entry inhibitor is under active investigation. In a single arm, open-labeled trial, maraviroc was used in intensification therapy in 15 HIV+ individuals who had undetectable plasma VL and detectable monocyte HIV DNA (>10 copies/10⁶ cells). After 24 weeks of treatment, there was a

decreased proportion of circulating CD16+ monocytes, a reduction in monocyte HIV DNA content and reduced soluble CD163. These changes were associated with significant improvement in neuropsychological performance in six subjects who entered the study with mild to moderate cognitive impairment, thereby suggesting that maraviroc intensification may have efficacy against HAND (Ndhlovu et al. 2014). In another study, neurocognitive performance was evaluated in conjunction with CSF HIV VL and inflammatory markers in HIV+ individuals as they were transitioned from a traditional ART regimen (tenofovir/emtricitabine/efavirenz) to a maraviroc-containing regimen with better CNS penetration (abacavir/lamivudine/maraviroc). Eight subjects completed the protocol and assessment at 24–36 weeks after treatment switch. Compared to baseline, abacavir/lamivudine/maraviroc treatment resulted in a trend, albeit non-significant, towards improvement in neurocognitive status and reduced TNF- α concentrations in the CSF (Tiraboschi et al. 2015). Because maraviroc is highly CNS-penetrating, these early results for maraviroc are promising and emphasize the potential importance of accessing primary immune cell reservoirs in the CNS (T lymphocytes, monocyte/macrophages) as critical therapeutic targets in HAND.

Cenicriviroc acts as a CCR5 and CCR2 chemokine co-receptor antagonist and is thereby hypothesized to block virus entry, inflammation and monocyte migration. A current clinical trial is investigating if dual CCR5 and CCR2 blockade will allow for reduced monocyte activation and entry into the CNS and thereby prevent cognitive decline. The trial is actively recruiting patients with a study design of a single arm, 24-week trial of cenicriviroc in HIV-1 suppressed patients on ART for 1 year or more with mild to moderate cognitive impairment. This clinical study is currently recruiting subjects (NCT02128828).

44.5.3 Potential Targeting of Immune Activation and Oxidative Stress

Neurocognitive impairment results from dendritic damage and neuronal loss, especially in the cortex, hippocampus, and basal ganglia (Ho et al. 1989; McArthur et al. 2003; Everall et al. 2009). HIV-induced neuronal injury in vitro is associated with the release of various toxins from HIV-infected macrophages, and NMDA receptor-mediated excitotoxicity may be the common injury pathway (Lipton 2004; Kaul et al. 2005). Monocyte and macrophage activation, either due to viral infection or inflammation, results in the production and release of known neurotoxins including glutamate, nitric oxide, carbon monoxide and TNF- α into the CNS compartment. HAND patients have elevated levels of multiple pro-inflammatory cytokines, including IL-1 β , TNF- α and IL-6 in the CNS and/or CSF (Oster et al. 1987;

Perrella et al. 1992; Achim et al. 1993; Foli et al. 1997). Immune activation of astrocytes could similarly amplify these potentially neurotoxic pathways. Inflammatory mediators modulate the permissibility of the blood brain barrier and the entry of infected monocytes into the CNS. Additionally, TNF- α contributes to inflammatory processes in the periphery, such as gut permeability and bacterial translocation, which has been linked to neurological complications of HIV (Ancuta et al. 2008; Brenchley et al. 2006a, b; Brenchley and Douek 2008). Therefore, reducing inflammation in the periphery as well as within the CNS could be expected to improve neurocognitive impairment in HIV-infected patients.

Systemic oxidative stress in HIV infection is driven by chronic immune activation and through direct effects of HIV proteins (Elbim et al. 1999; Repetto et al. 1996; Allard et al. 1998). Evidence of oxidative stress was first suggested after diminished levels of reduced glutathione were found in plasma, lymphocytes, PBMCs, and monocytes isolated from HIV+ individuals (Eck et al. 1989; Malvy et al. 1994; de Quay et al. 1992). Additionally, in vivo markers of oxidative stress correlate with systemic disease progression in HIV-infected individuals (Suresh et al. 2009; Wanchu et al. 2009). Suppression of HIV replication with ART significantly reduces immune activation and oxidative stress, however this suppression is incomplete (Roc et al. 2007; Bandaru et al. 2007; Eden et al. 2007). This persistence suggests that the pathways driving production of neurotoxins remain active and that neuronal damage is accumulating even during virologic suppression.

Specific work examining the role of the antioxidant response in modulating neurotoxicity and neurocognitive dysfunction led to the identification of heme oxygenase-1 (HO-1) as a key marker and potential regulator of HAND (Cross et al. 2011; Gill et al. 2014). HO-1 is an inducible, detoxifying enzyme that is critical for limiting oxidative stress, inflammation, and cellular injury within the CNS and other tissues. Prefrontal cortex HO-1 deficiency correlated with higher brain and CSF viral load, markers of immune activation, and a clinical diagnosis of HAND (Gill et al. 2014). Using an in vitro model, it was demonstrated that HIV infection reduces levels of HO-1 in macrophages and that this deficiency is linked to increased production of the excitatory neurotoxin, glutamate (Cross et al. 2011; Gill et al. 2014). And, as restoration of HO-1 reduced the production of glutamate by HIV-infected macrophages, therapeutics that can induce HO-1 expression in the CNS may have efficacy in preventing the progression of HAND in HIV+ individuals. Tecfidera (dimethyl fumarate) is a recently FDA approved therapeutic for multiple sclerosis. Dimethyl fumarate, and its primary in vivo metabolite monomethyl fumarate, has been demonstrated to effectively induce HO-1 expression, inhibit HIV replication and attenuate

macrophage-mediated neurotoxicity in an in vitro model of HAND (Cross et al. 2011). Future studies may continue to target components of the immune response and oxidative stress pathways in order to directly modulate the production of neurotoxins and limit HAND.

44.5.4 Anti-inflammatory Properties of Statins

The neuropathogenesis of HAND is attributed to chronic inflammation and the production of excitatory neurotoxins from activated monocytes, which are recruited to the CNS during HIV infection. Persistent monocyte/macrophage activation has been proposed as a critical target for adjunctive therapy to prevent HAND. Atorvastatin has been demonstrated to have anti-inflammatory properties on monocyte activation status in vitro and in ART treated HIV+ individuals (Ganesan et al. 2011). A clinical trial to determine if atorvastatin will reduce the inflammatory and activated phenotype of monocytes in HIV+ individuals on ART is currently recruiting subjects (NCT01600170). Ongoing research is also examining the role of other statins on monocyte-mediated inflammation and activation, especially in regards to reducing neurovascular risk factors in HIV+ individuals (Subramanian et al. 2012; Funderburg et al. 2014).

Outside of a novel therapeutic in HAND, statins have been prescribed in the treatment of hyperlipidemia, which, particularly in mid-life, appears to be associated with an increased risk of dementia (Anstey et al. 2008). Statins also promote cardiovascular and, presumably, cerebrovascular health through antioxidant and anti-inflammatory effects and improved endothelial function (Miida et al. 2007; Jain and Ridker 2005). A recently published systematic review of the role of statins in preventing cognitive decline and dementia reviewed randomized controlled trials and observational studies of various study designs. The conclusion of the analysis was that initiation of statins in late life does not prevent cognitive decline or dementia over the next few years (3–5 years) (Power et al. 2015). It could not be determined if mid-life or long-term statin use has beneficial effects on cognition in later years, however, the authors state that these findings have no bearing on existing recommendations regarding statin use for primary and secondary prevention of cardiovascular disease.

44.6 The Effect of Common Comorbidities in HAND

While HAND can develop at almost any stage of HIV infection, it is more common as immunosuppression and disease burden progresses. Some demographic risk factors have been identified however, including anemia, low body mass index,

increasing age, injection drug use and female sex (McArthur and Brew 2010). In the era of ART, HIV+ individuals are living longer and accumulating additional risk factors for neurocognitive impairment including those associated with aging, Alzheimer's disease and neurovascular events.

44.6.1 Hepatitis C Co-infection and HAND

Hepatitis C virus (HCV) infection is a worldwide problem that is often linked to HIV infection, with an estimated 30% of HIV+ patients having co-infection with HCV (Armstrong et al. 2006). HCV has been suspected to be an independent risk factor for neurocognitive impairment (Forton et al. 2005), however, studies attempting to determine the contribution of HCV to HAND in HIV co-infected cohorts have been limited. In a recently published study, the effect of HCV on neurocognitive performance in chronically HIV-infected patients enrolled in the CHARTER study demonstrated that HCV co-infection does not contribute to neurocognitive impairment (Clifford et al. 2015). Specifically, global deficit scores, the proportion of impaired individuals and the domains of neurocognitive functioning did not differ with HCV status. Among those with HCV, there was no association between neurocognitive performance and serum HCV RNA levels (Clifford et al. 2015). Notably, this CHARTER study excluded those HCV+ individuals with substantial HCV-associated liver damage. However, new treatments for HCV that are both more tolerable and effective are becoming available (Liang and Ghany 2013) with Sofosbuvir (Sovaldi) gaining FDA approval for treatment of HCV in HIV+ individuals.

44.6.2 Aging, APOE and Neurovascular Risk Factors and HAND

The number of older people living with HIV is growing due to the increased life expectancy of people with HIV undergoing ART but also due to the increasing number of people seroconverting at an older age. In 2013, an estimated 4.2 million people aged 50 years and older were living with HIV (Mahy et al. 2014). Those living with HIV have an increased risk of age-associated comorbidities including peripheral arterial disease, cardiovascular disease, and impaired renal function (Schouten et al. 2014; Canizares et al. 2014). Metabolic and systemic effects of ART can compound these risk factors (Samaras et al. 2007) and the risk for an ischemic stroke is higher in those with HIV+ (Sico et al. 2015). In addition to neurovascular events affecting cognition, genetic risk factors such as the *Apolipoprotein E* (APOE) $\epsilon 4$ allele may predispose to developing HIV-associated dementia, although this association remains controversial (Morgan et al. 2013; Valcour et al. 2004). Additionally, clinical

depression may present or be caused by cognitive impairment (Woods et al. 2009). Recognizing the numerous other factors contributing to neurocognitive function and decline in HIV+ individuals has already led to the examination of several potential therapeutics (see Sect. 44.4) and will undoubtedly lead to more therapeutic targets in the future.

44.6.3 Genetic Variation as Risk Factors in HAND

HAND is a complex phenotype and many studies to date have focused on the frontal cortex, (rather than white matter, hippocampus and basal ganglia) and investigated the most severe forms of HAND (HAD and HIV encephalitis). Host genetic variation is expected influence the neuropathogenesis of HAND, just as host genetics have been demonstrated to impact an individual's susceptibility to infection and rate of disease progression (Rappaport and Berger 2010; Naicker et al. 2012; Guernon et al. 2012). Genomic, transcriptomic, and epigenomic studies have highlighted the role of inflammation, immune regulation, metabolism and oxidative stress in the development of HAND (reviewed in Kallianpur and Levine (2014)). An important distinction between identifying genetic risk factors for HIV encephalitis (HIVE) and identifying risk factors for HAND, which is the more relevant goal in the era of ART. An autopsy study conducted through the National NeuroAIDS Tissue Consortium (Gelman et al. 2013), distinguished transcriptome patterns in individuals with HAND with HIVE from those with HAND without HIVE. Notably, HAND without HIVE is associated with low brain viral load, increased expression of endothelial-type transcripts, and absence of HIVE-specific transcriptomic features. A broad range of functions/pathways identified by grouping of genes identified by weighted-gene coexpression network analysis (WGCNA) includes genes involved in cancer and oligodendrocyte function were implicated in HAND pathogenesis (without HIVE).

Thus, although candidate genes have been suggested as either risk or protective factors in HAND, variations in study design and methodology and changing epidemiology (pre-ART vs. post-ART) have resulted in a lack of identification and validation of individual genes and associated variants as predictive markers for increased risk for HAND. As such, the quest for identifying genetic risk factors for HAND is somewhat similar to the quest for identifying risk factors for multiple sclerosis: no single gene/variant can fully account for heritable risk, but perhaps multi-genic influences on immune function, inflammatory responses, synaptic plasticity and neuronal function, and oxidative stress and mitochondrial function represent the driving forces for HAND that defy further identification of a signature genetic risk.

Future studies are necessary to explore the interplay of genes in different brain regions and they will have to tackle the more subtle changes associated with today's predominant forms of HAND, ANI and MND. These future studies may be able to identify additional therapeutic targets and improve our understanding of the complex neuropathology mediating HIV-associated cognitive impairment.

44.7 Review Questions

1. List 3 major disorders that fall under HAND that are discussed within this chapter.
2. Why can HAND not be mediated simply by uncontrolled HIV replication in the CNS compartment and the neuro-pathogenesis of this syndrome?
3. How is Functional MRI (fMRI) used to examine the symptoms of HAND?
4. How has ART been used for the treatment of HAND?
5. How is systemic oxidative stress created in patients with HIV infection?

44.8 Answers

1. Asymptomatic neurocognitive impairment (ANI), mild neurocognitive disorder (MND), and HIV-associated dementia (HAD)
2. Initiation of neuronal damage and clinically evident HAND can occur before AIDS criteria are met, as is evident by a prospective study that identified HAND in 8–34 % of aviremic patients who had no comorbidities and with a CD4 nadir of greater than 200 cells/ μ L (Cysique et al. 2006).
3. It can examine the hemodynamic response related to neural activity, and impairment of normal recruitment of brain regions that suggest brain dysfunction. Using this technique, it has been shown that HIV+ individuals have greater parietal activation for a simple attention task and greater frontal and parietal activation during more complex tasks (Chang et al. 2001).
4. With ART treatment, many patients have shown durable and complete suppression of HIV replication as determined by plasma viral RNA quantification, although a surprisingly high overall rate of HIV 'blipping' (defined as spontaneous expression of viral RNA copies (plasma, CSF)) in individuals on ART with sustained antecedent undetectable VLs with return to undetectable levels (Grennan et al. 2012) of up to 25 % has been reported.
5. Systemic oxidative stress in HIV infection is driven by chronic immune activation and through direct effects of HIV proteins (Elbim et al. 1999; Repetto et al. 1996; Allard et al. 1998).

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Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder in which memory and cognitive dysfunctions are the earliest symptoms. Pathologically, the disorder is characterized by neuron loss in hippocampus and multiple cortical regions, along with the formation of extracellular amyloid plaques and intracellular neurofibrillary tangles. The amyloid in this disorder is comprised largely of a peptide called A β , which is a degradation product of a protein with unknown function referred to as the amyloid precursor protein. The genetics of dominant inherited forms of Alzheimer's disease implicate increased production of the long form of the A β peptide as the critical feature leading to unavoidable development of the disease. This recognition has led many to consider anti-amyloid therapeutics as a means of slowing or arresting the disease. One approach that may become the first to test the so-called "amyloid hypothesis" is immunotherapy. Both vaccination and monoclonal antibody therapies have been tested with considerable success in mouse models of amyloid deposition. These approaches can prevent the formation of amyloid deposits, remove already existing deposits, and reverse the memory deficits associated with amyloid deposition in these mice. A human clinical trial was cut short due to an apparent autoimmune reaction in a fraction of the patients, but the results from this truncated trial still suggest that there may be therapeutic benefits of the approach. Current research is focusing on trying to understand the mechanisms by which antibodies directed against A β aid in clearing the amyloid deposits, how the vaccines might be constructed to overcome self-tolerance without evoking autoimmune reactions, and testing various monoclonal antibody preparations for efficacy in clinical trials. Even partial success in slowing the progression of this disease can have considerable societal and economic impact. Furthermore, verification of the amyloid hypothesis will encourage development of a number of other anti-amyloid therapies that may synergize with the immunotherapeutic approach.

Keywords

Active vaccination • Alzheimer's disease • Amyloid • Amyloid precursor protein • Antibody titer • Beta-pleated sheet • Cerebral amyloid angiopathy • Dementia • Immunotherapy • Monoclonal antibody • Passive vaccination • Senility

45.1 Introduction

Historically, the condition of dementia first described by Alzheimer in 1907 was called generically "presenile dementia." This nomenclature distinguished Alzheimer's as a disease, and not the dementia that occurred typically as humans

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reached advanced years, known commonly as “senility”. It was believed that all of us would develop senility after living long enough, and that this was a normal part of the aging process.

One of the defining characteristics of Alzheimer’s was its pathology, consisting of senile or neuritic plaques and intracellular inclusions termed neurofibrillary tangles (see below). In the last quarter of the last century it became increasingly clear that many cases of “senile dementia” also had these same plaques and tangles common to the presenile form described by Alzheimer. This led to terminology referring to “senile dementia of the Alzheimer-type” to describe these late-onset cases. Ultimately, it became recognized that the pathologies Alzheimer reported were associated with dementias at any age, and the term “Alzheimer’s disease” was used to describe any severe cognitive deterioration that was associated with plaque and tangle pathology. This nomenclature has held for roughly the last two decades.

Over this same period, the numbers of individuals diagnosed with Alzheimer’s disease increased dramatically. In part, this was due to increased public awareness that severe cognitive deterioration was not normal human aging (senility) but a disease with specific pathological determinants. A second reason was the increasing number of humans living to the age of risk for developing Alzheimer’s disease. Although his original patient was quite young (50s), the vast majority of Alzheimer victims are in their 70s and 80s when they begin to show symptoms. The average age of death of an Alzheimer patient is greater than the age of death in the general population. In the past, these were survivors whose physiology protected them from cardiovascular disease and cancers (the leading causes of death for the last 50 years). Today, the remarkable medical success in retarding the age-adjusted incidence of cardiovascular disease has expanded the population reaching these later years, leading to increased numbers of demented patients (currently reaching four million in the United States, or slightly more than 1 out of every 100 Americans). Medical care costs for Alzheimer patients, who average 10 years survival with the disease, total \$100 billion, or 7% of all medical costs in the US. The major reason this disease is so costly is that most are institutionalized for some period preceding their demise. It is estimated that if the onset of dementia could be delayed by as little as 5 years, this would save \$50 billion in medical costs (essentially the same as the new Medicare drug benefit). Clearly, effective therapeutic or preventative approaches to this disorder would provide clear benefits not only to the patients and their families, but the nation as a whole.

The pathology of Alzheimer’s disease was initially described using silver stains of histological sections. This resulted from the success of the emerging photographic industry in the early part of the last century, where silver chemistry was being worked out with great precision. The plaques and

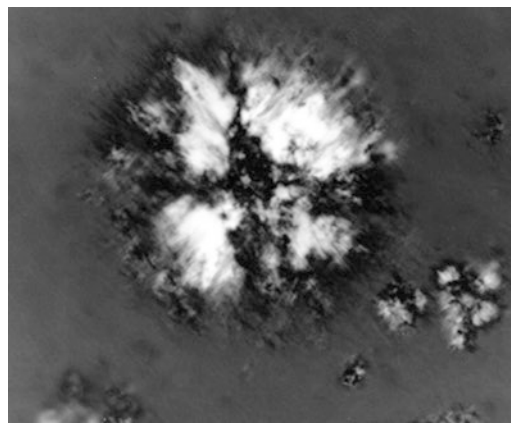


Fig. 45.1 Compacted parenchymal amyloid plaque in APP transgenic mouse hippocampus. Section was stained with Congo red and viewed with cross-polarized light. Total magnification $\times 400$

tangles described by Alzheimer were by definition, “argyrophilic,” meaning they bound silver molecules, which could then be reduced chemically to reveal stained regions.

The neuritic or senile plaques, also now called compact plaques, are comprised of fibrils of a short peptide called the A β peptide. This 40–42 amino acid peptide aggregates in a beta-pleated sheet structure to form fibrils, which are themselves aggregated into these dense plaques. These fibrils are referred to as “amyloid” because they stain with a dye called Congo red. There are many types of amyloids in the body, all of which share the properties of beta-pleated sheet structures, fibril formation and binding of Congo red dye. One feature of the Congo red dye is that it will shift the plane of polarized light when bound to beta sheet structures, leading to a characteristic red–green birefringence when viewed through crossed polarizing filters (Fig. 45.1). Surrounding these amyloid plaques is a zone of degenerating neuronal processes called neurites. These neurites are swollen, apparently drawn towards the amyloid plaques, and contain proteins or modifications of proteins (such as phosphorylation) not normally found in the larger processes of neurons. In addition to the compacted amyloid plaques, this amyloid material could often be found in association with blood vessels, where it was called cerebral amyloid angiopathy.

In the mid-1980s, George Glenner and his team (Glenner and Wong 1984) tried to determine the amino acid sequence of the amyloid deposits in Alzheimer’s disease. He had already found that other forms of amyloid in the body consisted of small fragments of much larger proteins that aggregated in this beta-pleated sheet secondary structure. When he tried to sequence the amyloid from the compacted plaques, he found variable results because much of the material had been truncated at the N terminal used to initiate the sequencing. However, the vascular deposits were more uniform, and he sequenced a 40 amino acid peptide called A β . This led within a year to the identification of a previously uncharacterized

parent molecule containing the A β peptide sequence called the amyloid precursor protein (APP). This molecule has a single transmembrane spanning domain and the bulk of the 1000 amino acid peptide is oriented towards the outside of the cell. A more detailed description of amyloid synthesis and its different form of aggregation is available in a separate chapter for "Alzheimer's disease."

Neurofibrillary tangles proved more difficult to identify at the molecular level. They also were fibrillar aggregates of a protein, and they also had a great deal of beta-pleated sheet structure, including staining by Congo red. However, their location within cells precluded them from being referred to as an amyloid. It is now widely accepted that these neurofibrillary tangles are comprised of abnormally and excessively phosphorylated forms of the microtubule associated protein tau. These tau filaments are largely insoluble by most known solvents, which slowed the identification of their major components.

A more detailed description of tau biology and neuroinflammation associated with AD pathology can be found in a separate chapter for "Alzheimer's disease."

A detailed account of the genetics of Alzheimer's disease is beyond the scope of this chapter. Others have detailed this quite thoroughly (Hardy 1997). However, there are at least three genetically identifiable categories of Alzheimer's disease. The first are those rare cases in which Alzheimer's is caused by a dominant genetic mutation (Table 45.1). While accounting for at most 1–2% of all cases, these have been very informative. Three genes are known to carry these mutations. One is the APP itself (Goate et al. 1991). Mutations in APP which modify its cleavage to favor either more A β or longer forms of A β (A β ending at amino acid 42 instead of amino acid 40) can cause an early onset form of Alzheimer's, with symptoms beginning in the 40s to 50s. A second gene that can carry a large number of different mutations and cause Alzheimer's disease is referred to as presenilin-1 (Sherrington et al. 1995). All tested mutations in this gene that cause Alzheimer's disease increase production

of the long form of A β . Presenilin-1 is a component of the complex that cleaves the variable C terminal of A β . It is also relevant that an extra gene dosage of APP can cause plaque and tangle pathology. Most Down's syndrome carriers have an extra copy of the APP gene located on human chromosome 21. By age 40, most will have plaque and tangle pathology upon autopsy, and many will develop a dementia condition in their late 40s and 50s.

A second genetic category includes normally occurring polymorphisms that increase the risk of developing the disease. While many polymorphisms have been mentioned, one that is consistently linked to increased risk is the Apolipoprotein E4 allele. Individuals carrying this allele have a threefold greater risk of developing the disorder (Corder et al. 1993; Poirier et al. 1993). One effect of this allele is to lower the age of onset, so these cases are often found in the 60s. The third category is comprised of those cases for which genetic linkage cannot be identified. It is important to note that the extreme age of onset complicates genetic analyses, as the likelihood of forebears reaching the typical age of onset is quite low. Thus, the size of the idiopathic group is not well defined. As more gene polymorphisms become consistently linked to increased risk, the size of this population may decline.

The recognition that the final common path for all known genetic causes of Alzheimer's disease, including Down's syndrome, is increased amounts of the longer A β variants led to the development of transgenic mice over-expressing mutated forms of human APP. While many unsuccessful mice were generated in the early 1990s, the first to demonstrate compacted amyloid plaques was the PDAPP mouse expressing a platelet-derived growth factor gene promoter-driven human APP (isoforms 695, 751, and 770) with the V717F (Indiana) mutation (Games et al. 1995). Shortly thereafter, a second APP mouse, the Tg2576, was found to have remarkably similar pathology (Hsiao et al. 1996). In the same month, a presenilin transgenic mouse developed was described (Duff et al. 1996). Although the presenilin transgenic mice alone had little pathology, our group found that the presenilin mutation greatly accelerated the accumulation of plaque pathology when crossed with the Tg2576 mouse (Holcomb et al. 1998).

There are now a variety of different APP transgenic mice that develop plaque pathology. The similarities are, in the opinion of these writers, more striking than their differences. All develop compacted plaques in the cerebral cortex and hippocampus. These plaques are associated with dystrophic neurites and the activation of astrocytes and microglial cells. All mice develop a memory dysfunction that is usually correlated with A β levels in mice of a given age (Morgan et al. 2000; Lewis et al. 2001). In none of the mice is there extensive neuron loss, as found in AD. Moreover, none of these mice develop tau pathologies, such as neurofibrillary tangles.

Table 45.1 The genetics of Alzheimer's disease

Type of Alzheimer's	Age of onset	Inheritance	Comments
Familial (<2 %)	40s–50s	Autosomal dominant	Mutations in amyloid precursor protein, presenilin-1 or presenilin-2 account for most cases
Inherited Risk (30–50 %)	60s–70s	Not dominant	Apolipoprotein E4 allele increases risk (earlier age of onset). Pathology more severe
Sporadic (50–70 %)	70s–80s	None identified	Increased risk; head trauma, decreased risk; education

Thus, these mice should be characterized as being models of amyloid deposition, not models of Alzheimer's disease.

When A β is added to tau transgenic mice, the results suggest that amyloid pathology may precipitate tau pathology. Either when APP mice are crossed with tau mice (Lewis et al. 2001) or exogenous A β is applied to tau transgenic mouse brain (Gotz et al. 2001), there is acceleration of the tau pathology. Later, a triple transgenic mouse has been generated that harbors APP, presenilin and tau mutations (Oddo et al. 2003), although neuron loss has not been reported to date. These transgenic models of select aspects of Alzheimer pathology have proven useful for preclinical evaluation of experimental therapies, which might be able to slow or halt the progression of Alzheimer's disease, including immunotherapy.

45.2 Early Studies

The first suggestions that anti-A β antibodies may be useful in ameliorating amyloid pathology came from work by Beka Solomon and colleagues in Israel. They published that anti-A β antibodies could block the formation of A β fibrils from monomeric forms in vitro (Solomon et al. 1996). They then proceeded to show that these antibodies could disaggregate preformed amyloid fibrils, and that the antibodies could achieve disaggregation at stoichiometries less than 1:1 (Solomon et al. 1997). This suggested that the antibodies were catalytically dissolving the fibrils, presumably by favoring the formation of non-beta sheet conformations. Inclusion of antibodies could block the in vitro neurotoxicity of A β . Subsequently, they identified a 4 amino acid sequence in the N terminal domain of A β they felt was the essential epitope for this mode of action (Frenkel et al. 1998). The catalytic disaggregation hypothesis remains one of the major proposed mechanisms for the action of anti-A β antibodies (Table 45.2).

The first observation that vaccination against A β might effectively lower A β deposition in vivo came from Schenk's

study (Schenk et al. 1999). These authors demonstrated that immunization of young PDAPP transgenic mice resulted in dramatic reduction in amyloid deposition as the mice aged. Even when started at midlife, the immunization protocol eliminated formation of amyloid deposits. Importantly, they observed the presence of activated microglia in the vicinity of the few remaining deposits. This led them to speculate that opsonization of the amyloid deposits led the microglia to phagocytose this material.

The same group subsequently demonstrated antibody-stimulated phagocytosis could occur in vitro (Bard et al. 2000). They placed microglia on sections of Alzheimer disease brain tissue, with and without anti-A β antibody present. Only in the cases where the antibody was present did they observe clearance of the amyloid deposits by the microglia. Although cultured primary microglia and cell lines phagocytose fibrillar A β added to cultures, antibody addition accelerates this process (Webster et al. 2001). Thus, a second mechanism by which anti-A β antibodies may clear amyloid deposits is by microglial activation and stimulation of phagocytosis (Table 45.2).

A third mechanism by which antibodies might reduce brain A β levels was suggested by DeMattos et al. (DeMattos et al. 2001). These authors noted that passive administration of a monoclonal antibody generated against A β resulted in large increases in circulating A β , analyzed by ELISA (enzyme-linked immunosorbent assay, a measurement that may be complicated by circulating antibody under some circumstances). They suggested that the circulating antibodies would sequester A β in the periphery, thereby increasing the brain to blood concentration gradient leading to a greater net efflux of A β from the brain to the periphery. It is important to recognize that the deposition of A β in mouse brain requires considerable over-expression of the APP transgene, always with a mutation that further increases the production of longer forms of A β . Thus it is conceivable that even minor shifts in the production and clearance of A β may have profound impacts on whether deposits accumulate or not.

For the most part, early studies of A β vaccination focused upon amyloid deposition. However, some of the APP mice were also being characterized behaviorally, and were found to have deficits in memory function that correlated with the extent of amyloid deposition. Thus, shortly after the publication that amyloid vaccination effectively reduced amyloid loads, our group and that of St-George-Hyslop et al. began immunizing APP transgenic mice to observe the impact on memory formation. To be honest, our group was concerned that the vaccine might provoke excessive inflammation in the brains of the transgenic mice (by over-activating microglia), and cause premature memory deficits. This was based on a widely-held belief that inflammation associated with amyloid deposits may be a pathogenic mechanism in Alzheimer's disease (Akiyama et al. 2000). However, our

Table 45.2 Mechanisms of Anti-A β Action

Catalytic Dissolution	Antibody binding converts A β secondary structure to form incapable of β -sheet fibril formation	Solomon et al. (1996) Solomon et al. (1997)
Microglial Activation	Opsonization of amyloid deposits activates microglia via effector molecules to clear deposits by phagocytosis or other mechanisms	Schenk et al. (1999a) Bard et al. (2000)
Peripheral Sink	Circulating anti-A β antibodies bind A β and reduce the free concentration in blood. This leads to increased net efflux from the brain	DeMattos et al. (2001)

testing specifically failed to identify premature memory loss with the immunization, and instead found protection from the development of memory deficits as the mice aged. Similar protection was found by the St-George-Hyslop group in the CRND8 APP transgenic mouse, and we arranged for tandem publication of these results (Morgan et al. 2000; Janus et al. 2000). Subsequent work found that some types of memory deficits could be reversed quite rapidly using passive immunization (Dodart et al. 2002; Kotilinek et al. 2002). This has led to the hypothesis that some pool of A β other than fibrillar deposits is responsible for some of the memory deficits observed in transgenic mice, and that this pool can be rapidly depleted by some anti-A β antibodies. Recent studies suggest that it is an oligomeric pool that is responsible for disruption of synaptic plasticity (Walsh et al. 2002; Cleary et al. 2005). Anti-A β antibodies appear capable of neutralizing the plasticity-disrupting influence of these oligomers (Klyubin et al. 2005).

It is not certain which of the proposed mechanisms of anti-A β immunotherapy is most correct. It is important to recognize that they are not mutually exclusive, and that all three may be working. In fact, the balance among these three mechanisms may vary for different types of immunotherapy.

45.3 Clinical Trial Experience with Active A β Vaccination

Based on the success of the A β vaccination approach in mouse models of amyloid deposition, Elan and Wyeth teamed to perform phase 1 and phase 2A trials in humans. Phase 1 trials resulted in no overt adverse events in human volunteers. Thus a phase 2 trial was initiated with 300 patients receiving a vaccine against A β (AN1792; using QS-21 as an adjuvant) and 60 patients receiving placebo inoculations. The original goal of the trial was to repeatedly vaccinate patients until a predetermined anti-A β antibody titer was reached. It was noted in the phase 1 trial that only a portion of the patients developed measurable antibody titers against A β (Schenk 2002). Even in the mouse studies, repeated inoculation was necessary to achieve high antibody titers (Dickey et al. 2001). This was further complicated by the advanced age of the patient population, which is known to cause increased variation in the response to vaccines.

Within several months of initiating the trial, the trial was interrupted due to the occurrence of multiple instances of adverse reactions in the patient population. This was characterized as aseptic meningoencephalitis, essentially swelling in the brain that was a form of autoimmune reaction presumably elicited by the vaccine (Orgogozo et al. 2003). Examination of tissue from two patients who ultimately came to autopsy revealed substantial T cell infiltration into the brain (Nicoll et al. 2003; Ferrer et al. 2004). In all, roughly

6% of the patients in the trial developed these symptoms, with the majority recovering after treatment with steroidal anti-inflammatory agents.

Although the inoculations with the A β vaccines were discontinued, the patients in the trial continued to be monitored both medically and cognitively. One cohort of patients in the trial, those in Zurich, appeared to have benefited from the vaccine. Hock et al. (Hock et al. 2003) found that those patients with the highest antibody titers appeared to remain stable cognitively. This stabilization appeared to extend 2 years after the last immunization. Importantly, it was the titers of antibodies that reacted with the amyloid deposits on brain sections that were associated with cognitive benefits (Hock et al. 2002). ELISA assayable titers did not associate with improved behavioral outcomes. When the entire study was analyzed, the linkage between antibody titers and cognitive benefit was less substantial (Gilman et al. 2005). It is unclear whether measurement of brain reactive antibody titers would improve this association as found for the Zurich cohort. Important caveats regarding the implications of this study stem from its truncation. Any results have to be considered within the context that the trial was not completed as planned and the potential benefits or failures must be considered in this context.

The histopathology of the patients in this trial is limited to three published reports. Two from patients that had a meningoencephalitic reaction (Nicoll et al. 2003; Ferrer et al. 2004) and one from a patient lacking any adverse reaction to the vaccine (Masliah et al. 2005). Although control cases were archival and the number of cases is very small, all three reports suggest there was less amyloid deposition than would be expected for an AD patient at each patient's stage of the disease. The reductions did not appear uniform throughout the brain. Moreover, the reductions appeared to be primarily in the neuritic plaque and diffuse A β deposits, but not in the amyloid deposits associated with the blood vessels.

A curious observation in the trial was the magnetic resonance imaging (MRI) findings of increased hippocampal shrinkage in those patients with the highest antibody titers (Fox et al. 2005). This occurred over the first year of the trial, and was also accompanied by ventricular enlargement. A similar observation was made within the Zurich cohort, however in this case, by the end of the second year, the hippocampal volumes were greater in those patients with the highest brain reactive antibody titers (Nitsch 2004). Nitsch has speculated that the initial loss of brain volume is secondary to two major changes; elimination of the bulk of the accumulated A β peptide and reduction of the inflammation and edema associated with the glial reaction to the amyloid deposits.

A 4.6-year follow-up study reports that there was no difference in percentage decrease in whole brain or hippocampal volume, or percentage increase in ventricular volume between antibody responders and placebo-treated patients,

either from the last visit in the phase 2a study or from the baseline MRI in the phase 2a study (Vellas et al. 2009). The samples size was limited to 7–8 per group in this follow-up study. The antibody responders maintained a low but detectable antibody titer and less cognitive decline as determined by the Disability Assessment for Dementia scale. Thus, this study supports the hypothesis that A β immunotherapy may have long-term functional benefits.

Table 45.3 depicts the A β —targeted clinical trials after the AN17982 trial. Second-generation vaccines were designed using a shorter peptide fragment, such as A β (1–6) or A β (1–7), in an attempt to prevent non-specific immune responses seen with the full-length A β vaccine. Using the second-generation vaccine, CAD106 was the first that reached the clinical phases of development (Wiessner et al. 2011). A recently completed phase II clinical trial has shown an A β -specific antibody response in 75 % of treated patients, without causing adverse inflammatory reactions. Vanutide cridifcar, developed by Janssen, has recently completed phase II trials with an additional phase II trial still ongoing (Ryan and Grundman 2009). However, the pharmaceutical company has abandoned the plans for further development of this vaccine. Affitope AD02 is another second-generation vaccine which completed a phase 2 trial (Schneeberger et al. 2010), but could not reach either primary or secondary outcome measures.

45.4 Other Forms of Active Immunization in Pipeline

Certainly, the AN1792 vaccine developed by Elan was relatively simple. It consisted of full length A β peptide, incubated in a physiological salt solution to promote fibril formation injected with a QS-21 adjuvant. One of the major issues in developing an effective A β vaccine is the recognition that A β is a self-protein to some extent. A β may be present in normal cells, albeit as a minor and short-lived product of APP processing. Thus vaccines may need to break self-tolerance (Monson et al. 2001). A second consideration is that an autoimmune reaction may have undesirable effects, such as that observed in those patients developing meningoencephalitis in the AN1792 trial. Presumably, these adverse reactions were due to development of a T cell response against A β , a self-antigen. A third consideration is that the relatively short A β peptide is not by itself a very potent immunogen. Thus, since the original publication by Schenk et al. (Schenk et al. 1999), there have been a number of alternative vaccine formulations investigated to produce anti-A β antibodies.

One of the first was an attempt to use mucosal vaccination as an alternative to injections (Weiner et al. 2000; Lemere et al. 2002). This was found to be an effective approach with

both production of high titer antibodies and clearance of amyloid deposits in transgenic mice. Another approach was to use a liposome-based therapy with the A β 1–16 peptide rather than full-length molecules (Nicolau et al. 2002). This palmitoylated vaccine construct was effective in breaking down self-tolerance to the vaccine in A β -overproducing mice. A different rationale was developed by a group at New York University (Sigurdsson et al. 2002). They were concerned that injecting full-length A β , a known neurotoxin, could produce adverse reactions by forming fibrils. This group has explored use of a truncated and modified A β peptide, which retains immunogenicity but not the capacity to form fibrils. Importantly, this vaccine can also reverse memory deficits in transgenic mice (Sigurdsson et al. 2004).

Another series of vaccines have used genetic engineering to produce a B-cell epitope towards A β and a T-cell response towards a non-self antigen. Agadjanyan et al. (Agadjanyan et al. 2005) used the first 15 amino acids of A β (where the vast majority of vaccine-generated antibodies bind; Dickey et al. 2001; McLaurin et al. 2002) coupled to a synthetic universal T cell epitope (PADRE). This succeeded in producing an immune response with high anti-A β antibody titers, but with no splenic T cell activation against the A β peptide. Presumably, a vaccine with these properties would not result in the autoimmune T cell reaction and would avoid the meningoencephalitic reaction found in some patients in the AN1792 trial. Importantly, these same authors found that the adjuvant chosen for the AN1792 trial, QS-21, biased the immune response toward a Th1 type of reaction (Cribbs et al. 2003). Given that Th1 responses are associated with autoimmune reactions, while Th2 responses suppress autoimmune responses, the choice of adjuvant may have contributed to the adverse events that were found with the active vaccine trial.

Another approach has been the development of DNA-based vaccines, where A β or a component is encoded genetically. Several of these have used viral vectors to deliver the vaccine. (Hara et al. 2004) administered adeno-associated virus encoding the A β peptide orally to mice. This resulted in epithelial cell expression of A β and prolonged elevation of anti-A β antibody titers, without eliciting T cell responses against A β (Hara et al. 2004). Kim et al. used an adenovirus vector encoding A β plus an adenovirus vector encoding GM-CSF to produce an immune response after intranasal administration (Kim et al. 2004). This response was found to have a Th2 bias, and was effective in reducing A β content in the brains of APP transgenic mice. Lavie et al. used a filamentous phage vector displaying only 4 amino acids from A β (EFRH; aa3–6) (Lavie et al. 2004). This vaccine produced a humoral response against A β , reduced brain amyloid loads and protected APP mice from cognitive deficits. Interestingly, another approach using an herpes simplex virus (HSV) amplicon to drive A β expression with a tetanus toxin fragment

Table 45.3 Clinical Trials of Alzheimer's Disease Therapy

Amyloid-related	Functions	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025
AGB101	SV2A modulator										Phase 2																
AN-1792	Full-length A β		Phase 2																								
Acitretin	ADAM10 enhancer										Phase 2																
Affitope AD02	A β (1–6)											Phase 2															
Avagacestat	GSI										Phase 2																
BAN2401	Anti-A β antibody												Phase 1, Phase 2														
Bapineuzumab	Anti-A β antibody							Phase 2, Phase 3																			
CAD106	A β (1–6)							Phase 2																			
CHF 5074	GSM											Phase 2															
Crenezumab	Anti-A β antibody											Phase 2															
E2609	BACE inhibitor																		Phase 2								
EIND005	A β oligomer inhibitor							Phase 2																			
EVP-0962	GSM													Phase 2													
Gammagard	IVIg								Phase 2																		
Gamunex	IVIg													Phase 2/3													
Gantenerumab	Anti-A β antibody												Phase 2/3, Phase 3														
JNJ-54861911	BACE inhibitor																	Phase 2									
NIC5-15	GSM							Phase 2																			
Octagam® 10%	IVIg										Phase 2																
Ponezumab	Anti-A β antibody										Phase 2																
Solanezumab	Anti-A β antibody							Phase 2, Phase 2/3, Phase 3																			
Thalidomide	NSAID																										
Vanutide erdifxar	A β (1–7)							Phase 2																			
Verubecestat	BACE inhibitor																										

SV2A synaptic vesicle glycoprotein 2A, ADAM10 a disintegrin and metalloproteinase domain-containing protein 10, GSI gamma-secretase inhibitor, GSM gamma-secretase modulator, BACE beta-site app converting enzyme, IVIG intravenous immunoglobulin, NSAID non-steroidal anti-inflammatory drug

produced in the central nervous system (CNS) inflammation, and may serve as a model for the autoimmune reaction apparently found in the AN1792 trial (Bowers et al. 2005).

Thus, there have been a number of clever alternative approaches to developing active immunization protocols for the possible treatment of Alzheimer's disease. Most either enhance the immunogenicity of the vaccination regimen, reduce the possible autoimmunity or both. Nonetheless, there are still potential problems with all of the vaccines. For example, the response will be variable in an aged population, as was clearly found for the AN1792 trial (Bayer et al. 2005; Vellas et al. 2009). A second consideration is the epitope specificity of the humoral response. Antibodies directed against one epitope may be more effective than others (for example, the apparently greater effectiveness of antibodies reacting with brain amyloid deposits (Hock et al. 2003)). A final consideration is that it is still not certain why the patients developed the meningoencephalitic reaction in the first place. Certainly, the argument that T cell activation against a self-antigen elicited an autoimmune reaction appears logical, but this is not at all proven. There is no good mouse model of the type of reaction found in the two human cases that have come to autopsy (the report by (Furlan et al. 2003) has been difficult to replicate). Active immunization reactions can be difficult to control; one cannot unvaccinate someone after the plunger has gone down. Still, if anti-A β immunotherapy does prove effective in halting or even slowing the progression of Alzheimer's dementia, it is likely that active immunization regimens will become a critical component to therapy for this disease, if only because of their reduced costs compared to passive immunization approaches.

45.5 Passive Immunization

The use of monoclonal antibodies as therapeutics has become commonplace. Antibodies or chimeric proteins against tumor necrosis-factor (TNF)- α are very effective in quelling certain inflammatory diseases. Trastuzumab (against HER2) is effective in reversing the growth of some breast tumors. As of 2015, there were 43 antibody therapeutics approved for use in the US (2 in 2015, 6 in 2014).

Monoclonal antibodies against A β appear equally effective in reducing brain amyloid deposits as active immunization against A β . Bard et al. (2000) were the first to demonstrate that systemic administration of anti-A β antibodies can lower amyloid loads (Bard et al. 2000). Importantly, they found that not all antibodies were equally effective in reducing brain A β content (Bard et al. 2003). In a striking demonstration that amyloid plaques are rapidly reversible structures in transgenic mouse brain, Bacskai et al. found that amyloid deposits imaged through a craniotomy window with multiphoton microscopy could be removed within 3 days after

topical administration of an anti-A β antibody (Bacskai et al. 2001). Injections of anti-A β antibodies directly into the brain parenchyma caused a time dependent clearance of A β deposits over a period of a week (Wilcock et al. 2003). Similar effects of monoclonal antibodies were found after intraventricular administration (Chauhan and Siegel 2002, 2003). In the triple transgenic mouse model, Oddo et al. found that anti-A β antibody injections into the hippocampus not only cleared amyloid deposits, but also reduced the hyperphosphorylation of tau at some sites (Oddo et al. 2004). Intriguingly, after 45 days, the amyloid deposits returned as did the tau hyperphosphorylation.

Within the passive immunization field, there have been discussions of antibody superiority based upon the epitope domain of the antibody (usually divided into N terminal, mid domain or C terminal) or the antibody subtype (IgG1, IgG2a, IgG2b etc.), with the conclusion that one type of epitope specificity or subtype is better than another. A third consideration is antibody affinity for A β . Unfortunately, none of these features has been manipulated independent of the others. Each individual antibody will vary in all of these properties, and, particularly for epitope specificity, it is very difficult to assume equivalence. Thus far, in the opinion of this author, each antibody needs to be considered a separate entity. Each antibody's ability to clear amyloid *in vivo*, inactivate oligomers, activate microglia, bind A β , increase circulating A β and activate effector molecules (such as Fc γ -receptors) must be measured directly. It is plausible that some antibodies may have superior profiles in clinical trials because of one or more of these properties. However, assuming that it is one of these properties, which confer superiority without measuring them, all will likely be misleading.

45.6 Clinical Trials of Passive Immunization

As of 2015, six monoclonal antibodies reached either phase 2 or 3 clinical trials: BAN2401 (phase 2, active), bapineuzumab (phase 2, completed), crenezumab (phase 2, active), ganetenerumab (phase 2, active), ponezumab (phase 2, completed), and solanezumab (phase 2/3, active) (Table 45.3). The other active A β -related clinical trials are for BACE inhibitors: E2609 (phase 2) and verubecestat (phase 2/3) (Table 45.3). No other A β -related clinical trials, such as active immunization, gamma-secretase inhibitors (GSIs), gamma-secretase modulators (GSMs), non-steroidal anti-inflammatory drugs (NSAIDs), or intravenous immunoglobulin (IVIG), are active.

Bapineuzumab and solanezumab are the first two monoclonal antibodies reached phase 3 clinical trials (Salloway et al. 2014; Doody et al. 2014). Both of them are humanized monoclonal antibodies targeting A β (1–6) or A β (1–28). Bapineuzumab was tested on two double-blind, randomized, placebo-controlled, phase 3 trials involving patients with

mild-to-moderate AD; one involving 1121 carriers of the apolipoprotein E (APOE) $\epsilon 4$ allele and the other involving 1331 noncarriers for 6.5 months. The monoclonal antibody treatment did not significantly improve cognitive functions as determined by the change from baseline in the ADAS-cog11 and DAD scores (Salloway et al. 2014). They rather observed significant edema as determined by brain MRI in antibody dose and APOE $\epsilon 4$ allele dose-dependent manner.

Solanezumab was tested on two double-blind trials (EXPEDITION 1 and EXPEDITION 2) involving patients with mild-to-moderate AD; one involving 1012 and the other involving 1040 patients for 18 months. Neither study showed significant improvement in the primary outcomes as determined by the change from baseline ADAS-cog11 score or ADCS-ADL scores (Panza et al. 2014b). Solanezumab is still active in phase 3 trials of AD patients (NCT01127633, EXPEDITION EXIT, and NCT01900665, EXPEDITION 3), but not recruiting patients.

BAN2401 is currently active on double-blind, randomized, placebo-controlled, phase 2 trials and recruiting up to 800 MCI and mild AD patients (NCT01767311). BAN2401 is also humanized antibody against A β .

Crenezumab recognizes multiple forms of aggregated A β , including oligomeric and fibrillar species and amyloid plaques with high affinity, and monomeric A β with low affinity. Crenezumab was tested on two phase 2 trials (ABBY and BLAZE), none of the study met the primary endpoints of change on ADAS-cog and CDR-SOB or PET amyloid imaging, but did report a separation on the secondary endpoint of cerebrospinal fluid (CSF) A β (Watt et al. 2014; Jindal et al. 2014). Crenezumab is also being tested in a prevention paradigm. In a landmark, adaptive, 5-year study that started in 2013, crenezumab is the first immunotherapy to be evaluated as part of the study (Garber 2012; Corbyn 2013).

Gantenerumab is a fully human monoclonal IgG1 antibody designed to bind with subnanomolar affinity to a conformational epitope on A β fibrils. The epitope encompasses both N-terminal and central amino acids of A β . One phase 2 trial was started on 2010, which was extended to a phase 3 trial enrolling 799 people (SCarlet RoAD). The study was discontinued by the end of 2014, followed by a report of no efficacy on primary or secondary endpoints in this trial (Jindal et al. 2014; Panza et al. 2014c). In March 2014, Roche started a new phase 3 trials of gantenerumab on 1000 patients with a clinical diagnosis of mild AD. Gantenerumab, together with solanezumab, is also being investigated by the Dominantly Inherited Alzheimer Network (DIAN) in a Phase 2/3 trial aimed at preventing dementia in 210 people who are on the path to AD due to an inherited autosomal-dominant mutation in APP, PS1, or PS2 (Corbyn 2013; Panza et al. 2014a, c; Mills et al. 2013).

Finally, ponezumab is a humanized IgG2 δ A monoclonal antibody that binds the free carboxy terminal amino acids 33–40 of the A β 1–40 peptide. Ponezumab was tested on a phase 2 trial (NCT00722046). Development of ponezumab

was discontinued due to no effect on the primary endpoints of change in brain or CSF A β burden.

45.7 Circulating Antibodies

Several groups have found that some individuals have titers of antibodies against the A β peptide. Unfortunately, consensus regarding a relationship to Alzheimer's disease has not yet emerged as there are reports of increased titers with disease (Nath et al. 2003), decreased titers with disease (Weksler et al. 2002) or no effect of disease (Hyman et al. 2001). It should be noted that the antibody titers observed are extremely low compared to those observed after immunization. Moreover, it is not clear the degree to which endogenous A β may interfere with the detection of anti-A β antibodies by ELISA (Li et al. 2004).

Still, this has led to a short, open label trial of human intravenous immunoglobulin (IVIG), an FDA approved product that has benefit in several types of disorders, including multiple sclerosis. Dodel et al. reported that monthly administration of intravenous immunoglobulin to 5 Alzheimer patients reduced A β in cerebrospinal fluid, increased A β in serum and improved cognition (Dodel et al. 2004).

Three phase 2/3 trials were conducted on IVIG therapy: gammagard, gamunex and octagam[®] 10% (Table 45.3). Gammagard was tested on two pivotal phase 3 trials. One was Alzheimer's Disease Cooperative Study, enrolling 390 patients with mild to moderate Alzheimer's disease for 18 months. The study did not show significant changes in primary endpoints: cognitive and global function as assessed by the Alzheimer's Disease Assessment Scale-Cognitive Subscale and the Alzheimer's Disease Cooperative Study Activities of Daily Living, CSF measurements, cerebral metabolism by fluorodeoxyglucose-positron enhanced tomography (FDG-PET), and brain amyloid load by PET (Loeffler 2013). The second phase 3 trial was subsequently terminated.

Gamunex is also one of several IVIG products and is currently actively tested on phase 2/3 trial by enrolling in 350 mild to moderate AD patients (NCT01561053).

Finally, octagam[®] 10% has been tested on two phase 2 trials. One was a multicenter, placebo-controlled phase 2 trial at seven sites in the USA and five in Germany (Dodel et al. 2013). This trial missed its primary endpoint of change in plasma A β levels, and was negative for most of its secondary biomarker outcomes. A second, single-center phase 2 study reports possible effects on the Clinical Dementia Rating Sum of Boxes and brain atrophy as seen on MRI (Kile et al. 2015). The study is still active but not recruiting new patients.

Overall, one feature of immunoglobulin therapy is that the circulating levels of IgG increase dramatically. This results in feedback regulation of a number of immune pro-

cesses, as the levels gradually return towards normal. This may also explain the rather curious finding that administration of a proteasome adjuvant with glatiramer acetate alone is adequate to reduce amyloid deposits in transgenic mice (Frenkel et al. 2005). This does not require the presence of antibodies, and appears related to the activation of resident microglial cells near the amyloid deposits. Thus, it is conceivable that immunomodulation may be just as important as a specific immune reaction in the benefits of immunotherapy in APP transgenic mice. Clearly it will be critical to identify if the same holds in human trials.

45.8 Future Directions

In addition to anti-A β immunotherapy, tau has been a target for the future immunotherapy (Pedersen and Sigurdsson 2015). For example, AADvac1 was the first anti-tau vaccine to enter phase 2 clinical trials (NCT02579252) (Godyn et al. 2016). AADvac1 composed of a tau peptide fragment linked to keyhole limpet hemocyanin (Kontseikova et al. 2014). More pre-clinical and clinical trials on the immunotherapy of tau protein are on the horizon for a future direction.

45.9 Review Questions

1. What are the 2 main neuropathological hallmarks of Alzheimer's disease? Compare and contrast their anatomical location and protein composition.
2. Describe the major types of genetic mutations that are associated with Alzheimer's disease.
3. What is the "Amyloid Hypothesis" of Alzheimer's disease?
4. Review 3 proposed mechanisms of action for how anti- β -amyloid antibodies might remove amyloid deposits.
5. What is the difference between active and passive vaccination?
6. List 3 barriers to the use of active vaccination clinically.
7. List 2 advantages of passive immunotherapy over active immunotherapy?
8. List 2 disadvantages of passive immunotherapy over active immunotherapy?

45.10 Answers

1. The two main neuropathological hallmarks of Alzheimer's disease are amyloid plaques and neurofibrillary tangles. Amyloid plaques are extracellular, and are composed of the β -amyloid peptide, while neurofibrillary tangles are

intracellular in neurons, and are composed of abnormally phosphorylated tau protein.

2. Mutations in the amyloid precursor protein, presenilin-1 or presenilin-2 can be inherited in some families. These genes are dominant, so inheritance of one gene copy causes the symptoms of Alzheimer's disease. The gene apolipoprotein-E has been identified as a risk factor. Inheritance of the apolipoprotein-E4 allele is associated with an increased probability of developing Alzheimer's disease.
3. The "Amyloid Hypothesis" posits that the symptoms of Alzheimer's disease result from the accumulation of extracellular amyloid in the brain of affected individuals.
4. The 3 proposed mechanisms include (1) catalytic dissociation of amyloid fibrils by the antibodies directly, (2) activation of brain microglial cells causing phagocytosis of β -amyloid and (3) efflux of β -amyloid from the brain to the circulation as antibodies in the circulation bind β -amyloid, which reduces free β -amyloid concentrations in the blood and causes a change in the equilibrium between brain and blood β -amyloid concentrations.
5. In active vaccination, a protein is injected together with an adjuvant (a molecule that enhances immunity), leading to the activation of the host immune system and the production of antibodies against the injected protein. In passive vaccination, the antibodies are injected directly.
6. (1) The production of antibodies is variable, resulting in different numbers and types of antibodies in different individuals. (2) There may be side effects due to activation of undesired components of the immune system. (3) Once the protein is injected into the host, the immune response cannot be stopped if adverse events ensue.
7. (1) Because a therapeutic agent is administered directly in passive vaccination, its pharmacokinetics can be measured, controlled and standardized. (2) If adverse events ensue, injections of the therapeutic antibody may be stopped. Neither is true of active vaccination.
8. (1) Production and storage of the therapeutic antibody for passive immuno-therapy is more complicated and expensive than active vaccination. (2) Administration of passive immunotherapy must be repeated over long durations as symptoms continue. In contrast, active vaccination is administered once, or in as a short series of boosters.

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Abstract

Although patterns of neuronal degeneration are unique in PD and ALS, both disorders share common pathways and processes that support and possibly initiate neurodegeneration. Most of these processes are associated with induction, propagation, or consequences of neuroinflammation. Increased numbers of microglia that express reactive phenotypes and proximate dying neurons reflect the neuroinflammatory cellular response. Neuroinflammation amplifies oxidative stresses via reactive oxygen, nitrogen, and carbon species that react with biomolecules and increase molecular modifications of lipids, proteins, and nucleic acids. These reactive modifications eventually become deleterious to biochemical and cellular processes resulting in dysregulation of cellular functions and further neuronal injury and death. Whether neuroinflammatory responses are causal or consequential remains to be determined. Nevertheless, the importance of inflammatory responses to neurodegeneration is underscored in animal models, whereby attenuation of neuroinflammation by genetic manipulation or pharmacological agents mitigates neurodegeneration and increases neuronal survival. As such, immunological strategies that target neuroinflammatory processes represent promising candidates for therapeutic intervention in neurodegenerative disorders. These strategies embrace the capacity of regulatory T cells to protect neurons either directly via neurotrophic factors, or indirectly by modulation of microglial function to attenuate neuroinflammatory responses and by induction of astrocyte-derived neurotrophic factors.

Keywords

Dopaminergic neurons • Glatiramer acetate • Macrophage • Microglia • MPTP • NADPH oxidase • Peripheral benzodiazepine receptors • pk11195 • SNpc • SOD1 • Striatum • α -synuclein • T lymphocyte or T cell • TDP-43

46.1 Introduction

Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS) are progressive neurodegenerative disorders ranking 2nd and 5th in disease prevalence within the United States

(Relja 2004). PD is characterized by progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and reductions in their terminals within the dorsal striatum (see Przedborski, Sect. 22.2.4.2). These lead to profound and irreversible striatal dopamine loss. Neurological signs and symptoms consist of tremor, disturbances in gait, balance and coordination that commonly progress to cognitive and autonomic impairments. Cell loss modeling indicates that 100–200 SNpc neurons degenerate/day in PD (Orr et al. 2002). For ALS (known also as Lou Gehrig's disease) motor dysfunctions are characterized by gradual degeneration of spinal cord motor neurons eventually leading to progressive

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weakness, paralysis of muscle, and death (see Simpson et al., Sect. 32.2.4.3). The mechanisms for neurodegeneration in both disorders relate, in part, to the abnormal accumulation, oligomer formation and misfolding of α -synuclein (α -syn) in PD and superoxide dismutase 1 (SOD1) or TAR DNA-binding protein 43 (TDP-43) in ALS. These protein aggregates form at synapses and axons leading to signaling abnormalities and neuronal dysfunction. Neuroinflammatory responses, mitochondrial function, glutamate transport toxicity, and free radical formulation are linked to protein toxicities and lead to neuronal destruction in both. It is hypothesized, that changes in the balance between factors promoting aggregation, clearance and synthesis of α -syn and SOD1 or TDP-43 underlies disease pathogenesis.

Microglial activation and alterations in lysosomal function are linked to oligomer accumulation in the membrane and extracellular and can be recognized by antibodies that often promote their clearance (Masliah et al. 2005). Anti- α -syn antibody-mediated clearance leads to decreased accumulation of aggregated α -syn in neuronal cell bodies and synapses with reduced neurodegeneration. Antibodies may also recognize abnormal α -syn associated with the neuronal membrane and promote α -syn degradation through lysosomal-autophagy pathways. Thus, the generation of humoral and cellular responses against aggregated proteins by vaccination may be effective in reducing the neuronal accumulation of α -syn aggregates. This approach, if administered appropriately, could improve disease outcomes (Miller and Messer 2005). Indeed, vaccination strategies with α -syn or SOD1 have been developed for treatments that highlight anti-amyloidogenic responses. To such ends, antibodies specific for peptides or conformations of misfolding neurodegenerative disease proteins can be engineered for affinity and stability then delivered intracellularly as intrabodies. For SOD1-linked familial ALS, or TDP-43 in sporadic disease, aberrant oligomerization of mutant proteins affects disease. Moreover, the formation of soluble oligomers suggests a general, unifying picture for SOD1 or TDP-43 aggregation in neuronal destruction. In the case of ALS, vaccination with recombinant SOD1 or passive immunization with antibodies against SOD1 was successful in animal models for ALS and PD (Urushitani et al. 2007; Gros-Louis et al. 2010; Masliah et al. 2005, 2011). The approach is based on reductions of toxic mutant proteins, but could also elicit secondary tissue destructive effects. Work from our own laboratory has shown that vaccine induction of the adaptive immune system can have both destruction and protective disease outcomes for PD and ALS (Benner et al. 2004; Reynolds et al. 2007, 2009; Kosloski et al. 2013; Olson et al. 2015). Immunoregulatory treatments (e.g. glatiramer acetate (GA), vasoactive intestinal peptide (VIP), granulocyte macrophage-colony stimulating factor (GM-CSF), and 1,25-dihydroxyvitamin D3) that modulate protective T cell

responses can be part of successful immunotherapeutic strategies. However, those treatments that elicit destructive immune responses can lead to opposite outcomes. In this chapter, we review the roles of the innate and adaptive immune system in the pathogenesis of PD and ALS and the recently developed means to harness both for therapeutic benefit (Fig. 46.1). To these ends, we provide a balanced review for the role of immunity in the pathogenesis of PD and ALS and the means to modulate it for therapeutic benefit. The strengths and concerns for each of these approaches are outlined and discussed.

46.2 Protein Misfolding and Modifications

46.2.1 α -Syn, SOD1 and TDP-43 Biology and Biochemistry

α -Syn is a major constituent protein in Lewy bodies, protein aggregates found in brains of Parkinson's disease patients (Spillantini et al. 1997). The expression of several mutations, including duplication or triplication of genes encoding for α -syn induce early-onset PD (Polymeropoulos et al. 1997; Kruger et al. 1998; Singleton et al. 2003; Farrer et al. 2004; Chartier-Harlin et al. 2004; Ibanez et al. 2004). Misfolding of this protein and lack of clearance are thought to play a major role in progression of PD. Two major cellular mechanisms of clearance include degradation by the ubiquitin-proteasome and the autophagic-lysosomal pathways. The former is supported by mutations in PD patients for parkin (*PARK2*) (Waters and Miller 1994) and UCH-L1 (*PARK5*) (Liu et al. 2002), proteins with ubiquitin E3 ligase and ubiquitin C-terminal hydrolase activities, respectively. In addition, blocking proteasome activity mitigates the degradation of α -syn (Webb et al. 2003; Machiya et al. 2010). The latter mechanism is supported in α -syn overexpressing cells by the presence of α -syn in organelles with morphological features of autophagic vesicles, increased clearance of α -syn with rapamycin, an autophagy stimulator, and increased accumulation of aggregated α -syn when autophagy is blocked (Webb et al. 2003; Lee et al. 2004). Moreover, α -syn clearance seemingly occurs in an aggregation stage-specific manner where the more toxic oligomeric intermediates are susceptible to clearance, but mature fibrillar inclusion bodies are not. Preformed α -syn fibrils are able to resist clearance by both the proteasome and autophagy in HEK cells expressing α -syn (Tanik et al. 2013). In this system, α -syn fibrils impair autophagy at the level of autophagosome clearance. Neutralization of the acidic compartments leads to the accumulation of α -syn aggregates and exacerbates α -syn-mediated toxicity and cell death (Lee et al. 2004). Wild type and A53T mutant α -syn increase numbers of LC3-II puncta associated with mitochondria and decrease

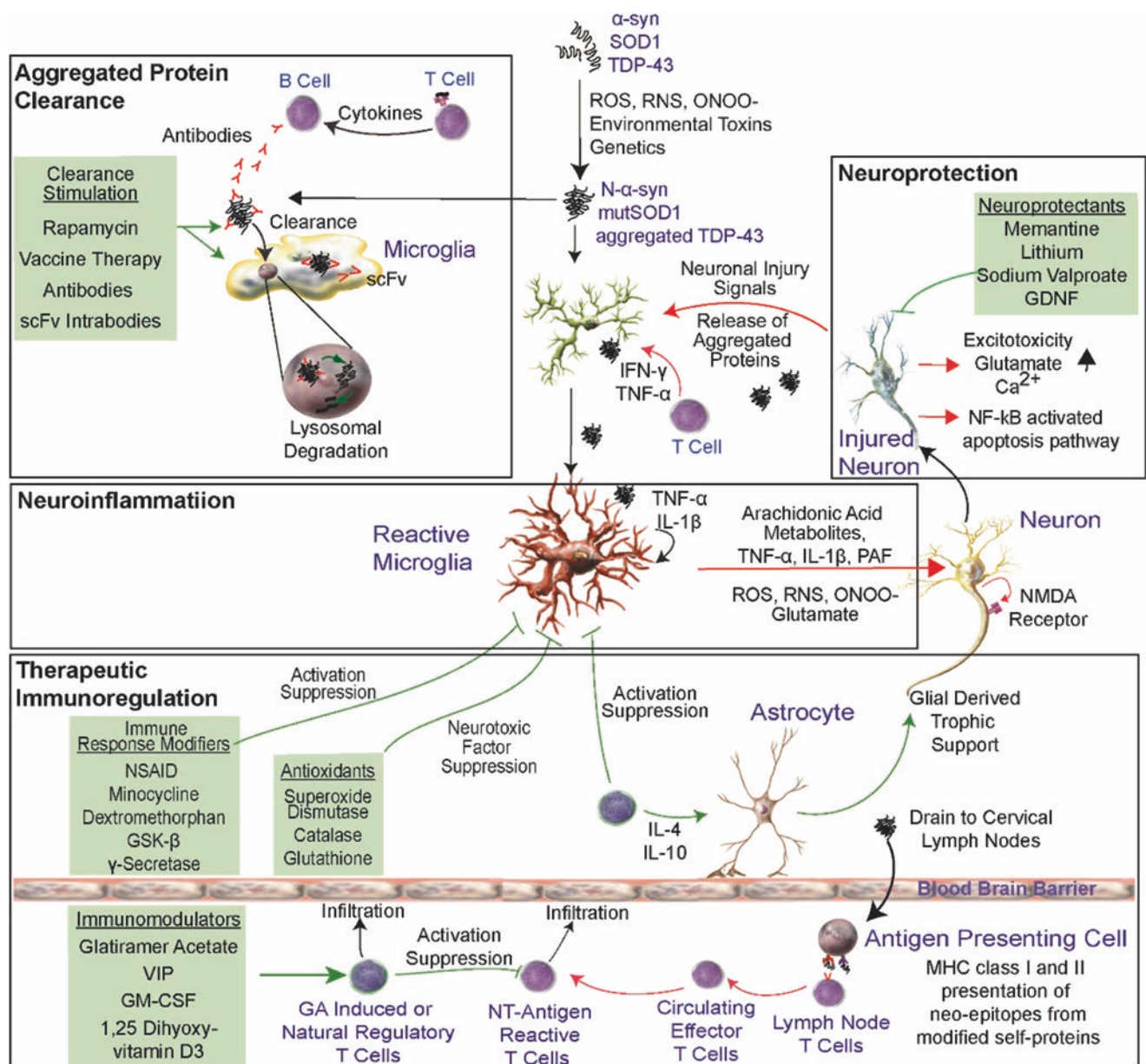


Fig. 46.1 Immunotherapeutic strategies for PD and ALS. There are several different targeted approaches currently available, or that can be imagined for the treatment of PD or ALS. The first of these is targeting the aggregated or misfolded proteins themselves to prevent oligomer-induced microglial activation. Vaccine-induced antibodies or intracellular-produced single chain antibodies (scFv, intrabodies) directed against misfolded/aggregated proteins, or drugs (e.g., rapamycin) that stimulate microglial/macrophage phagocytosis and lysosomal degradation may prove of therapeutic benefit by clearance of extracellular or intracellular misfolded/aggregated proteins. Another approach may use drugs that directly inhibit neurotoxicity (e.g., by inhibiting excitotoxicity or apoptosis) or promote neuroprotection (e.g., glial cell line-derived neurotrophic factor, GDNF), thus slowing disease progression. Finally, significant efforts are being made towards modulation of the immune response to misfolded/aggregated proteins. This may be accomplished by use of immune response modifiers and antioxidants that attenuate microglial activation. With the realization that disease can

either be exacerbated by effector T cells specific for neo-epitopes from modified self-CNS proteins (e.g. NT-modified proteins) or be ameliorated by regulatory T cells (natural or antigen-induced), therapeutic immunoregulation by immunomodulators or adjuvants to induce or upregulate regulatory T cell responses can inhibit exacerbated adaptive and innate immune responses and interdict further neurodegeneration. *α-syn* α-synuclein, *GA* glatiramer acetate, *GM-CSF* granulocyte macrophage colony stimulating factor, *GSK3-β* glycogen synthase kinase 3-β, *IFN-γ* interferon-γ, *MHC* major histocompatibility complex molecule, *mutSOD1* mutant superoxide dismutase-1, *N-α-syn* nitrated α-synuclein, *NMDA* N-methyl-D-aspartic acid, *NSAID* nonsteroidal anti-inflammatory drugs, *NT* nitrotyrosine, *ONOO-* peroxynitrite, *PAF* platelet-activating factor, *RNS* reactive nitrogen species, *ROS* reactive oxygen species, *SOD1* superoxide dismutase-1, *TDP-43* TAR DNA-binding protein 43, *TNF-α* tumor necrosis factor-α, *VIP* vasoactive intestinal peptide

numbers of mitochondria which are associated with decreased neuronal culture viability (Choubey et al. 2011). Dysregulation of protein degradation via autophagy and proteasomal functions may not be entirely distinct processes, but rather may be linked. Beclin-1, a protein involved in autophagy regulation, interacts with the E3 ubiquitin ligase parkin (*PARK2*) (Lonskaya et al. 2013). This interaction is decreased in both sporadic PD and the A53T PD model. The use of tyrosine kinase inhibitors increases the interaction of beclin-1 and parkin, and protects tyrosine hydroxylase positive neurons in the substantia nigra. In addition, PC12 cells transfected with human α -syn treated with the autophagy inhibitor 3-MA have decreased proteasome function as well (Yang et al. 2013).

In ALS, SOD1-positive aggregates are observed in the spinal cords from autopsied familial ALS (FALS) patients as well as from mutant SOD1 transgenic mice and are thought to play a critical role in toxicity and neuronal cell death (Kabashi and Durham 2006; Bruijn et al. 1997b; Watanabe et al. 2001; Shibata et al. 1996; Puttaparthi et al. 2003). Clearance of SOD1 aggregates involves proteasome activity, as inhibition of proteasome-mediated proteolysis promotes SOD1 aggregation in tissues from mutant SOD1-G93A mice, whereas restoration of activity reverses aggregate formation (Puttaparthi et al. 2003). In spinal cords of ALS patients, proteasome activity is decreased compared to healthy controls (Kabashi et al. 2012). Of interest, cytoplasmic, but not nuclear, proteasome activity is impaired from motor neurons of mutant SOD1 mice. Impairment of the 26S proteasome by conditional knockout of Rpt3 leads to ALS-like symptoms of decreased motor function, decreased body weight and decreased forelimb and hind limb strength (Tashiro et al. 2012). Basophilic inclusions are also increased with accumulations of TDP-43, FUS, optineurin and ubiquitin-2 in neurons. Additionally, clearance of mutant SOD1 by autophagy is comparable to that of the proteasome pathway as shown under conditions where mutant SOD1 is not toxic, the inhibition of macroautophagy induces mutant SOD1-mediated aggregation and cell death (Kabuta et al. 2006). Thus enhancing proteasomal or lysosomal function may be potential therapeutic strategies to halt disease progression in those afflictions associated with aggregation of misfolded proteins (Fig. 46.1).

More recently, sporadic and familial ALS patients without SOD1 mutations exhibit neuronal aggregates of the DNA binding protein, TDP-43 (Neumann et al. 2006; Mackenzie et al. 2007). Normally TDP-43 is found in the nucleus, however in ALS patients, TDP-43 aggregates accumulate in the cytoplasm. TDP-43 is also degraded by both the proteasome and autophagy (Wang et al. 2010). Pharmacological blockage of the proteasome leads to TDP-43 translocation to the cytoplasm. Monomeric and nuclear TDP-43 are primarily degraded by the proteasome, but aggregates and cytoplasmic

TDP-43 are cleared by autophagy (Urushitani et al. 2010; Scotter et al. 2014). The data show the importance of proper protein clearance by both the proteasome and autophagy/lysosome in the neuropathology of PD and ALS. New directives in genomics, proteomics and bioimaging have allowed monitoring of the effects of misfolded proteins in disease and provide new future therapeutic directives.

46.2.2 Protein Nitration and Oxidation

Postmortem analyses of PD patients consistently demonstrate the increased presence of nitrated residues as biomarkers for oxidative stress. Levels of serum nitric oxide and peroxynitrite are increased in PD patients compared to controls (Kouti et al. 2013). Protein modifications are among the many biomarkers detected in the brains of PD patients. Compared to brains and CSF from control donors, levels of nitrated proteins are elevated in PD patients (Aoyama et al. 2000). Most notable are 3-nitrotyrosine (NT) modifications of proteins that comprise Lewy bodies (LB); neuronal inclusions that are considered hallmarks of PD and consist primarily of α -syn, ubiquitin, and lipids (Giasson et al. 2000; Duda et al. 2000). These modifications suggest increased participation of inflammatory responses and reactive molecular species in PD; however, whether those modifications occur before or after inclusion into LB remain unclear. Also S-nitrosylated forms of parkin (*PARK2*) have been isolated from the temporal cortex from PD patients (Yao et al. 2004). S-nitrosylation of parkin can regulate its E3 ubiquitin ligase activity as well as mitochondrial quality control (Ozawa et al. 2013). Other oxidative post-translation modifications are also detected in tissues from PD patients. For example, carbonyl modifications, which are reflective of protein oxidation, are increased in SN, basal ganglia, globus pallidus, substantia innominata, cerebellum and frontal pole compared to controls and patients with incidental LB disease (ILBD), a putatively pre-symptomatic PD disorder (Alam et al. 1997). Whether the involvement of the latter two regions reflects a consequence of L-DOPA treatment or a more global consequence of the inflammatory spread of oxidative stress in PD is unclear. There are also increased oxidized forms of the PD-related proteins UCH-L1 (*PARK5*) and DJ-1 (*PARK7*) in the brains of PD patients (Choi et al. 2004, 2006). In addition, increased expression of neural heme oxygenase-1 by nigral astrocytes provides additional evidence for oxidative damage to proteins in PD (Schipper et al. 1998).

In ALS, abundant evidence points to the effects associated with peroxynitrite in affected tissues. Significant increases in levels of NT moieties are detected on CSF proteins from ALS patients (Shaw and Williams 2000; Aoyama et al. 2000), including Mn superoxide dismutase (Mn-SOD),

which are only slightly increased in patients with AD and PD (Chou et al. 1996; Aoyama et al. 2000). Moreover, NT immunoreactivity is associated with motor neurons of the spinal cord and axons (Calingasan et al. 2005), and co-localizes to axonal conglomerates and spheroid neurofilament accumulations of upper and lower motor neurons (Chou et al. 1996) and with A β 40 depositions within abnormal neurons (Calingasan et al. 2005). Oxidized SOD1 has been isolated from human central nervous system tissue (Auclair et al. 2013) and from immortalized lymphocytes. SOD1 appears to be oxidized by hydrogen peroxide at cysteine 111 (Bosco et al. 2010; Auclair et al. 2013). Oxidized SOD1, much like mutant SOD1, alters the conformation of Bcl2 by exposing the BH3 domain which can damage the mitochondria (Guareschi et al. 2012) and can inhibit proteasome activity (Le Pecheur et al. 2005). Whether TDP-43 is modified by nitration or oxidation in ALS patients remains unreported.

46.3 Microglial Inflammatory Responses

46.3.1 Innate Immunity and Disease

PD is characterized by activation of microglial cells found in and around degenerating neurons. Reactive microglia are commonly seen within the SNpc of PD brains investigated at autopsy (Croisier et al. 2005). A sixfold increase in the numbers of reactive microglia phagocytosing dopaminergic neurons has been shown (McGeer et al. 1988a) and correlates with the deposition of α -syn (Croisier et al. 2005). These reactive microglia over-express HLA-DR of the human MHC II complex, complement receptor type 3 (CR3, CD11b/CD18, Mac-1, Mo 1), CD23 (Fc ϵ RII, Fc receptor II for IgE), CD11a (lymphocyte function-associated antigen-1, LFA-1), CD54 (ICAM-1) and CD163 (scavenger receptor) (Hunot et al. 1999; Imamura et al. 2003; Pey et al. 2014). They also secrete a plethora of proinflammatory cytokines such as interferon- γ (IFN- γ) tumor necrosis factor- α (TNF- α), interleukin 1- β (IL-1 β), and upregulate proinflammatory enzymes such as inducible nitric oxide synthase (iNOS), lipocortin-1 and cyclooxygenase (COX) 1 and 2 (Hunot et al. 1999; Knott et al. 2000). MHC class II positive microglia also are significantly increased in the substantia nigra, putamen, hippocampus, transentorhinal cortex, cingulate cortex and temporal cortex of the PD brain (McGeer et al. 1988a; Imamura et al. 2003). Reactive microglia serve as *in vivo* indicators of neuroinflammatory responses and contribute significantly to progressive degenerative processes. In early-stage PD imaging, PK11195 ligand binding to peripheral benzodiazepine receptors, which are upregulated on reactive midbrain microglia and also serve as markers of neuroinflammation, inversely correlates with binding of 2- β -carbomethoxy-3 β -

(4-fluorophenyl) tropane (CFT) to the dopamine transporter (DAT) in the putamen as a measure of surviving dopaminergic termini, and also with the severity of motor impairment (Croisier et al. 2005). While the ability of nonsteroidal anti-inflammatory drugs (NSAIDs) to mitigate the severity of PD is in question (Ton et al. 2006; Bornebroek et al. 2007; Samii et al. 2009; Driver et al. 2011; Manthripragada et al. 2011), the fact that some studies suggest these drugs decrease the risk of developing PD point to a putative role for neuroinflammation in PD (Chen et al. 2003, 2005; Hernán et al. 2006; Wahner et al. 2007; Gagne and Power 2010; Gao et al. 2011). Also supporting the importance of inflammation in PD are biochemical and histological evidence in PD brains showing increased levels of carbonyl and nitrotyrosine protein modifications, lipid peroxidation, DNA damage, and reduction of glutathione and ferritin, the end results of inflammatory processes (Hald and Lotharius 2005). Postmortem samples of SNpc from sporadic PD patients also show elevated levels of the protein gp91^{phox}, the main transmembrane component of NADPH-oxidase, which co-localizes with microglia and is associated with the production of ROS. Likewise, in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice (see Przedborski, Sect. 22.2.4.2), large increases in gp91^{phox} immunoreactivity also co-localize in the SNpc with activated (Mac-1 immunoreactive positive) microglia, but not with astrocytes or neurons. Studies of post-mortem brains from three human subjects who, after 3–16 years previously had injected MPTP and developed parkinsonian syndromes, exhibited accumulations of activated microglial cells around dopaminergic neurons (Langston et al. 1999); however, histories of other self-administrative events remain unknown. Thus, an initial acute insult to dopaminergic neurons is thought to lead to a secondary and perpetuated neuroinflammatory response. This neuroinflammatory reaction, serves to alter homeostatic neural mechanisms or exacerbate disease processes by production of proinflammatory factors (Fig. 46.1).

In ALS patients, an abundance of data indicates the active involvement of the immune system in disease processes. Activated macrophages and perivascular microglia (mononuclear phagocytes, MPs) are found in affected tissues in ALS patients (Henkel et al. 2004; Graves et al. 2004). Several postmortem studies of tissues from ALS patients exhibiting neuronal loss compared to those of controls, demonstrate the increased presence of activated microglia in the ventral horn of the spinal cord, corticospinal tract, motor cortex, and brainstem (Henkel et al. 2004), and are thought to be involved in phagocytosis of degenerating neurons since they are found in close proximity in tissues showing early signs of degeneration. Macrophages and amoeboid microglia within those affected tissues from ALS patients exhibit upregulated HLA-A, -B, -C, -DR, -DP and -DQ molecules, CR3 (CD11b/CD18), CR4 (CD11c/CD18),

macrophage-colony stimulating factor (M-CSF) receptor (CSF-1R), β 2 integrins, CD68, MCP-1 and COX-2, which strongly suggest those microglia are in a reactive state (Kawamata et al. 1992; McGeer et al. 1993; Akiyama et al. 1994; Maihofner et al. 2003; Henkel et al. 2004). Furthermore, in vivo PET imaging using PK11195 to visualize upregulated peripheral benzodiazepine receptors by activated microglia show increased levels in the motor cortex, dorsolateral prefrontal cortex, thalamus, and pons of ALS patients. Recent works in mutant SOD1 (mSOD1) transgenic mice (see Simpson et al. Sect. 32.2.4.3) suggest a promising therapeutic strategy targeting the innate immune system. In the transgenic SOD1-G93A mice, the COX-2 inhibitor sulindac extended survival (Kiaei et al. 2005b). However, mixed and conflicting results are reported concerning the use of NSAIDs and the risk of developing ALS (Fondell et al. 2012; Tsai et al. 2015). The presence of wild type cells in the wild type/SOD1 Tg mouse chimeras enhances survival, whereas mutant SOD1 acting within non-neurons was toxic, no matter whether chimeric construction used was delivered via embryonic cells or bone marrow transplantation to irradiated recipients (Clement et al. 2003; Corti et al. 2004). To assess the role of SOD1 expression in MP cells on survival, mice transgenic for mutant SOD1 and flanking LoxP sequences (LoxSOD1) were crossed with mice expressing Cre under control of the CD11b promoter, an integrin exclusively expressed by myeloid cells (Boillee et al. 2006). Those crosses exhibited diminished SOD1 expression in microglia and macrophages, but not other cells, and extended survival compared to LoxSOD1 controls. Although no discernible differences in the activation phenotype of microglia or macrophages could be demonstrated, SOD1 expression was significantly diminished. Finally, reconstitution of SOD1-G93A/PU.1^{-/-} mice with wild-type bone marrow cells, established donor-derived microglia within the CNS, slowed motor neuron loss, prolonged disease duration and survival compared to mice treated with bone marrow cells from SOD1 transgenic animals (Beers et al. 2006). Taken together, these data suggest that targeting innate immunity and expression of SOD1 in the CNS of ALS represent a plausible therapeutic modality for ALS (Fig. 46.1).

46.3.2 Oxidative Stress

Once activated, microglia can produce noxious factors including pro-inflammatory cytokines, chemokines, quinolinic acid, and excitatory amino acids among others (Espey et al. 1997; Brown and Bal-Price 2003; Lee et al. 2002). In addition to these factors, activated microglia produce free radicals and reactive molecular species which are potentially toxic to invading organisms. NADPH oxidase (expressed by

microglia, macrophages and neutrophils) and myeloperoxidase (expressed predominately by neutrophils) are the enzymatic complexes, in their respective myeloid lineage cells, which produce radicals and reactive molecules. Among the cells in the CNS, microglia are responsible for generating a major portion of free radicals (Fig. 46.2) (Klegeris and McGeer 2000).

Reactive species of oxygen, nitrogen, and carbon are thought to play an active role in PD and ALS, wherein the very utilization and production of neurotransmitters produces harmful reactive species. The metabolism of dopamine produces H₂O₂ which can lead to inflammation and tissue damage by feeding into the ROS cycle and/or by dopamine-quinone modification of protein sulfhydryl groups via nucleophilic additions (Stokes et al. 1999). This is particularly important in PD where the ability to clear reactive oxygen species is reduced (see Sect. 46.3.2.3 Glutathione). Glutamate signaling through the NMDA receptor involves the generation of hydroxyl radicals in a NOS-dependent manner (Culcasi et al. 1994; Ayata et al. 1997). The excess of hydroxyl radicals can lead to neuronal death, which can be attenuated by antagonists of NMDA receptors (Mailly et al. 1999). In the MPTP model, NMDA antagonists alleviate parkinsonian symptoms (Steece-Collier et al. 2000), but this has not translated to PD patients (Addy et al. 2009). However, production of the majority of reactive oxygen species is mediated through the superoxide radical produced by NADPH-oxidase; the inhibition of which mitigates neurodegeneration in numerous model systems including MPTP (Wu et al. 2003). Increased nitro-tyrosine levels are detected in the SOD1-G37R model of ALS, but not hydroxyl radical markers 2,5 DHBA and MDA (Bruijn et al. 1997a). In addition, knockout of NADPH oxidase protects neurons in transgenic mice expressing mutant human SOD1-G93A (Wu et al. 2006). Lastly, the NMDA receptor antagonist [³H] MK-801 has higher binding in ALS patient brains compared to controls, suggesting that NMDA receptor expression or affinity is altered (Gredal et al. 1996). Combined, these data show that reactive molecules play substantial roles in the pathogenesis of PD and ALS.

Nitric oxide (NO) is a biological messenger molecule that has numerous physiological roles in the CNS and is associated with the protective killing function of macrophages and microglia. In contrast to the physiological roles of normal NO levels, excessive NO produced under pathological settings can act as a potent neurotoxin in a number of neurodegenerative models (Dawson and Dawson 1998). Excess NO reacts with super oxide species to form peroxynitrite, which readily crosses cell membranes and contributes to nitrotyrosine formation on proteins such as α -syn and SOD1 (see Sect. 46.2.2. Protein Nitration). In sporadic PD and some animal models, neuronal NOS (nNOS) and iNOS are both upregulated, while genetic ablation or pharmacological

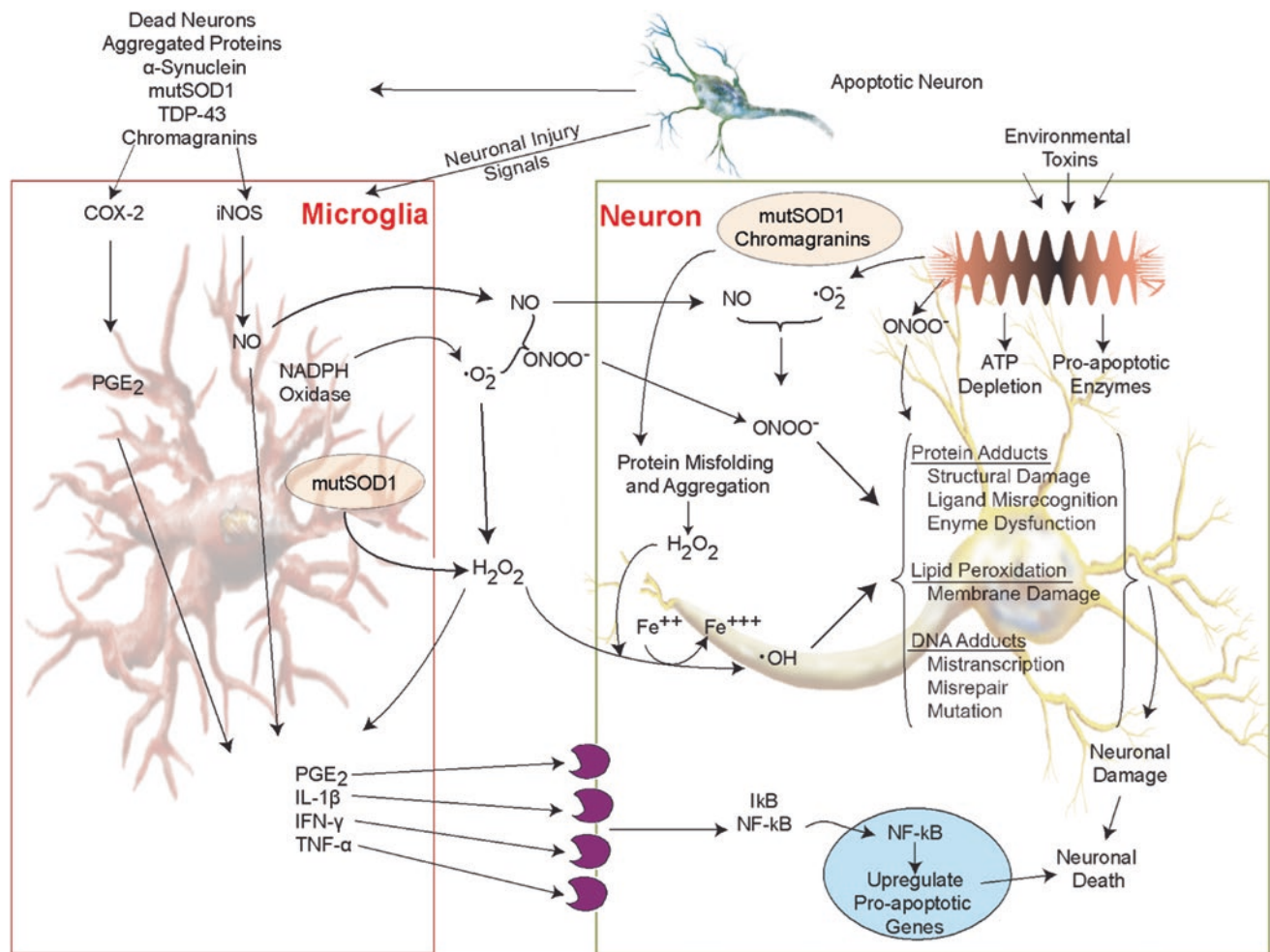


Fig. 46.2 Neuroinflammatory and oxidative stress pathways in PD and ALS pathogenesis. Free radicals can arise several diverse ways, such as glial cell activation, mitochondrial dysfunction, and protein aggregation. Increased microglia activation is attributable to increased neuronal cell death and cell debris including aggregated proteins. In ALS and related-animal models, mutated SOD1 (mutSOD1) expression or aggregated TDP-43 increases aggregated proteins, neuronal death and microglial activation. Microglial derived NO and superoxide (O_2^-) species react in extracellular spaces to form peroxynitrite (ONOO^-). Peroxynitrite readily crosses cell membranes where it contributes to lipid peroxidation, DNA damage and nitrotyrosine formation in α -synuclein and other cellular proteins. Damaged proteins are targeted

to cellular proteasomes for degradation via the ubiquitination pathway. Excess NO produced by activated microglia can lead to nitration or S-nitrosylation of cellular proteins, including parkin. Such modifications may diminish E3 ubiquitin ligase activity necessary for efficient protein turnover by proteasomes. Excessive protein damage caused by oxidants and disruptions in the ubiquitin pathways may overload or inhibit protein degradation quality control measures leading to the accumulation of damaged proteins in cells. When reactive species exceed anti-oxidant defenses, oxidative stress is generated; destroying molecular structures, such as proteins, lipids and DNA, causing irreversible and detrimental damage, neuronal cell injury and death. Adapted from Gao et al. (2003)

inhibition of excess NO production is neuroprotective in the MPTP model (Przedborski et al. 1996; Liberatore et al. 1999; Hancock et al. 2008). Patients with higher UPDRS scores are associated with decreased *NOS2* (the gene for iNOS) methylation, which is suggestive of increased expression (Searles Nielsen et al. 2015). In ALS patients, expression of nNOS and endothelial NOS (eNOS) as determined by immunohistochemistry is significantly higher than that found in controls (Kashiwado et al. 2002), and iNOS expression among spinal cord infiltrates is noted in both ALS patients and the SOD1-G93A transgenic mouse model of ALS (Wong and Strong 1998; Almer et al. 1999).

46.3.2.1 DNA Modifications

Modification of nucleic acids by free radicals and reactive species can induce chromosomal aberrations with high efficiency, suggesting that chromosomal damage exhibited in neurons of PD patients might be related to an abnormally high oxidative stress. Among the most promising biomarkers of oxidative damage to nucleic acids is nucleoside 8-hydroxyguanosine (8-OHG) for RNA or 8-hydroxy-2'-deoxyguanosine (8-OHdG) for DNA. 8-OHG is an oxidized base produced by free radical attack on DNA by C-8 hydroxylation of guanine and is one of the most frequent nucleic acid modifications observed under conditions of oxidative

stress. In PD patients, levels of 8-OHG nucleic acid modifications are commonly increased in the caudate and SN compared to age-matched controls (Zhang et al. 1999). Immunohistochemical characterization of these modifications indicates that the highest levels of 8-OHG modifications are found in neurons of the SN and to a lesser extent in neurons of the nucleus raphe dorsalis and oculomotor nucleus, and occasionally in glial cells. Given that 8-OHG nucleic acid modifications are rarely detected in the nuclear area, mostly restricted to the cytoplasm, and that immunoreactivity is significantly diminished by RNase or DNase and ablated with both enzymes (Zhang et al. 1999), suggests that targets of oxidative attack include both cytoplasmic RNA and mitochondrial DNA. Of particular interest are the findings that concentrations of 8-OHG in CSF of PD patients are higher than in age-matched controls; however, serum concentrations of 8-OHG appear highly variable (Abe et al. 2003). Similarly, 8-OHdG levels are increased in CSF of PD patients compared to age matched controls (Isobe et al. 2010). Further evidence for the role of oxidized nucleic acids in PD comes from the upregulation of the enzymes that repair oxidized bases. 7,8-Dihydro-8-oxoguanine triphosphatase (MutT homolog 1 (MTH1) or nudix (nucleoside diphosphate linked moiety X)-type motif 1) hydroxylase 1) cleaves oxidized dNTPs to dNMPs to monophosphates preventing their incorporation). 8-Oxoguanine DNA glycosylase (OGG1) performs base excision repair on 8-OHdG. The mutY Homolog (MUTYH) glycosylase excises oxidized adenosine pair with guanine bases in DNA. All three enzymes are upregulated in PD compared to age matched controls (Shimura-Miura et al. 1999; Fukae et al. 2005; Arai et al. 2006). Additionally, in the MPTP model, striatal neurodegeneration is more severe in MTH1 null mice compared to WT (Yamaguchi et al. 2006). Together, these data suggest that 8-OHG or 8-OHdG are markers of oxidative damage in PD.

In ALS, 8-OHdG modified DNA is detected in spinal cord tissues (Calingasan et al. 2005) and CSF (Ihara et al. 2005) from sporadic ALS (SALS) and familial ALS (FALS) patients, while the level of nuclear 8-OHdG increased in motor cortex of SALS patients, but not FALS patients (Shibata et al. 2000). This is also recapitulated in the SOD1-G93A transgenic mouse model of ALS where 8-OHdG is increased in the spinal cord, frontal cortex and striatum (Aguirre et al. 2005).

46.3.2.2 Lipid Peroxidation

4-Hydroxy-2-nonenal (HNE) is a reactive α,β unsaturated aldehyde that is a major product during the oxidation of lipid membrane polyunsaturated fatty acids, which form stable adducts with nucleophilic groups, such as thiols and amines, on proteins. HNE modification of membrane proteins forms stable adducts that can be used as biomarkers of cellular damage due to oxidative stress. Compared to post-

mortem tissues from control subjects, immunochemical staining on surviving dopaminergic nigral neurons from PD patients show the presence of HNE-modified proteins on 58 % of the neurons compared to only 9 % from controls. Weak or no staining was detected on oculomotor neurons in the same midbrain sections from PD patients (Yoritaka et al. 1996), and the presence of HNE-modified proteins was confirmed on LB from PD and diffuse LB disease patients, but not controls (Castellani et al. 2002). HNE is increased in the caudate nucleus, but not in the putamen or the frontal cortex (Mythri et al. 2011), suggesting that lipid oxidation occurs in specific regions in patients with synucleinopathies. HNE species are typically more stable than reactive oxygen species, thus they easily spread from the site of production to modify targets at a distant site. HNE modifications of DNA, RNA, and proteins have various adverse biological effects such as interference with enzymatic reactions, induction of heat shock proteins, and are considered to be largely responsible for cytotoxic effects under conditions of oxidative stress (Toyokuni et al. 1994). The cytotoxic effects of HNE modifications may be in part due to inhibition of complexes I and II of the mitochondrial respiratory chain; induction of caspase-8, -9, and -3, cleavage of poly(ADP-ribose) polymerase (PARP) with subsequent DNA fragmentation; inhibition of NF- κ B mediated signaling pathways; or diminution of glutathione levels. Consistent with an abundance of data showing the dysregulation of proteasomal function in PD, direct binding of HNE to the proteasome also inhibits the processing of ubiquitinated proteins. Concentrations that induce no acute change in cell viability in vitro initially cause a decrease in the proteasomal catalytic activity to the extent that it induces accumulation of ubiquitinated and nitrated proteins, reductions in glutathione levels and mitochondrial activity, and increased oxidative damage to DNA, RNA, proteins, and lipids (Hyun et al. 2003). In addition to disruption of the proteasome, dopamine catabolism is impaired in the presence of HNE and malondialdehyde (MDA) (Rees et al. 2007). This leads to the accumulation of the reactive dopamine intermediates.

In SALS patients, HNE levels are significantly elevated in the sera, CSF, the ventral motor neurons and surrounding glia (Shibata et al. 2000; Simpson et al. 2004). HNE levels in serum and CSF are directly correlated with the extent of the disease, but not with the rate of disease progression. One HNE-modified protein was the astrocytic glutamate transporter EAAT2 (GLT-1) (Pedersen et al. 1998), suggesting a role for HNE-mediated impairment of glutamate transport, increased glutamate levels and excitotoxic-induced neurodegeneration in ALS. In the SOD1-G93A mouse model of ALS, three HNE-modified proteins were identified in the spinal cord, dystrophin related protein 2 (DRP-2), Hsp70 and α -enolase, proteins involved in axonal outgrowth, proper protein folding and glycolysis respectively (Perluigi et al. 2005).

46.3.2.3 Glutathione

Production of ROS and NO in neurons is buffered primarily by the glutathione (GSH) system. GSH content in the SNpc of PD patients is decreased by 40–50 %, but not in other regions of the brain, not in age-matched controls, and not in patients with other diseases affecting dopaminergic neurons (Sian et al. 1994a). This diminution continues with progression and severity of disease, suggesting a correlation with concomitant increases in reactive species (Pearce et al. 1997). GSH depletion has been suggested as the first indicator of oxidative stress during PD progression, possibly occurring prior to other hallmarks of PD including the decreased activity of mitochondrial complex I (Andersen 2004). Also, elevated GSSG/GSH ratios in PD patients (Sian et al. 1994a) argue strongly for a role of oxidative stress in this disease (Dringen 2000). An increase in glutathione peroxidase immunoreactivity, exclusive to glial cells surrounding surviving dopaminergic neurons, is observed in PD brains (Damier et al. 1993). Interestingly and possibly more important, the SN and striatum have lower levels of GSH relative to other regions of the brain, which include, in increasing order: SN, striatum, hippocampus, cerebellum, and cortex (Kang et al. 1999). Although varying in different regions of the brain, all GSH levels diminish by about 30 % in the elderly, suggesting a possible link with the age-associated risk factor for PD. GSH depletion cannot be explained by increased oxidation of GSH to GSSG as levels of both are diminished in the nigra of PD patients (Chen et al. 1989; Sian et al. 1994a). Diminished GSH levels do not appear to be caused from failure of GSH synthesis as γ -glutamylcysteine synthetase is unaltered, as are glutathione peroxidase and glutathione transferase activities (Sian et al. 1994b). Glutathione peroxidase is found near or co-localized with Lewy bodies in PD patients' brains (Power and Blumbergs 2009). In addition, *in vitro* glutathione peroxidase can accelerate the formation of and become entangled in α -syn fibrils (Koo et al. 2013). This suggests that glutathione peroxidase may interact directly with α -syn as part of PD pathogenesis. Glutathione transferase is increased in the synaptosomal fractions in PD patients compared to controls (Shi et al. 2009). Glutathione transferase also increases over the progression of PD, suggesting an attempt by neurons to detoxify oxidized proteins. Other possible mechanisms for diminished levels include increased removal of GSH from cells by γ -glutamyltranspeptidase (Sian et al. 1994b) and the formation of adducts of glutamyl and cysteinyl peptides of GSH with dopamine. Nevertheless, depletion of GSH may render cells more sensitive to toxic effects of oxidative stress and potentiate the toxic effects of reactive microglia (Chen et al. 2001). This is born out in rats where selective depletion of GSH leads to neurodegeneration of nigral neurons (Garrido et al. 2011). Inhalation of GSH by PD patients

decreases UPDRS scores showing that increasing GSH has a clinical benefit (Mischley et al. 2015) and suggesting that strategies that increase GSH may prove to be a valuable target.

Direct evidence for perturbations of the glutathione system in ALS is more limited compared to PD. One report demonstrated increased levels of oxidized NO products, higher GSH levels, and lower GSSH levels, thus lower GSSH/GSH ratios in the CSF of SALS patients (Tohgi et al. 1999). Through MRI imaging, GSH levels were determined to be lower in the motor cortex of ALS patients compared to healthy controls (Weiduschat et al. 2014). However, no change in total, oxidized or reduced GSH were detected in the erythrocytes of ALS patients compared to controls (Baillet et al. 2010), but glutathione peroxidase levels were decreased in erythrocytes of ALS patients compared to controls (Cova et al. 2010). In addition, reports of increased GSH-binding sites in the spinal cord of ALS patients could reflect an upregulation of glutathione receptors (Bains and Shaw 1997). These data suggest a decrease in GSH function in ALS, though more work is needed to test GSH expression and activity in the spinal cord and motor neurons of ALS patients. This could be especially important in familial ALS caused by mutant SOD, since this mutant protein is also part of the antioxidant system of the cell.

46.3.3 Modulation of Innate Immunity

46.3.3.1 PPAR- γ

Immune suppression through receptor modulation has been another approach attempting to alleviate or reverse PD progression. The peroxisome proliferator-activated receptor- γ (PPAR- γ), a nuclear receptor involved in carbohydrate and lipid metabolism, is upregulated in the ipsilateral substantia nigra of MPTP-treated hemiparkinsonian Rhesus monkeys compared to control and the contralateral substantia nigra (Swanson and Emborg 2014). Agonists of peroxisome PPAR- γ inhibit inflammatory responses in a variety of cell lines, including monocyte/macrophages and microglial cells (Breidert et al. 2002). *In vivo* administration of PPAR- γ agonists modulates inflammatory responses in the brain. Pioglitazone, a PPAR- γ agonist used currently as an anti-diabetic agent, has anti-inflammatory effects in animal models of autoimmune disease, attenuates glial activation, and inhibits dopaminergic cell loss in the SN of MPTP-treated mice (Breidert et al. 2002). Pioglitazone also rescued diminution of rearing events and increases in freeze time and duration for MPTP-intoxicated mice to traverse a beam (Laloux et al. 2012; Quinn et al. 2008), suggesting that this PPAR- γ agonist can also reverse some motor dysfunction. Pioglitazone also improves the clinical score,

decreases CD68⁺ cell number, and protects neurons in the striatum and the substantia nigra of MPTP-treated monkeys (Swanson et al. 2011). In addition, another full PPAR- γ agonist, rosiglitazone, has been shown to protect nigral neurons and decrease TNF- α and COX-2 expression in the striatum (Lee et al. 2012). LSN862 and GW855266X, partial agonists of PPAR- γ , protect nigral neurons and mitigate microgliosis in the MPTP mouse and 6-hydroxy dopamine (6-OHDA) rat models, respectively (Swanson et al. 2013; Sadeghian et al. 2012). Treatment with pioglitazone and GW855266X after 6-OHDA, also protects neurons in the substantia nigra as well as decreases microgliosis (Sadeghian et al. 2012). In addition, the PPAR- γ antagonist GW9662 decreases the viability of nigral neurons and further decreases neurons in the substantia nigra following MPTP intoxication (Martin et al. 2012). In cynomolgus macaques, pioglitazone reduces dyskinesia caused by L-DOPA treatment (Huot et al. 2015). Diabetic patients who were prescribed glitazone drugs had decreased incidence of PD when controlling for smoking, head injury, disease severity and other medications (Brauer et al. 2015). However a phase 2 clinical trial testing the efficacy of pioglitazone in treating PD failed to decrease UPDRS scores (Investigators. 2015) or serum IL-6, serum 8-OHdG, or PGC-1 α (Simon et al. 2015). This data suggests that PPAR- γ receptor agonists can decrease neuroinflammation and neurodegeneration in PD models, however, this has yet to be translated into a PD therapy.

Pioglitazone has not been studied to the same extent in ALS as PD, but data suggests that PPAR- γ agonists are protective in ALS models. Pioglitazone mitigates the loss of neurons in the lumbar spinal cord and the increase in microglia and astrocytes in the SOD1-G93A transgenic mouse model (Kiaei et al. 2005a; Shibata et al. 2008; Schutz et al. 2005). Pioglitazone increases survival time, improves motor performance, mitigates increased NF- κ B, iNOS, nitrotyrosine residues, phosphorylated p38, and I κ B α in the lumbar spinal cord of those mouse models of ALS. A clinical trial using pioglitazone to treat ALS was unable to increase survival time in riluzole and pioglitazone-treated group compared to those treated with riluzole alone. In fact, pioglitazone treatment increased the hazard for death by 21 %, however, this was not significant (Dupuis et al. 2012) and may be due to pioglitazone-induced metabolic dysregulation (Jawaid et al. 2014).

46.3.3.2 Minocycline and Modulators of Microglial Activation

Minocycline, a semisynthetic second generation tetracycline, easily penetrates the blood–brain barrier and has been shown recently to effectively protect from neurodegeneration in several disease models including ALS, PD, cerebral ischemia, Huntington's disease, multiple sclerosis, and spinal

cord injury (Domercq and Matute 2004), all of which involve microglial activation as the principle effector of secondary neurotoxicity. Tetracyclines prevent cell death in models of neurodegeneration by both attenuation of innate and adaptive immunity and blockage of apoptotic cascades (Domercq and Matute 2004). Minocycline specifically inhibits microglial activation and proliferation, the induction of caspase-1 and -3, iNOS, and COX-2 (Chen et al. 2000; Domercq and Matute 2004; Wang et al. 2004). Minocycline also attenuates adaptive immunity by reducing the expression and activity of matrix metalloproteinases, which alter blood–brain barrier permeability (Brundula et al. 2002; Popovich et al. 2003; Zhu et al. 2002; Domercq and Matute 2004). Minocycline can also alter the blood–brain barrier by inhibiting the function of the p-glycoprotein transporter (Milane et al. 2007). In the MPTP mouse model of PD, oral administration of minocycline attenuates microglial activation, protects dopaminergic neurons in the SNpc, and restores motor function (Peng et al. 2006). Administration of minocycline was also effective in ALS transgenic mice, which delayed onset of disease, increased motor performance, and extended survival by 2–3 weeks compared to non-treated mice (Zhu et al. 2002; Kriz et al. 2002). Minocycline decreases M1-type responses (decreased number of CD11b⁺ cells and decreased expression of CD86, CD62 TNF- α , IFN γ , and IL-1 β) and increased M2 responses (increased CD206, arginase-1, IL-4, IL-10, and Ym1) in the SOD1-G93A transgenic model of ALS (Kobayashi et al. 2013). Based on these results, and early human trials showing minocycline is safe in humans (Pontieri et al. 2005), a phase III clinical trial was initiated to treat ALS patients. However, patients given minocycline had a trend toward more rapidly progressing and severe disease compared to placebo control (Gordon et al. 2007). Other modalities that have been used successfully to attenuate microglia activation in models of neurodegeneration include non-steroidal anti-inflammatory agents (Dairam et al. 2006), COX-2 inhibitors such as rofecoxib (Hewett et al. 2006) and parecoxib (Reksidler et al. 2007), and dextromethorphan (Zhang et al. 2006) among others (Fig. 46.1).

Free radical production by activated microglia accompanied by dysregulation of antioxidants (e.g., SOD1, glutathione and catalase) diminish the protective potential by establishing a pro-oxidative stress environment. In turn, these antioxidants may be used therapeutically to modulate the microglial response. While SOD-1 and GSH levels decline in PD patients (Poirier et al. 1994; Kunikowska and Jenner 2003), gastrodin (Wang et al. 2014), melatonin (Zaitone et al. 2013), and exercise (Tsou et al. 2015) protect nigral neurons, as well as increase GSH and SOD expression in the MPTP model. Despite this success in animal model, these strategies to reduce inflammation in PD patients have met with limited success to date.

46.4 Adaptive Immunity

46.4.1 Cell-Mediated Immunity

While naïve T cells are typically precluded from CNS entry, neuroinflammation aggressively recruits activated components of the adaptive immune system to sites of active neurodegeneration by increasing expression of cellular adhesion molecules and inducing chemokine gradients. Moreover, glial cells secrete toxic factors that disrupt blood brain barrier function. Nonetheless, evidence indicates a far more complex relationship between the CNS and immunological system than previously realized. Further challenging this view of the “immune privileged” status of the CNS are severe combined immunodeficiency (SCID), RAG2 knockout and CD4⁺ depleted mice which have exacerbated neuronal loss following facial neuron axotomy (Serpe et al. 1999, 2003). However, reconstitution of these mice with splenocytes and more specifically CD4⁺ T cells help protect damaged facial nerves. Rodents and humans that have sustained CNS injuries also have expanded T cell repertoires against myelin-associated antigens, yet do not appear to be at increased risk for the development of CNS autoimmunity (Jones et al. 2002, 2005; Popovich et al. 1996). Any functional consequence of such T cell responses against CNS antigens following injury remains to be determined.

Evidence exists for associations between HLA type and PD. Early reports identified significant association of PD with expression of HLA-Aw24, -B14, -B16, -B17, -B18, -A2, and -A28 (Hoffman et al. 1977; Elizan et al. 1980), whereas later works failed to detect significant deviations of HLA haplotypes among PD patients compared to unaffected controls (Marttila et al. 1981; Lees et al. 1982). Interestingly, a study analyzing HLA class I and II alleles among 45 German PD patients demonstrated a significant increase in the representation of the DQB1*06 allele suggesting an association between idiopathic PD and the immune system (Lampe et al. 2003). The association of HLA-DQB1 alleles in PD patients was verified by genome-wide association studies (Nalls et al. 2014). Meta-analysis of genome-wide association databases has also indicated that HLA-DRB1*04 and HLA-DRB1*08 are also associated with increased incidence of PD (Ahmed et al. 2012).

The association of any one HLA type with ALS is controversial. While some studies found no statistical association with ALS (Bartfeld et al. 1982; Norris et al. 1986; Woo et al. 1986), others found increased incidences of ALS patients that express HLA-A3, -A12, -Bw40, -Bw35 (Antel et al. 1976; Jokelainen et al. 1977; Hoffman et al. 1978; Kott et al. 1979). Additionally, milder disease progression may be associated with HLA-Bw40 (Jokelainen et al. 1977), whereas more aggressive disease is associated with HLA-Bw35 (Hoffman et al. 1978). The discrepancies between these studies may be

explained by different populations examined and/or the relatively small numbers of patients in these studies.

In PD patients, increased numbers of CD8⁺ T cells are found in close proximity to activated microglia and degenerating neurons within the SN; however, those numbers are consistently low (McGeer et al. 1988b). In the MPTP mouse model, numbers of CD8⁺ and CD4⁺ T cells are significantly increased as late as day 21 post-intoxication with mean CD4/CD8 ratios of 0.33 ± 0.07 (range 0.19–0.64) (Kurkowska-Jastrzebska et al. 1999), lower than CD4/CD8 ratios typically found in the peripheral circulation. Whether these infiltrating T cells are activated, antigen-specific, or migrating in response to microglial inflammation has yet to be determined, but the presence of major T cell subsets at levels exceeding those typically found in the CNS and in lower ratios found in the periphery suggests a more substantial role in PD than that associated with surveillance. In that vein, numerous aberrations in peripheral lymphocyte subsets are detectable in PD patients. Compared to age-matched controls, numbers of total lymphocytes, CD19⁺ B cells and CD3⁺ T cells were diminished in both drug-naïve and -treated PD cohorts (Bas et al. 2001). Among CD3⁺ T cells, numbers of CD4⁺ T cells were diminished; whereas, numbers of CD8⁺ T cells were not significantly changed. The frequencies of cells within CD4⁺ T cell subsets are differentially diminished, with a greater loss of naïve helper T cells (CD45RA⁺) and either unchanged or increased effector/memory helper T cell subset (CD29⁺ or CD45RO⁺) (Bas et al. 2001). The decrease in CD4⁺ cells correlates with more severe PD disease symptoms (Stevens et al. 2012). Increased frequencies of T cells with effector-type phenotype were noted in PD patients compared to age- and environment-matched controls (Saunders et al. 2012). Moreover, increasing frequencies of CD4⁺ T cells that expressed CD45RO⁺ or FAS⁺ were correlated with diminishing motor dysfunction as determined by increased UPDRS part III scores, whereas those that expressed CD31 or $\alpha 4\beta 7$ integrin diminished with increased UPDRS scores. The relative balance of the different CD4⁺ subsets is also changed with increased levels of Th1 and Th17 cells and decreased frequencies of Th2 and Tregs (Chen et al. 2015). Not only is T cell number altered during PD, but function may also be changed. Regulatory T cells (Tregs) from PD patients have less suppressive ability than Tregs from age- and environment-matched controls (Saunders et al. 2012). Experiments in the MPTP model demonstrated a detrimental role for CD4⁺ cells. SCID mice (Benner et al. 2008) and mice deficient in CD4⁺, but not CD8⁺ cells (Brochard et al. 2009) show no significant loss of dopaminergic neurons in the SN of MPTP-treated mice. These data from PD patients and mouse models suggest that alterations of T cell frequencies and function play a determinative role in the development and progression of PD.

The increased mutual co-expression of CD4 and CD8 by CD45RO⁺ T cells, as well as upregulation of CD25 (α -chain of the IL-2 receptor), TNF- α receptors, and significant down-regulation of IFN- γ receptors suggests that at least some T cell subsets are activated in PD patients. However, evaluation of these parameters to assess whether activated T cell phenotypes are derived from any one T cell subset or many subsets have yet to be incorporated into one study. Interestingly, a significantly greater number of micronuclei and unrepaired single strand DNA breaks, shown to result from exposure to higher levels of oxidative stress and inflammation (Cerutti 1985), are detected in lymphocytes and activated T cells from PD patients compared to age-matched controls (Petrozzi et al. 2002; Migliore et al. 2002). Moreover, 8-OHG is increased in peripheral leukocytes as well as decreased levels of glutathione peroxidase and vitamin E in PD patients compared to controls (Chen et al. 2009). Lymphocytes from PD patients also have increased total basal and mitochondrial ROS compared to controls (Prigione et al. 2009).

In ALS patients, lymphocytes, as well as myeloid cells, are consistently found within or around affected tissues (Graves et al. 2004). Tissues with lymphocytic infiltrates include spinal cords, brain, muscles, and associated vessels (Graves et al. 2004). Most reports have identified the infiltrating lymphocytes as T cells, with a minor presence of B cells. Most indicate that both CD4⁺ and CD8⁺ T cells are present within affected tissues. One report indicated the presence of CD8⁺ T cells, with rare CD4⁺ cells in the anterior and lateral corticospinal tracts and anterior horns (Troost et al. 1990), while another reported the presence of primarily CD4⁺ T cells with rare CD8⁺ cells in muscle (Troost et al. 1992). Although not rigorously examined, a consensus suggests that these T cells are in an activated state by the presence of upregulated surface markers such as MHC I and II molecules (Troost et al. 1992) and CD40 ligand (CD40L) (Graves et al. 2004). Of particular interest is the finding that PCR amplification of the third complementarity-determining region (CDR3) to examine the T cell receptor (TCR) repertoire of infiltrating T cells in cerebral spinal fluid (CSF), spinal cords and brains of ALS patients showed increased utilization of TCRBV2 (variable region 2 of the T cell receptor β -chain) transcripts, which was independent of HLA haplotype (Panzara et al. 1999). This result suggests there is specific expansion of T cells with a T cell receptor containing variable region 2 in ALS patients.

Early studies of ALS patient peripheral blood revealed a general loss of T cells as demonstrated by reduced numbers of E-rosetting T cells (a function mediated by CD2), and later confirmed by flow cytometric analysis showing significant losses of total CD2⁺ T cells with concomitant increase with increased numbers of surface Ig⁺ B cells (Provinciali et al. 1988). Other studies have failed to show significant differences in T or B cell frequency or function (Appel et al.

1986). Analyses of T cell subsets in ALS patients relative to normal controls show variable results, and include diminished or increased frequencies of CD4⁺ cells, diminished or unchanged CD8⁺ T cell frequencies (Mantovani et al. 2009; Chen et al. 2014). Of interest, ALS patients exhibit increased frequencies of both CD4⁺ and CD8⁺ T cells that co-express IL-13, a phenotype functionally associated with immunoregulatory functions (Shi et al. 2007). Increased frequencies of CD4⁺IL-13⁺ T cells are inversely correlated with clinical score, while the CD8⁺IL-13⁺ T cells directly correlate with the rate of disease progression (Shi et al. 2007). Additionally, numbers of CD4⁺ T cells that express IL-9, IL-17, IL-21 and IL-22 are increased in ALS (Saresella et al. 2013). ALS patients with more severe disease progression have diminished Treg numbers (Mantovani et al. 2009; Henkel et al. 2013). Of interest, in a minor subset of ALS patients with persistent motor conductance blockage, but not those without blockage, frequencies of CD3⁺, CD4⁺, and CD8⁺ T cells, as well as CD16⁺ mononuclear cells are increased, suggesting a relationship between peripheral blood abnormalities and pathogenic processes associated with conduction blockage in ALS patients (Tanaka et al. 1993). However, unlike the MPTP model of PD, depletion of CD4⁺ T cells in the SOD1 transgenic model of ALS leads to faster disease onset (Beers et al. 2008).

46.4.2 Humoral Immunity

The possibility that humoral immunity may play a role in either the initiation or regulation of PD has arisen from experimental studies triggering dopaminergic neuron degeneration, including the observation of C1q⁺ microglia (Depboylu et al. 2011), and neurons that stain with anti-IC3b and anti-C9 in the SN of PD patients (Loeffler et al. 2006). In both idiopathic and genetic cases of PD, pigmented dopaminergic neurons and Lewy bodies showed significant increased immunolabeling with IgG, but not IgM, and were associated with increased numbers of activated microglia expressing the high affinity IgG receptor Fc γ RI (Orr et al. 2005). One of the curiosities in PD is the presence of naturally occurring autoantibodies reactive to α -syn. Depending on the study, PD patients either have increased (Papachroni et al. 2007), decreased (Besong-Agbo et al. 2013) or no significant change (Heinzel et al. 2014) in the concentration of these antibodies in the serum compared to healthy controls. These autoantibodies are possibly associated with clearance processes for extracellular Lewy bodies, and as such may reflect a protective mechanism. It is also possible that these autoantibodies are reacting with α -syn on the surface of neurons which could mediate neuronal death. In addition, increased levels of autoantibodies to myelin oligodendrocytic glycoprotein (MOG), myelin basic

protein (MBP), myelin-associated glycoprotein (MAG), and proteolipoprotein (PLP) are detected in PD patients compared to healthy controls (Papuc et al. 2014). As with α -syn, the role these autoantibodies play in PD symptomology or disease progression remains unclear.

Perhaps the most studied of immune-associated aspects in ALS patients are those of humoral immunity. Global alterations of humoral immunity include increased IgG, IgA and immune complexes in the serum (Hoffman et al. 1981; Saleh et al. 2009); and the presence of immune complexes have been detected within kidney, spinal cord and motor cortex of ALS patients (Oldstone et al. 1976; Palo et al. 1978; Donnenfeld et al. 1984). Circulating immune complexes and complement component C3 are increased in ALS patients (Chen et al. 2014). The same is true of SOD1-G93A transgenic mice, wherein IgM and complement C3 are detected on motor neurons (Chiu et al. 2009). These humoral aberrations and depositions of humoral products provoked an increased impetus for an autoimmune approach to ALS etiology. Thus, many studies have assessed the possible antigenic reactivities of those antibodies. Those reactivities from ALS patients' sera and CSF comprise epitopes associated with nervous and non-nervous tissues as well. Nervous system specificities in ALS encompass those directed against moieties from spinal cord, motor neurons (Engelhardt and Appel 1990), neurofilaments (Niebroj-Dobosz et al. 2006) myelin (Edgington and Dalessio 1970), and Ca^{2+} channels (Smith et al. 1992; Kimura et al. 1994). The plethora of data showing antibody reactivities to gangliosides especially among IgM antibodies (Pestronk et al. 1989; Li and Pestronk 1991; Ben Younes-Chennoufi et al. 1992) is thought to reflect a possible pathogenic role in motor neuron diseases; however the mechanism by which those antibodies exact their toll has not been ascertained. ALS patients have increased IgG levels that bind oxidized SOD1 (van Blitterswijk et al. 2011). Interestingly, high levels of IgM that are reactive to oxidized SOD1 are associated with longer survival, whereas higher concentrations of IgG reactive to SOD1 are associated with shorter survival. Co-culture with neuronal tissues and passive transfer of sera or immunoglobulin from patients and immunized animals to rodent recipients has revealed functional consequences of these reactivities relative to ALS clinical signs. Passive transfer yields increased presence of human IgG in spinal cord motor neurons and neuromuscular junctions with increased miniature end-plate potential (MEPP) frequency (Appel et al. 1991), increases in the rate of spontaneous neurotransmitter release, and axonal degeneration and denervation in most muscles (Uchitel et al. 1992). Within 12–24 h of IgG transfer, increases are found in the densities of synaptic vesicles, CSF glutamate concentrations, Ca^{2+} levels in axon terminals of neuromuscular junctions and synaptic boutons on spinal motor neurons (Engelhardt et al. 1995, 1997; La Bella et al. 1997; Pullen

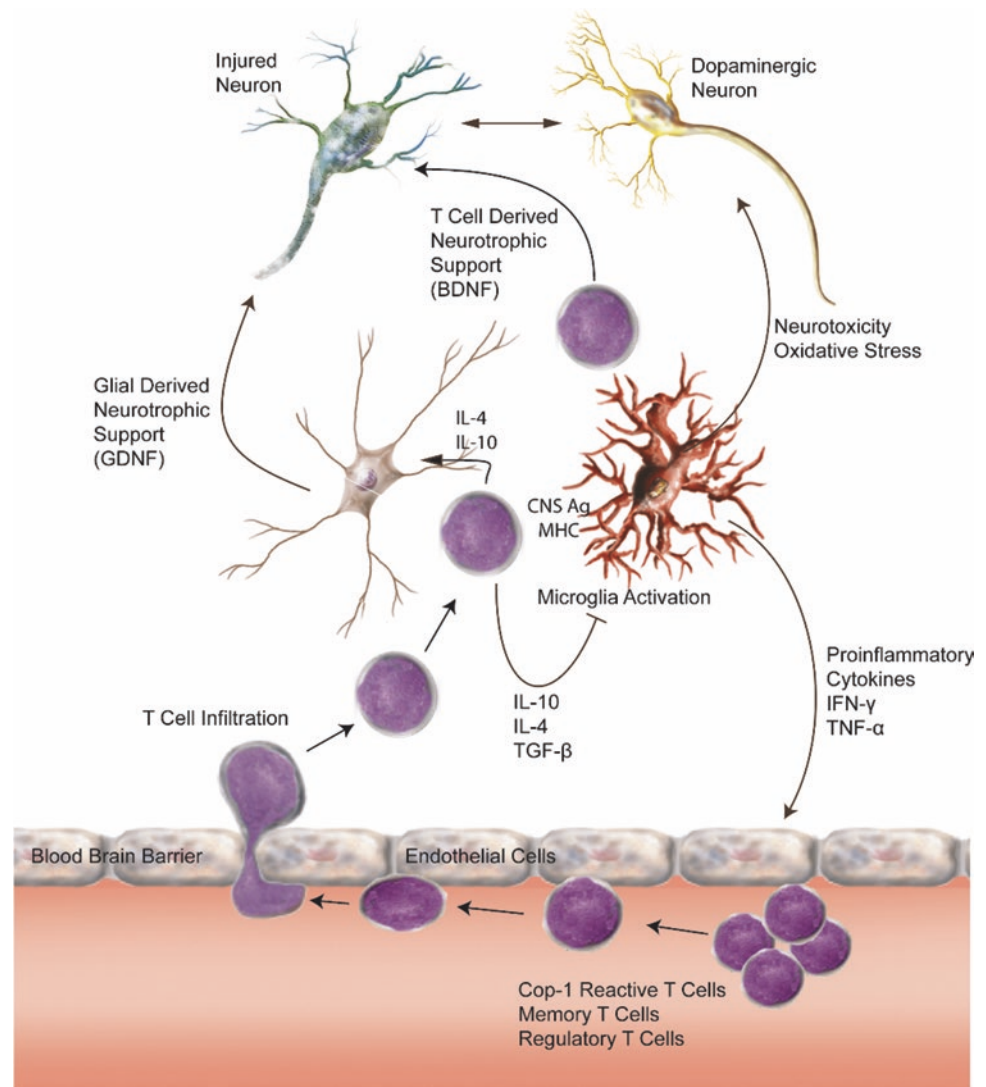
et al. 2004). Antibodies are internalized with increased phosphorylation of neurofilament H (Engelhardt et al. 1995) while Golgi system and rough endoplasmic reticulum dilate with concomitant increased Ca^{2+} levels that are precipitously depleted after 24 h (Engelhardt et al. 1997; Pullen et al. 2004). By 72 h after transfer, CSF glutamate and aspartate levels increase, without appreciable change in glutamine and glutathione levels (La Bella et al. 1997). By 8 days post-transfer, areas of neuronal cell necrosis are evident (Pullen et al. 2004) and sensitivity to L-type Ca^{2+} channel blockers is retained for up to 4 weeks post transfer (Fratantoni et al. 2000). Thus, passive transfer of immunoglobulin from ALS patients appears to lead to long-lasting effects of motor neurons at the neuromuscular junction and may reflect early stage events in immune-mediated pathogenesis of ALS.

46.5 Therapeutic Immunoregulation

46.5.1 Cell-Mediated Immunomodulation

Another potential therapeutic avenue for PD involves T cell-mediated immune responses (Fig. 46.1). Activation of T cells directed against antigens expressed at the injured areas of the CNS can be neuroprotective under acute and chronic neurodegenerative conditions (Kipnis et al. 2002). However, immunization with such antigens typically leads to development of autoimmune disease. Immunization with copolymer-1 (Cop-1, glatiramer acetate, Copaxone) or passive transfer of Cop-1 specific T cells is beneficial for protecting neurons from secondary degeneration after injurious conditions (Kipnis et al. 2000; Benner et al. 2004). Cop-1 reactive T cells have partial cross-reactivity with myelin basic protein (MBP) and other self-antigens expressed in the brain (Arnon and Sela 2003). Therefore, immunization with Cop-1 leads to increased accumulation of T lymphocytes in areas of injury within the brain and spinal cord and is neuroprotective without causing any adverse effects; however, the molecular mechanism of this response is not fully understood. T cells reactive to Cop-1 could be a source of brain-derived neurotrophic factor (BDNF) and other neurotrophic factors (Kipnis et al. 2000) or can induce production of neurotrophins by microglia or astroglia. Moreover, Cop-1 is a strong inducer of Th2 cells that secrete anti-inflammatory cytokines (Wang et al. 2011; Ibarra et al. 2007; Sela and Mozes 2004; Aharoni et al. 1997, 2000; Arnon and Sela 2003). In the MPTP model of PD, adoptive transfer of T cells from donors immunized with Cop-1, but not those from ovalbumin immunized donors, are neuroprotective for dopaminergic neurons within the substantia nigra as well as the striatal termini, and attenuates reactive microglial neuroinflammation (Benner et al. 2004). Additionally, adoptive transfer of those T cells prevents the loss of nigral N-acetylaspartate (NAA) levels

Fig. 46.3 T cell-mediated neuroprotection in a PD model. In MPTP-intoxicated mice, regulatory T cells infiltrate the inflamed nigrostriatal pathway where they encounter cross-reactive CNS antigens (such as myelin basic protein or reactive species-modified proteins) presented in the context of MHC by resident microglial cells. In response, activated T cells secrete anti-inflammatory cytokines such as IL-4, IL-10, and TGF- β that suppress toxic microglial activities. Neurotrophin expression may occur directly from T cells or T cell-derived IL-4, and IL-10 may induce neurotrophin production in neighboring glia. These activities lead to neuroprotection indirectly by suppression of microglial responses and directly through the local delivery of neurotrophins



associated with MPTP-induced neurodegeneration as determined by quantitative proton magnetic resonance spectroscopic imaging (^1H -MRSI) (Boska et al. 2005). Suppression of microglial-associated inflammation was associated with T cell accumulation within the SNpc, induction of a type 2 helper T cell (Th2) response with production of anti-inflammatory cytokines (IL-4, IL-10), and increased expression of glial cell-derived neurotrophic factor (GDNF) by astrocytes, but not by infiltrating T cells or microglia (Benner et al. 2004). CD4 $^+$ T cells, rather than CD8 $^+$ T cells, are primarily responsible for the bulk of the neuroprotective capacity, while passive transfer of anti-Cop-1 antibodies provide no apparent neuroprotection (Laurie et al. 2007). In addition, adoptive transfer of anti-CD3 activated CD4 $^+$ CD25 $^+$ regulatory T cells (Tregs), but not activated effector T cells, ameliorates MPTP-induced neuroinflammation and dopaminergic neurodegeneration with as few as 3.5×10^6 Tregs mediating virtual complete neuroprotection (Reynolds et al. 2007). Moreover, Tregs attenuate reactive microglia responses in vivo

and responses to inflammatory stimuli including nitrated α -syn as well as enhancing GDNF and BDNF gene expression and production by astrocytes.

Immunization with Cop-1 extends survival by 25 % in the SOD1-G93A transgenic mouse model of ALS (Angelov et al. 2003), however, the extended survival was not verified by others (Habisch et al. 2007; Banerjee et al. 2008), though onset of motor symptoms was delayed (Habisch et al. 2007). Much like PD, Cop-1 is thought to have a therapeutic effect in ALS by inducing regulatory T cells (Th2, Th3, or Tregs) in an epitope specific or non-specific manner. These activated Tregs extravasate in response to neuroinflammation from neurodegenerative processes; secrete anti-inflammatory cytokines in response to cross-reactive self-epitopes (e.g. myelin basic protein) to attenuate reactive microglia; suppress the inflammatory response; and induce neurotrophic responses by T cells and/or other glia (Fig. 46.3). This notion is supported by Cop-1-treated ALS patients that exhibit increased IgA, IgG and IgM reactivities to Cop-1 and

increased Th2/Th1 cytokine ratios in treated patients' serum (Mosley et al. 2007). In addition, the adoptive transfer of wild type Tregs into SOD1-G93A transgenic mice improved survival and delayed onset of motor dysfunction (Banerjee et al. 2008), suggesting the protective capability of increasing Treg numbers or function this ALS model. Treatment with Cop-1 is well-tolerated by ALS patients who exhibited both humoral and T cell-mediated immune responses (Gordon et al. 2006). However, Cop-1 was unable to delay the decline of motor function or the time to death, tracheostomy, or positive pressure ventilation compared to controls (Meininger et al. 2009). Despite the lack of success with Cop-1, induction of Tregs may prove to be a possible target for the treatment of ALS and other neuroinflammatory diseases.

The recent development of novel immune-mediated therapeutic vaccine strategies for disease intervention and attenuating neuroinflammation are predicated on the ability of Tregs, either induced or naturally occurring, to modulate both innate and adaptive immune responses and suppress inflammation both in autoimmune disease and in models of neurodegeneration. Treatment with vasoactive intestinal peptide (VIP) is emerging as a therapeutic tool to generate Tregs both *in vitro* and *in vivo*. VIP originates as a neuropeptide that functions as both a neurotransmitter and neuromodulator in many organ systems, including the central and peripheral nervous systems (Said 1976). VIP-containing neurons are present in the CNS in areas that influence the immune system as well as in lymphoid organs, and are thought to be involved in the recruitment of immune cells, many of which express receptors for VIP (Kaltreider et al. 1997; Reubi et al. 1998, 2000). VIP is also produced by lymphocytes, preferentially Th2 T cells, in response to different mitogenic or inflammatory stimuli (Delgado et al. 2005; Gomariz et al. 1990, 1992; Leceta et al. 1996). Th2-derived VIP also acts as an autocrine regulator by promoting Th2 responses *in vivo* (Delgado et al. 1999, 2000, 2002; Goetzl et al. 2001; Vassiliou et al. 2001; Voice et al. 2003). Additionally, VIP treatment was efficacious for the induction of Tregs in a variety of inflammatory disorders including arthritis, graft versus host disease, and experimental allergic encephalitis (EAE) (Delgado et al. 2005; Fernandez-Martin et al. 2006). VIP has also been used to expand regulatory T cells *ex vivo* and elicit conversion of CD4⁺CD25⁻ T cells to Tr1 regulatory T cells. Thus, VIP can be utilized to generate Tregs specific for self-antigens that can in turn promote antigen-specific tolerance and suppress development of autoimmune disorders in a variety of animal model systems (Fig. 46.1) (Delgado et al. 2005; Fernandez-Martin et al. 2006; Gonzalez-Rey et al. 2006). Splenocytes and Tregs from VIP-treated mice protect tyrosine hydroxylase-expressing neurons in the substantia nigra and striatum of MPTP-intoxicated mice as well as decrease the numbers of

activated microglia and degenerating neurons (Reynolds et al. 2010). Direct intra-nigral injection, and to a lesser extent intraperitoneal injection of VIP also led to neuroprotection, decreased microgliosis, and decreased levels of TNF- α , IL-1 β and iNOS expression in the MPTP mouse model (Delgado and Ganea 2003). In the 6-OHDA rat model of PD, intraperitoneal administration of VIP decreased lipid peroxidation, DNA fragmentation, SOD and catalase activity and NO levels (Tuncel et al. 2012). In the 6-OHDA rat model, VIP administration also increased synaptic density in the striatum compared to rats treated with 6-OHDA alone, suggesting that VIP plays a role in repairing or forming new synapses (Korkmaz et al. 2012). VIP's neuroprotective effect is mostly mediated by binding VIP receptor 2 (VIPR2, VPAC2). This was determined by creating agonists specific for each of the 2 VIP receptors and testing the neuroprotective and anti-inflammatory effects in the MPTP model of PD (Olson et al. 2015). VIP is also effective in the SOD1-G93A transgenic mouse model. Intraperitoneal injections of the lipophilic VIP-derivative stearyl-norleucine-VIP extended survival, increased the time until weight loss, and delayed onset of motor dysfunction compared to controls (Goursaud et al. 2015). Moreover, astrogliosis and microgliosis were diminished and proinflammatory mediators such as IL-1 β , NO, and TNF- α were diminished, while the expression in the spinal cord of IL-10, BDNF, and GDNF were promoted suggesting that VIP decreased inflammation.

Selective activation of particular subsets of dendritic cells (DCs) with 1,25-dihydroxyvitamin D3 (the active metabolite of vitamin D3) or GM-CSF (Fig. 46.1) can not only activate lymphocytes, but also induce T cell tolerance to self-antigens or to specific antigens tandemly administered as immunization or tolerization regimens. DCs also can affect B cell function, antibody synthesis, and isotype switching. DCs stimulated with GM-CSF or 1,25-dihydroxyvitamin D3 may exert tolerogenic functions through arresting type 1 helper T cells (Th1), skewing the Th1/Th2 balance, and generating Tregs (Gangi et al. 2005; Gregori et al. 2002; Vasu et al. 2003). In addition to its effects on DCs, 1,25-dihydroxyvitamin D3 can also increase autophagy (Li et al. 2015) and increase the number of cells expressing GDNF (Sanchez et al. 2009), both of which may be neuroprotective. The neuroprotective ability of vitamin D3 in PD was tested in a clinical trial that showed no worsening of clinical signs as determined by Hoehn and Yahr, and UPDRS II scores, whereas the placebo-treated group showed significant worse scores (Suzuki et al. 2013). Moreover, total Parkinson's disease questionnaire 39 (PDQ39) and PDQ39 bodily support scores improved or did not diminish in patients treated with vitamin D3, however, significantly declined in the placebo controls. This suggests that vitamin D3 may slow the decline in PD for the short term. In the SOD1-G93A transgenic mouse model of ALS,

a 10-fold increase in dietary vitamin D3 increased movement ability, motor performance, and paw grip endurance (Gianforcaro and Hamadeh 2012). Together, these data suggest that vitamin D3 plays a role in symptom alleviation in animal models as well as PD and ALS.

In addition to the effects on DCs, GM-CSF is protective in the MPTP and paraquat models of PD. Pretreatment with GM-CSF prior to MPTP intoxication or adoptive transfer of GM-CSF-induced Tregs protected dopaminergic neurons in the SN, diminished microgliosis, and restored spontaneous movement (Kim et al. 2009; Kosloski et al. 2013). Administration of GM-CSF into the SN leads to neuroprotection and decreased microgliosis following paraquat treatment (Mangano et al. 2011). Due to the neuroprotective capacity in the MPTP model, a Phase I clinical trial (NCT01882010) is underway to determine the safety of GM-CSF in PD patients and the effect on PD patient immune profiles.

46.5.1.1 Modulation of Humoral Immunity

Using another vaccine strategy to elicit humoral immune responses directed at cephalic epitopes, immunization of human α -syn transgenic mice with mutant human α -syn produced high affinity anti- α -syn antibodies with concomitant diminution of human α -syn inclusions in neurons and synapses, and diminished neurodegeneration (Masliah et al. 2005). Moreover, anti- α -syn antibodies recognized abnormal human α -syn and supported degradation of α -syn aggregates. In addition, in the MPTP model of PD, Fc γ receptor and RAG2 knockout mice are more resistant to loss of dopaminergic neurons than wild type mice suggesting an important role for humoral responses in neuroprotection (Lira et al. 2011). However, as stated in Sect. 46.4.2, not all PD patients have increased anti- α -syn antibodies (Heinzel et al. 2014; Besong-Agbo et al. 2013). In PD mouse models, the addition of exogenous anti- α -syn antibodies decreases α -syn fibrils (Games et al. 2013, 2014; Lindstrom et al. 2014). In addition, human intravenous immunoglobulin that contain an unknown mix of antibodies from thousands of donors, can bind monomeric and oligomeric α -syn. However, these antibodies were not able to significantly decrease α -syn fibril formation in vitro (Patrias et al. 2010; Smith et al. 2012). Several ongoing clinical trials using humanized anti- α -syn antibodies to clear truncated and aggregated α -syn (NCT02157714 and NCT02095171). Along those same lines, mimetopes for α -syn have been used in vaccine strategies for clearing extraneuronal α -syn deposits (Schneeberger et al. 2012; Mandler et al. 2014). Several clinical trials using these strategies (NCT01568099, NCT01885494, NCT02618941, and NCT02216188) are actively ongoing or have been completed, but not yet published.

Similarly, immunization of SOD1-G37R transgenic mice with recombinant mutant SOD1 reduced the amount of

mutant SOD1 in spinal cords of immunized mice, increased numbers of surviving motor neurons, and extended life span by greater than 4 weeks, which significantly correlated with increasing anti-SOD1 antibody titers (Urushitani et al. 2007). In contrast, this strategy failed to protect SOD1-G93A mice, which exhibit a more severe and aggressive phenotype than SOD1-G37R mice, however ventricular infusion of purified anti-human SOD1 antibody alleviated clinical signs and significantly extended life span by 4 %. These data provide support for immunotherapeutic strategies that target extracellular burdens of aggregated or misfolded toxic proteins such as α -syn and SOD1 in neurodegenerative disorders (Fig. 46.1).

46.5.1.2 Other Vaccine Strategies

One quasi-immunotherapeutic strategy targets intracellular accumulation of toxic proteins in neurons by using single chain antibodies (scFv) or intrabodies (Chen et al. 1994) (Fig. 46.1). This strategy is accomplished intracellularly by preventing misfolding of proteins to toxic forms, interfering with misfolded protein interactions, or enhancing degradation of the toxic form. Clearly, keys to this strategy are the production of scFv with affinities that retain epitope specificities in vivo and efficacious delivery to vulnerable or affected neurons. For delivery, genes encoding the scFv must be transfected into target cells and successfully expressed as a functional intrabodies. Several scFv to α -syn have been prepared (Emadi et al. 2004, 2007; Maguire-Zeiss et al. 2006; Barkhordarian et al. 2006; Miller et al. 2005). Several of those scFv recognize different conformations of α -syn, and upon co-incubation with monomeric α -syn in vitro, decrease the rate of α -syn aggregation, inhibit formation of oligomers and protofibrils, and inhibit toxicity. In transfected cells, scFv are stably expressed for greater than 3 months with minimal toxicity, bind intracellular α -syn, increase the amount of detergent soluble α -syn species, while decreasing the amount of insoluble species, and counter the reduced cell adhesion which characterizes cells that overexpress α -syn (Messer and McLearn 2006). When scFv reactive to α -syn are expressed in neurons, total, oligomeric and phosphorylated α -syn is diminished in transgenic mice expressing human α -syn (Spencer et al. 2014). Thus, anti- α -syn scFv have the potential to provide a therapeutic modality to control intracellular accumulation of toxic protofibrillar forms of α -syn, and possibly diminish or delay disease progression (Fig. 46.1). Similar findings were found in ALS mouse models. The expression of scFv against SOD1 decreases the number of SOD1 aggregates and increased cell survival (Ghadge et al. 2013). In addition, the expression of scFv in SOD1-G93A transgenic mice increases survival and time until disease onset (Patel et al. 2014). Thus, this data indicates that in PD and ALS models, scFv reduce the effects of protein misfolding in neurons and suggests a potential beneficial role as a therapeutic strategy in PD and ALS.

Therapeutic vaccine approaches using neuronal antigens represent a potential efficacious interdictory modality for slowing or halting the progression of neuroinflammation and secondary neurodegeneration and removing neurotoxic aggregates (Fig. 46.1). These approaches should be considered in strategies with other anti-inflammatory or antioxidant therapies for a combinatorial modality to protect against neuroinflammation and consequent neurodegeneration in PD and ALS. Since these interventions have failed to produce results individually and elements of innate and adaptive immunity contribute to inflammation, perhaps a combination would be more effective at ameliorating detrimental immune responses.

46.6 Neuroprotective Strategies

46.6.1 Growth Factors

The role of neurotrophins in reducing neurodegeneration and promoting neuroregenerative processes presents an exciting possibility for therapy in PD (Fig. 46.1). A study of lentiviral delivery of GDNF showed trophic effects on degenerating nigrostriatal neurons in a primate model of PD (Kordower et al. 2000). Results indicated the augmentation of dopaminergic functions in aged monkeys and reversal of functional deficits with complete prevention of nigrostriatal degeneration in MPTP-treated monkeys. These data indicate that GDNF delivery using a lentiviral vector system can prevent nigrostriatal degeneration and potentially induce regeneration in primate models of PD, showing the potential for a viable therapeutic strategy for PD patients. However, clinical trials of intraputamenally infused GDNF in PD patients are controversial with one 2-year phase I trial showing improved UPDRS scores and increased ^{18}F -dopa uptake in the putamen and no untoward effects in a limited cohort (Patel et al. 2005), while phase II trials were halted after 6 months due to lack of efficacy and adverse effects in patients (Nutt et al. 2003; Lang et al. 2006). Of note, several parameters that affect delivery of GDNF were changed between the trials. Several different strategies to deliver GDNF have been developed including delivery by installation of GDNF genes, GDNF-engineered mesenchymal stem cells, nanoformulated GDNF, AAV-GDNF gene constructs, continuous intracerebral administration of methionylated GDNF (Aly and Waszczak 2015; Hoban et al. 2015; Agbay et al. 2014; Peng et al. 2014; Wakeman et al. 2014); the latter two strategies are in clinical trials (NCT01621581 and NCT00006488).

Growth factors have also been used in the treatment of ALS. Intrathecal administration of BDNF has been used in different clinical trials of ALS patients; however, no significant beneficial effects were demonstrated (Kalra et al. 2003;

Beck et al. 2005). More recently, human progenitor cells over-expressing human GDNF were injected into the cisterna magna of SOD1-G93A transgenic mice, and differentiated into astrocytes, released GDNF, and protected motor neurons from degeneration; however microgliosis, loss of limb function, or survival were not affected (Suzuki et al. 2007; Park et al. 2009). Thus, strategies for treatment of PD and ALS with neurotrophic factors provide promising potential for neuroprotective benefit, however, to date, these strategies have yet to be proven efficacious in neurodegenerative diseases.

46.6.2 Neuroprotectants

Many diverse mechanisms, factors, and pathways are involved in neurodegenerative disorders, thus several different therapeutic methods have been developed to target a specific factor or an entire intricate pathway with the intent of ameliorating, preventing, or reversing neuronal cell damage. Inflammation and oxidative stress form a commonality between many neurodegenerative diseases; therefore most therapeutic modalities currently under investigation target MP activation to decrease the magnitude of the inflammatory responses. The targets of these therapies include, but are not restricted to, enhancement of neurotrophic factors such as GDNF, upregulation of anti-inflammatory cytokines (IL-4, IL-10, and TGF- β 1), inhibition of enzymatic activities that encourage neurotoxicity (GSK-3 β , γ -secretase), Ca^{2+} and glutamate excitotoxicity blockers that inhibit NMDA receptor function, antagonism of A2A receptors that stimulate release of GABA in the globus pallidus and suppress neuronal cytotoxicity (memantine, lithium, sodium valproate), and attenuation of inflammation by anti-inflammatory drugs (NSAID, minocycline) or β 2 adrenergic receptor agonists (norepinephrine, salmeterol). Anti-inflammatory and/or anti-oxidative therapies could be used in conjunction to form combinational therapies targeting multiple sites of oxidative stress that contribute to inflammatory responses and progressive degenerating disease (Fig. 46.1).

It should be noted that these therapies have shown promise in animal models, but have yet to be translated into patients. The exact reasons for this discrepancy between models and patients are unknown (Athauda and Foltynie 2015), but could be related to translating effects from an acute model to the progressive disease in patients. It should also be noted that patients receiving treatments may need more than palliative therapy to recover motor function. Perhaps neuroregeneration, as well as neuroprotective therapy is required for full recovery of motor function.

46.7 Genetics and Immunity

Recent evidence has shown that genetics may contribute to the onset of neurodegenerative disorders (Li et al. 2002). Familial PD is associated with several mutations in proteins such as α -syn, parkin, DJ-1 and others (Hardy et al. 2003). Genome-wide association studies (GWAS) of PD patients implicate several genes, including *STK39*, *BST1*, *SNCA*, *LRK2*, *CCDC62* and *MAPT* as being associated with PD (International Parkinson Disease Genomics Consortium et al. 2011; Lill et al. 2012; Nalls et al. 2014). Further analysis revealed some immune-related genes are associated with increased risk of PD. Besides the HLA alleles discussed above (4.1 Cell-Mediated Immunity) and *BST1* which promotes pre-B cell growth, linkages to the age at onset (AAO) for PD have been identified on chromosomes 1 and 10. The latter is significantly associated with glutathione s-transferase omega-1 (*GSTO1*) (Li et al. 2003); a provocative finding since *GSTO1* is thought to be involved in the post-translation modification of IL-1, a major component in the regulation of inflammatory responses (Laliberte et al. 2003). One factor associated with the chromosome 1p peak is the embryonic lethal abnormal vision 4 (*ELAVL4*) gene (Noureddine et al. 2005), a human homologue of the *Drosophila* ELAV (Good 1995) and essential for temporal and spatial gene expression during CNS development. Additionally, *ELAVL* gene products are known to bind to AU-rich response elements (ARE) in the 3'-untranslated region (3'UTR) of inflammation-associated factors (Good 1995). Interestingly, PD patients homozygotic for allele 1 at position -511 of the IL-1 β gene have an earlier onset of the disease than those homozygotic for allele 2, which produces higher amounts of IL-1 β . Thus, higher production of IL-1 β may provide some neuroprotective effect for dopaminergic neurons (Nishimura et al. 2000; Mizuta et al. 2001).

In ALS, one of the genes associated with the familial form of the disease is *SOD1* (Rosen et al. 1993), which has an important role in eliminating toxic levels of superoxide radicals. Gene expression analyses of post-mortem specimens showed differentially expressed genes associated with the immune response in ALS, most notably IL-1 receptor accessory protein (IL-1rap), MHC class II, thromboxane synthase, Fc ϵ R γ -chain, and the cytokine regulated upon activation, normal T-cell expressed and secreted (RANTES) (Malaspina and de Bellerche 2004; Wang et al. 2006). Of interest, as most of these analyses are performed with tissues from end-stage patients, only approximately 10 % of the differentially expressed genes from ALS patients are associated with inflammation/immune function, whereas the majority of genes pertain to stress-activated pathways (Malaspina and de Bellerche 2004). This is in contrast to mice that express the G93A mutation of human *SOD1*, wherein the majority of early changes are associated with inflammation/immune function genes (Malaspina and de Bellerche 2004). At this

time, the exact role of genetics in ALS is unclear. Approximately 17 gene regions are thought to be associated with ALS, but the genes in these regions which are responsible for this association are not known (Keller et al. 2014). To address this, ICSNPathway (Identify candidate Causal SNPs and Pathways) analysis of an ALS genome-wide association study revealed several pathways which may be affected in ALS including chromatin remodeling, interphase, nucleosome assembly, and classical complement activation (Lee and Song 2015).

46.8 Review Questions

- The cellular target for immunotherapy that would be most beneficial to inhibit secondary neurodegeneration is the
 - astrocyte
 - neuron
 - microglia
 - oligodendrocyte
- The primary producer of reactive oxygen species by microglia is
 - dopamine synthesis
 - NADPH oxidase*
 - myeloperoxidase
 - superoxide dismutase
- In PD, postmortem samples show increased
 - reactive microglia.
 - loss of striatal dopaminergic termini
 - loss of dopaminergic neuronal bodies within the substantia nigra pars compacta
 - reactive oxygen species modified proteins
 - all of the above
- CD45RO+ T cells that mutually express CD4 and CD8 more likely indicate those cells are
 - dead
 - recent thymic emigrants.
 - anergic
 - activated
- An example of free radical modification of RNA
 - 8-hydroxyguanosine (8-OHG)
 - 4-Hydroxy-2-nonenal (HNE)
 - 3-nitrotyrosine (NT)
 - 8-hydroxy-2'-deoxyguanosine (8-OHdG)
- Free radical modification of DNA is best exemplified by
 - 8-hydroxyguanosine (8-OHG)
 - 4-hydroxy-2-nonenal (HNE)
 - 3-nitrotyrosine (NT)
 - 8-hydroxy-2'-deoxyguanosine (8-OHdG)
- One molecular marker of nitric oxide modification of proteins is
 - 8-hydroxyguanosine (8-OHG)
 - 4-hydroxy-2-nonenal (HNE)

- (c) 3-nitrotyrosine (NT)
 (d) 8-hydroxy-2'-deoxyguanosine (8-OHdG)
8. A protein marker resulting from modifications due to lipid peroxidation is
 (a) 8-hydroxyguanosine (8-OHG)
 (b) 4-hydroxy-2-nonenal (HNE)
 (c) 3-nitrotyrosine (NT)
 (d) 8-hydroxy-2'-deoxyguanosine (8-OHdG)
9. Immunotherapeutic strategies using scFv directed against α -synuclein targets are designed to
 (a) prevent misfolding to the toxic form of the protein
 (b) interfere with misfolded protein interactions
 (c) enhance degradation of the toxic protein form
 (d) *all of the above*
10. Passive transfer of sera or immunoglobulin from ALS patients to rodent recipients results in increases in the following sequelae with the exception of
 (a) *denervation of striatum*
 (b) denervation of muscles
 (c) human immunoglobulins in spinal cord motor neurons
 (d) human immunoglobulins in neuromuscular junctions
 (e) miniature end-plate potential (MEPP)
11. A ligand utilized for in vivo imaging of reactive microglia via upregulated peripheral benzodiazepine receptors is
 (a) CFT
 (b) DA
 (c) GT1b
 (d) MPTP
 (e) *PK1195*
12. The ultimate effect of reactive species on cellular function include
 (a) ligand misrecognition
 (b) enzyme dysfunction
 (c) membrane damage
 (d) mutation
 (e) *all of above*
13. Glatiramer acetate is an immunomodulatory drug that is FDA approved and clinically indicated for
 (a) amyotrophic lateral sclerosis
 (b) *remitting/relapsing multiple sclerosis*
 (c) Parkinson's disease
 (d) Alzheimer's disease
 (e) Huntington's disease
14. Regulatory T cells capable of attenuating microglial responses are more likely to produce and secrete
 (a) *IL-10*
 (b) IL-2
 (c) IFN- γ
 (d) TNF- α
15. A first indicator of oxidative stress during progressive disease in PD, occurring prior to other hallmarks including loss of mitochondrial complex I activity, has been suggested to be
 (a) reactive microglia
 (b) diminished dopamine
 (c) increased astrocytosis
 (d) *depletion of glutathione*
 (e) all of the above
16. IL-2/IL-2R interactions are found localized to
 (a) T cell-T cell interactions
 (b) striatum
 (c) frontal cortex
 (d) cerebellum
 (e) *all of the above*
17. The possible mechanism by which glatiramer acetate functions is
 (a) competition with myelin-basic protein (MBP) for binding to major histocompatibility complex (MHC) molecules
 (b) competition of GA/MHC with MBP/MHC for binding to the T-cell receptor
 (c) partial activation and tolerance induction of MBP-specific T cells
 (d) induction of GA-reactive T-helper 2- (TH2)-like regulatory cells
 (e) *all of the above*
18. Levels of glutathione least concentrated in
 (a) *substantia nigra*
 (b) striatum
 (c) hippocampus
 (d) cerebellum
 (e) cortex

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Abstract

This chapter has presented a number of approaches that may be effective in augmenting the limited repair capacity of the mammalian central nervous system (CNS). In comparison to the CNS, the regenerative capacity of the peripheral nervous system can be quite robust and a number of investigators have sought to identify differences in the hope of identifying molecular targets that may be exploited to enhance CNS regeneration. Another promising approach has been cell transplantation in order to replace lost cells within the damaged or diseased CNS. The choice of cell type will be critical in developing cell-based regeneration and repair strategies. For example, if stem cells (pluripotent or multipotent) are selected, the cells must be competent to generate specific cell types targeted for each disease. Furthermore, if pluripotent cells are selected, this increases the risk of teratoma formation. Cellular reprogramming or direct conversion of somatic cells into functional “induced” neuronal cell types may also be a feasible approach. However, there may be limitations on the number of cells that can be effectively generated using this methodology.

Keywords

3-D scaffolds • Adipose stem cells • Astrocyte • Bioengineering • Biomaterials • Cellular reprogramming • Embryonic stem cells • Genetic engineering • Glia • Glial cell • Induced pluripotent stem cells • Mesenchymal stem cells • Microglia • Multipotent stem cells • Neuron • Neuroprotection • Neuroregeneration • Neurotrophic factors • Oligodendrocyte • Regeneration • Schwann cell • Tissue engineering

47.1 Introduction

The field of regenerative medicine in the context of the central nervous system (CNS) is focused on the ability to repair, replace and regenerate neural tissues, affected due to some disease condition, injury, or due to the aging process. The

underlying goals are to develop therapies capable of restoring function of the CNS.

The nervous system is composed of two major parts, the central nervous system (CNS) and the peripheral nervous system (PNS). The major components of the vertebrate CNS include the brain, spinal cord, and retinas (and optic nerves). The CNS integrates sensory information from the environment as well as internally and is responsible for coordinating the organism’s activities and behaviors. The PNS consists of the ganglia and nerves outside of the CNS and a principal function is to connect the CNS with limbs and organs, serving as the communication relay. Disease or damage to the nervous system can lead to severe functional and behavioral deficits. The neuroregenerative capacity of the PNS and the CNS are quite different and can vary widely across species

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(Horner and Gage 2000). While regeneration of cut or damaged axons readily occurs in the mammalian PNS, as well as in the CNS of lower vertebrates such as frogs, newts and some fish, the adult mammalian CNS has limited capacity for regeneration.

A prominent difference in the regenerative capacity of the mammalian PNS versus the CNS is associated with the glial cells that form the myelin sheath on the axons, the Schwann cells and oligodendrocytes, respectively. The myelin sheath formed by these two glial cell types serves as an electrical insulator and facilitates the propagation of action potentials along the length of many axons throughout the nervous system. While there are a number of similarities between these two glial cell types, significant differences are clearly evident with respect to their role in neural regeneration (Martini et al. 2010). The Schwann cells of the PNS are derived from the neural crest and following peripheral nerve injury provide a conducive environment for axonal regeneration for peripheral neurons, as well as CNS neurons projecting through the PNS. Following peripheral nerve injury, proliferating Schwann cells produce and secrete a number of trophic factors with growth promoting activities including, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial cell line-derived neurotrophic factor (GDNF) (Bunge 1994; Johnson et al. 1988). These neurotrophic factors promote the survival of the injured neurons and are also involved in regulating gene expression programs involved in axon regeneration and formation of the growth cone (Reichardt 2006; Kwok et al. 2014). In addition, they produce extracellular matrix molecules (ECM) such as laminin and fibronectin that promote neurite outgrowth and also express integrin ECM receptors and numerous cell adhesion molecules including, neural cell adhesion molecules (NCAMs) and N-Cadherin (Thornton et al. 2005).

A principal reason for the absence of a prominent regenerative capacity in the adult CNS resides within the glial environment. The myelin producing cells of the CNS, the oligodendrocytes are derived from oligodendrocyte progenitor cells (OPCs) generated from neuroepithelial cells lining the ventricles and central canal of the brain and spinal cord, respectively. The limited regenerative capacity of the adult mammalian CNS is in large part due to the oligodendrocytes, along with astrocytes (another major glial cell component of the CNS), that produce a number of inhibitory molecules at the site of injury that suppress regeneration and the reestablishment of function. Lesions in the CNS result in the formation of a glial scar and the production of myelin-associated molecules that inhibit axon regrowth including chondroitin sulfate proteoglycans (CSPGs), Nogo-A, myelin-associated glycoprotein (MAG), oligodendrocyte-myelin glycoprotein (OMgp), ephrins (B3 and A3), semaphorins (4D, 5A, and 3F), and myelin glycolipid sulfatide (Giger et al. 2010; Silver et al. 2015). Identification of corresponding receptor systems

for many of these molecules have demonstrated that the functional receptors mediate growth cone collapse and growth inhibition.

Advances in the fundamental understanding of neurodevelopment can provide insight in development of strategies for rescue and repair of the damaged and diseased nervous system. Experimental studies during early neural development as well as clinical observations suggest that when compared to the adult mammalian CNS, the immature CNS provides a more permissive environment such as that can support regeneration. For example, axonal regeneration across a lesion site can occur in fetal mouse and rat spinal cord. Results for successful spinal cord regeneration across a crush or transection in the neonatal opossum spinal cord (before 12 days postnatal) have led to reestablishment of synaptic connections and behavioral recovery (Saunders et al. 1998). During this permissive period of CNS regeneration there is a notable lack of myelin. Furthermore, the end of this “critical period” for CNS regeneration is marked by the appearance of neurite growth inhibiting molecules associated with the appearance of the oligodendrocytes (Varga et al. 1995). As such, a number of strategies to stimulate CNS regeneration have focused on neutralizing these inhibitory myelin oligodendrocyte-associate factors.

An additional limiting factor for successful axon regeneration appears to be a relatively poor intrinsic regenerative capacity within adult mammalian CNS neurons (Lu et al. 2014). Indeed, there is a difference in physiological neurite outgrowth during development compared to axon regeneration in mature neurons. One might surmise that axonal regeneration recapitulates axonogenesis during development since both processes require the formation of a growth cone for process extension. However, although intrinsic mechanisms play a significant role in axon outgrowth during development, extrinsic environmental cues are also essential in regulating this process and in determining target recognition (Kolodkin and Tessier-Lavigne 2011). Understanding how intrinsic and extrinsic cues regulate regeneration in the mature CNS has been a focus for regenerative medicine for many years (Fawcett et al. 2012; Harel and Strittmatter 2006). The earlier work of Aguayo and colleagues demonstrated that providing a permissive environment that of a peripheral nerve graft, supported the regeneration of some adult CNS axons (David and Aguayo 1981; Keirstead et al. 1989). Additional studies have demonstrated that, following lesions in the CNS, undamaged axons can sprout new neurites and form functional synaptic connections with considerable specificity within relatively localized regions. Furthermore, under the appropriate circumstances some severed CNS axons can regrow considerable distances (several centimeters) and form synaptic connections, however the majority of severed axons fail to regenerate. A number of recent studies have elucidated several molecular signaling

pathways that are involved in regulating the ability for adult axonal CNS regeneration (Lu et al. 2014). The PTEN/mTOR pathway appears to be a general signaling pathway regulating axon regeneration (Park et al. 2009). The JAK/STAT and DLK/JNK pathways may also be involved in providing signals from the damaged or lesioned axon to the neuronal soma in order to initiate an injury response leading to regeneration. Identification of these as well as other molecular pathways may provide insight into mechanisms for promoting regeneration of adult CNS axons following injury or disease.

47.2 Development of Experimental Strategies to Regenerate and Repair the CNS

The lack of a robust regenerative capacity in the CNS, along with limited treatment options for patients with CNS injury or other neurodegenerative conditions has in part led to the development of the fields of regenerative medicine and CNS tissue engineering. A number of different strategies are actively being pursued to enhance CNS regeneration.

One approach takes into consideration the regenerative capacity of the PNS by grafting a segment of peripheral nerve into the CNS. Earlier studies by Aguayo and colleagues were among the first to demonstrate that CNS axons, those of the retinal ganglion cells (RGCs), were able to regenerate effectively through an autologous peripheral nerve graft (Keirstead et al. 1989). In these studies a segment of the optic nerve was removed and replaced by a peripheral nerve graft taken from the sciatic nerve. The other end of the sciatic nerve was then sutured into the normal visual target, the superior colliculus. Following regeneration of at least some RGC axons, electrophysiological recordings revealed light evoked activity in the superior colliculus. However, behavioral recovery was not demonstrated after regeneration of host projections. These early studies provided compelling evidence for the regenerative capacity of adult mammalian CNS axons, under the appropriate conditions. Thus, providing a regenerative conducive environment (in this case a grafted peripheral nerve containing Schwann cells) is an important consideration for development of CNS regenerative therapies.

47.2.1 CNS Regenerative Failure and the Glial Environment

Studies using animal models of CNS injury have provided excitement in the field of CNS regenerative medicine by modulating and boosting the intracellular signaling pathways involved in axon outgrowth as well as manipulating the extracellular barriers associated with the glial scar. A number of

approaches have been employed to neutralize myelin-associated inhibitory factors that suppress CNS axon regeneration following injury. Approaches have included development of function-blocking antibodies, Nogo-receptor-blocking peptides and fusion proteins, as well as extensive *in vivo* studies generating knockout mice to regulate levels of expression of the myelin-associated inhibitory molecules. These strategies have been used in a number of experimental models including spinal cord injury, stroke, and autoimmune diseases. Studies conducted in adult non-human primates subjected to a unilateral spinal cord cervical lesion showed functional and behavioral recovery of some motor skills following intrathecal administration of a function blocking anti-Nogo A antibody (Freund et al. 2009). These strategies have been implemented in clinical trials for acute spinal cord injury (Abel et al. 2011) and ozanezumab, a humanized monoclonal antibody against Nogo-A has been used in amyotrophic lateral sclerosis (ALS) patients (Meininger et al. 2014).

47.2.2 Inflammation and CNS Regeneration

Most neurodegenerative diseases and CNS injury are accompanied by a local inflammatory response. Characteristics of the neuroinflammatory response include invasion of circulating immune cells (macrophages and lymphocytes) and induction of inflammatory mediators including a variety of cytokines and other reactive molecules. Since many of these molecular entities are produced locally around the site of injury or inflammation, they have become targets for possible therapeutic intervention. Neurons and glial cells (oligodendrocytes, astrocytes and microglia) can produce inflammatory mediators, as well as their respective cytokine receptors throughout the CNS. Their low levels of expression in the healthy brain suggest they may contribute to normal homeostatic functions within the CNS. Under pathological conditions, microglia and macrophages are recruited to the site of injury. Damaged cells and their associated debris serves to stimulate the resting microglia to transition into migratory macrophages that produce cytokines and trophic factors that can exert both damaging as well as protective effects on cells within the local environment. Differentiation of macrophages towards “classically” (M1) activated state can be neurotoxic and induce extensive retraction of damaged axons (Horn et al. 2008; Kigerl et al. 2009). As such, depletion of M1 macrophages appears to have neuroprotective effects in models of spinal cord injury. Interestingly, macrophages can also mediate sprouting of injured CNS axons likely through release of neurotrophic and growth factors and/or by indirectly activating glial cells within the scar region (Benowitz and Popovich 2011). Provision of neurotrophic factors by transplanted microglia and macrophages may help to explain their activity in promoting axon

outgrowth in different models of spinal cord injury (Rapalino et al. 1998). Differentiation of macrophages towards the “alternatively” (M2) activated state has been associated with neuroprotection and enhancement of neurite outgrowth from damaged CNS neurons. Compared with the M1 phenotype, the M2 macrophages appear more proficient at CNS repair (Silver et al. 2015). Therapeutic applications using autologous macrophage transplantation appears promising in clinical trials (Jones et al. 2010). Methods that modify the local injury environment to favor the conversion of macrophages towards the M2 phenotype are likely to have benefits in CNS regeneration and offer additional interesting therapeutic targets.

47.2.3 Adult Neural Stem Cells and Endogenous Repair of the CNS

Many neurodegenerative diseases result in significant neuronal loss. Neuronal cell death is also frequently associated with severe neural insults, such as traumatic brain injury (TBI), chronic traumatic encephalopathy (CTE), stroke, and spinal cord injuries. Development of strategies to replace dead or dying neurons is likely to rely, in part, on important advances in understanding the fundamental mechanisms of endogenous neurogenesis. As such, a fundamental concept of regenerative medicine in the CNS is to develop strategies to improve neuronal survival and replace cells lost as a result of neurological disease or injury.

The discovery of neural stem cells within the adult mammalian brain has focused considerable interest into harnessing the endogenous capacity of these cells for CNS regeneration and repair. The original concept of adult neurogenesis in the mammalian CNS was quite controversial and was met with considerable skepticism. However, it is now well established that new neurons are generated throughout life in specific neurogenic regions of the dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ) (Altman and Das 1965; Gage et al. 1998; Lois and Alvarez-Buylla 1993; Reynolds and Weiss 1992; Aimone et al. 2014). Since the original reports a number of studies have demonstrated that adult neurogenesis in the DG plays a role in hippocampal-dependent learning and memory (Aimone et al. 2014), while adult neurogenesis in the SVZ results in production of new olfactory interneurons necessary for the normal function of the olfactory bulb and associated behaviors (Sakamoto et al. 2014). Considerable research efforts have elucidated mechanisms regulating proliferation and the differentiation of these adult stem cells into specific neural cell types. Furthermore, studies have also investigated adult neurogenesis in the context of neurodegenerative conditions such TBI and experimentally induced focal lesions (Perederiy et al. 2013; Sun 2016). Studies have demonstrated that different

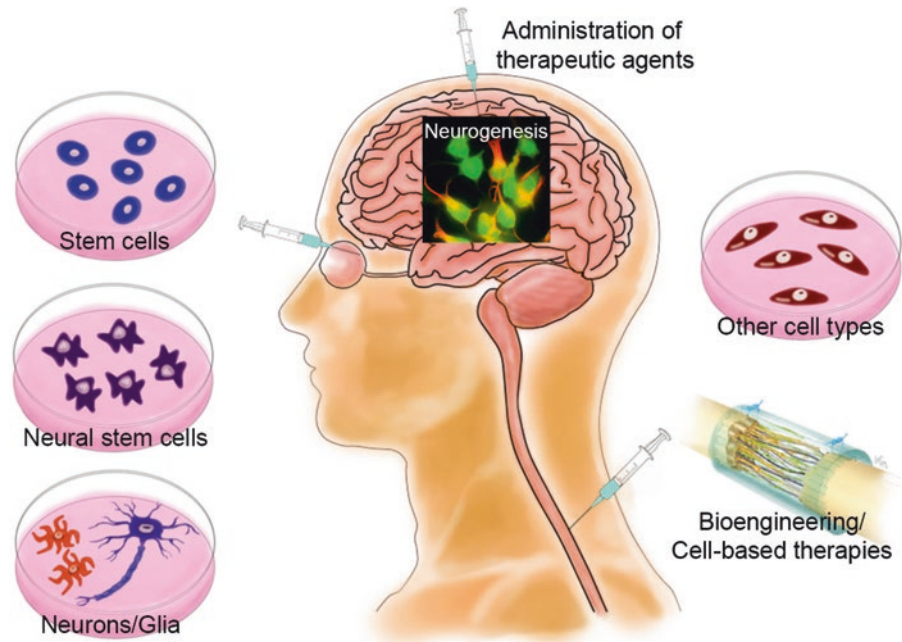
models of TBI significantly increased cell proliferation in the DG and SVZ of rodents (Sun 2016). However, due to a number of challenges including, difficulties in obtaining human brain tissue and cell birth dating studies, evidence of TBI-induced neurogenesis in the human brain is lacking. Nevertheless, mobilizing and augmenting endogenous adult neurogenesis is an attractive strategy towards repopulating the damaged or diseased CNS. Since the natural regenerative capacity of the adult CNS is quite limited, exogenous methods must be employed. With this type of strategy in mind, a number of studies have shown that different growth factors can enhance adult neurogenesis in vitro and in vivo in animal studies. Factors that have a demonstrated enhancement of neurogenesis and synaptic plasticity include, BDNF, basic fibroblast growth factor-2 (FGF-2), epidermal growth factor (EGF), NGF, vascular endothelial growth factor (VEGF) and insulin-like growth factor (IGF) (Vivar et al. 2013). In addition to neurotrophic and growth factors, a number of drugs currently in clinical trials for treating neurological conditions, including TBI, enhance neurogenesis in animal studies. These include, erythropoietin, statins, and imipramine (Sun 2016; Xiong et al. 2013). However, it should be noted that in addition to an enhancement of neurogenesis, these exogenous agents in many cases also exert neuroprotection which may also contribute to enhanced recovery in behavioral and functional studies. Nevertheless, these findings should prove valuable in designing clinical strategies to improve the prospects of endogenous adult neural stem cell-based therapies.

47.3 Stem Cells for CNS Repair Strategies

The successful isolation of human embryonic stem cells and the development of induced pluripotent stem cell technologies is making it possible to recapitulate developmental processes, as well as modeling neurodegenerative conditions. Furthermore, the advent of cellular reprogramming technologies has made it possible to implement rational strategies to generate specific cell types with a goal of developing cell-based therapies for CNS disorders. Additionally, advances in biomaterials and in 3-D scaffold fabrication techniques is making it feasible to mimic the neural stem cell niche. In this section I provide an overview of approaches merging stem cells, scaffolds, drug delivery systems, gene therapy, cellular engineering and biomaterials to develop experimental regenerative strategies for the CNS (Mallapragada et al. 2015; Sandquist et al. 2016).

Current therapies targeted for CNS-related disorders often rely on the use of pharmacological-based methods, which in many cases are not without serious side effects. As such, the implementation of cell-based therapies has gained considerable interest. In recent decades the field of stem cell biology and cell transplantation has come to the

Fig. 47.1 Central nervous system regeneration and repair strategies. Injections and administration of therapeutic agents to enhance regeneration (neuroprotection, stimulating axon growth, neutralizing inhibitory factors). Stimulating endogenous neurogenesis. Cell-based therapies using stem cells (ESCs, iPSCs), neural stem cells, neurons and glial cell types. A variety of other somatic stem cells, as well as other cell types may be used for cellular therapies. Coupling of engineering approaches with cell-based therapies is another strategy for CNS regeneration



forefront of biomedical research with aspirations of development of cures for various neurological disorders.

One approach is for transplanting cells that can replace the lost populations of neurons or glia (Fig. 47.1). Alternatively, transplanted cells may also provide trophic support via autocrine and paracrine signaling related to their cellular secretomes. An alternative approach involves harnessing the natural abilities for adult neurogenesis in order to generate new neurons. While another route takes advantage of bioengineering approaches, where cell-based approaches are combined with biomaterials, scaffolds and nanoneuro-medicine concepts, in order to develop novel therapies that target neurological conditions.

47.3.1 Pluripotent Stem Cells

Stem cells are defined by their potentially unlimited capacity for self-renew and ability to differentiate into multiple cell types. These properties are attractive to the field of regenerative medicine, which seeks to replace cells lost to neurodegenerative conditions, injury, or due to the natural aging process. Stem cells from a number of different sources are being used in neuroscience research in search of therapeutic treatment strategies targeting neurodegenerative diseases including Alzheimer's, Parkinson's, ALS and damage such as that from stroke as well as CTE. In general, stem cells are broadly categorized by their differentiation potential, from pluripotent cells, which are able to generate cells from all three primary germ layers (endoderm, mesoderm and ectoderm), to multipotent cells, which differentiate into a limited number of tissue specific cell types (Fig. 47.2).

47.3.1.1 Embryonic Stem Cells

Thomson and colleagues (Thomson et al. 1998) were the first to report the isolation and characterization of pluripotent human embryonic stem cells (ESCs) isolated from the inner cell mass of the blastocyst. Considerable interest has focused on the potential of ESCs to differentiate into various neural cell fates and thus, they have become useful as a source of cells for CNS transplantation and tissue engineering. However, derivation of specific cell types in some cases requires complicated and expensive schemes to generate a relatively small proportion of the desired cells and hence considerable effort is being devoted to overcome these obstacles.

Differentiation conditions to drive ESCs into a number of different neural cell types have been elucidated and these cells tested for possible therapeutic benefits in a number of neurodegenerative conditions; including Alzheimer's, Parkinson's, and Huntington's disease, spinal cord injury, TBI, stroke, as well as blinding ocular disease with varying success.

Generation of mesencephalic precursor cells and dopaminergic neurons derived from ESCs has been effective in recovering some motor function in models of Parkinson's disease (Bjorklund et al. 2002; Kim et al. 2002; Kriks et al. 2011; Yang et al. 2008). Implantation of ESC-derived neural precursors has also improved behavioral deficits in models of Huntington's disease (Song et al. 2007). In addition, transplantation of ESC-derived neural stem cells into the cortex of rodent models of Alzheimer's disease has resulted in some improvements in cognitive function (Wang et al. 2006). Embryonic stem cell-derived neural stem cells and neurons have also provided functional improvements in models of stroke (Daadi et al. 2008; Yanagisawa et al. 2006) and epilepsy (Cunningham et al. 2014). Preclinical studies using

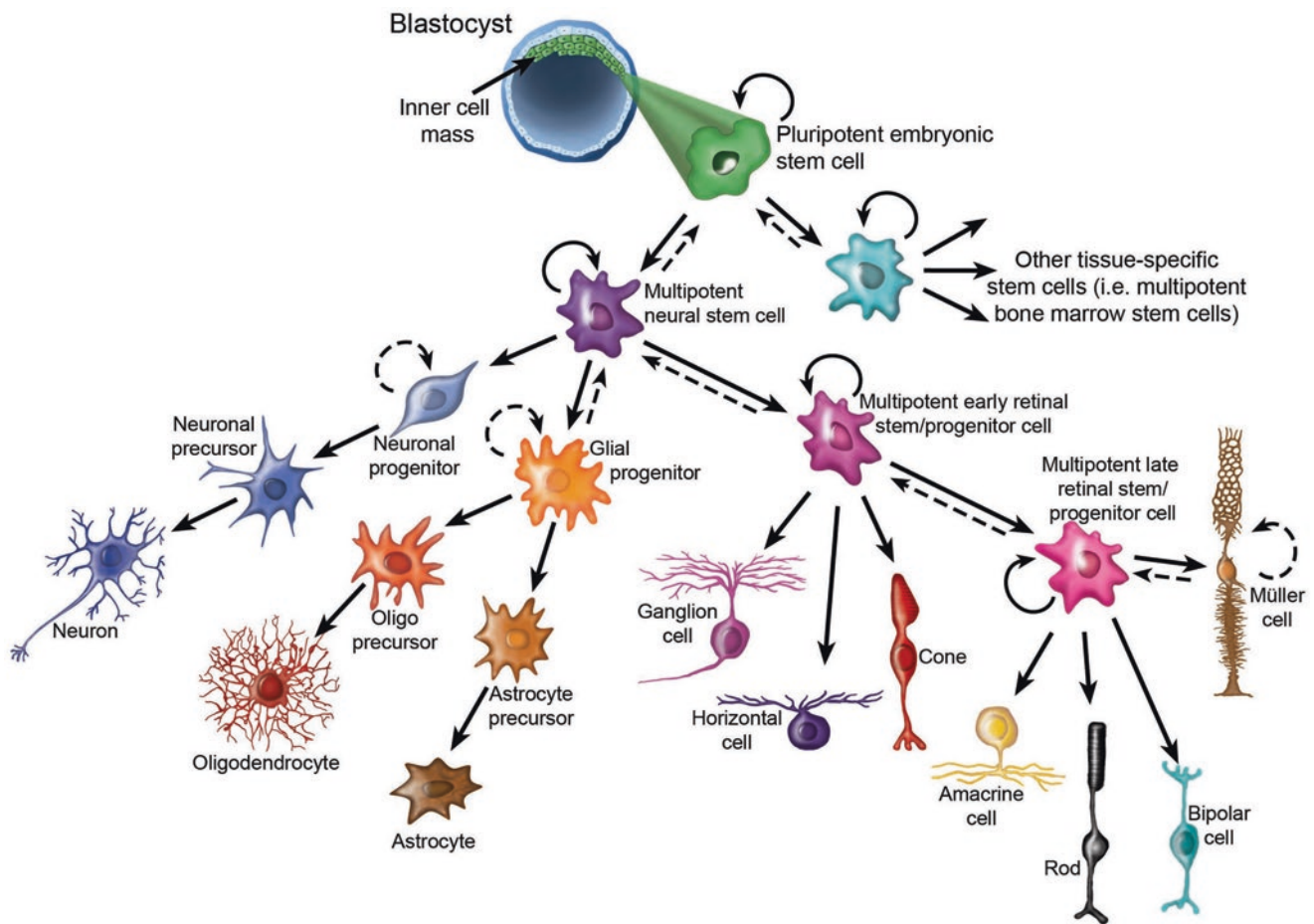


Fig. 47.2 Stem cell hierarchy and multipotency of neural and retinal stem cells. Pluripotent embryonic stem cells (ESC) are derived from the inner cell mass of a blastocyst (in humans the blastocyst stage is about 4–5 days post fertilization). Under appropriate culture conditions the ESCs can produce multipotent tissue specific stem cells from all three germ layers (ectoderm, mesoderm and endoderm). This illustration depicts the generation of differentiated cell types from multipotent neural and retinal stem cells. Multipotent neural stem cells generate the three major neural cell types present

within the central nervous system (CNS): neurons, oligodendrocytes and astrocytes. Multipotent retinal stem cells produce the seven major cell types found within the vertebrate retina which include: ganglion cells, horizontal cells, cone and rod photoreceptors, amacrine cells, bipolar cells and Müller glial cells. Arrows curling back onto the same cell represent the ability for self renewal, while reversed arrows represent possible reprogramming events (*Source*: Reprinted from Sandquist et al.(2016), Copyright 2016, with permission from Springer)

human ESC-derived OPC transplants in a rat model of spinal cord injury demonstrated significant improvement in locomotor activity and histological examination of the injured spinal cords showed improved axon survival and extensive remyelination around the axons (Okamura et al. 2007). Clinical trials are underway utilizing human ESC-derived OPC transplants for acute spinal cord injury. Promising clinical trials for wet age-related macular degeneration and Stargardt's macular dystrophy (Schwartz et al. 2012) are employing transplantation of retinal pigmented epithelial (RPE) cells derived from human ESCs. In this paradigm, the human ESCs are directed along an in vitro differentiation paradigm taking into consideration the normal developmental profile to generate RPE cells.

The stem cell debate: While ESCs offer hope for development of new therapies, their use in biomedical research is

still hotly debated and raises considerable ethical and political concerns. Many believe that ESC research may lead to discoveries of new medical treatments for a number of debilitating neurological conditions that would potentially alleviate the suffering of thousands of patients. However, it still necessitates the destruction of a human embryo. This debate as to whether the potential benefits of human ES research outweigh ethical objections still remains a critical moral dilemma. The recent discovery of reprogramming somatic cells to pluripotent stem cells (see below) may serve as an alternative method of deriving stem cells with minimal ethical concerns. Nevertheless, many biomedical researchers strongly support the continued study of all stem cell types to determine similarities and differences and it still remains unclear which cell source will be the most useful for cell replacement therapies in the future.

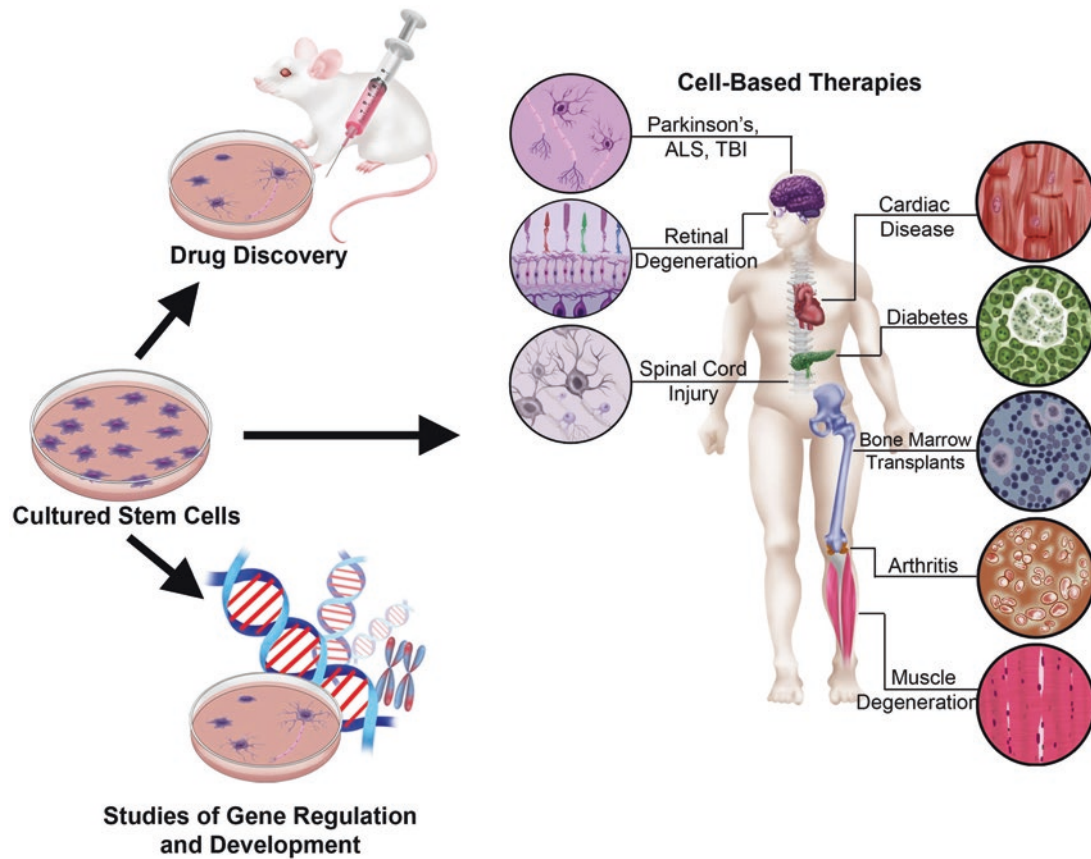


Fig. 47.3 Stem cell research. Recent advances in stem cell biology have revolutionized research opportunities in drug discovery. The ability to isolate and generate stem cell and somatic cell lines associated with specific diseases is providing effective in vitro models for pre-clinical testing. Stem cell research is also providing powerful tools to help decipher the molecular mechanisms of gene regulation and

development. Another important goal of stem cell research is to elucidate the pathways for generating specific cell types that can be used for cell transplantation to treat a variety of degenerative diseases and injuries (Source: Reprinted from Sandquist et al.(2016), Copyright 2016, with permission from Springer)

47.3.1.2 Induced Pluripotent Stem Cells

The development of induced pluripotent stem cell (iPSC) technology was pioneered by S. Yamanaka and colleagues (Takahashi et al. 2007; Takahashi and Yamanaka 2006) who demonstrated that adult cells can be converted to pluripotent stem cells, thus removing many of the ethical barriers associated with ESC research. Induced pluripotent stem cells are created by genetically reprogramming adult cells into an “embryonic-like” pluripotent state by forced expression of genes and factors involved in maintaining pluripotency. Induced pluripotent stem cells hold significant promise in the field of regenerative medicine as they have become useful tools for drug development, for modeling diseases and have considerable appeal to generate patient specific-pluripotent stem cells (Fig. 47.3). Furthermore, since iPSCs have the ability of self-renewal, they can propagate indefinitely, as well as differentiate into a variety of cell types in the body (including, neurons, glia, heart, pancreatic, liver cells, etc.), thus representing a single source of cells that could potentially be used to replace cells lost to disease or damage.

These unlimited supplies of patient-specific cells could be used to generate autologous transplants, thus reducing graft-host immune rejection. The iPSC technology has recently advanced to the stage of clinical trials for retinal degenerative conditions.

The field of stem cell biology has taken advantage of the normal developmental process of neural pattern formation of the CNS. Understanding and elucidating normal development has provided a rational strategy to differentiate pluripotent stem cells into specific neural cell types. Neuronal differentiation from iPSCs (as well as ESCs) is commonly achieved through a developmental profile that combines embryoid body cultures, retinoic acid stimulation and activation, and Sonic Hedgehog pathway agonists. Using specific factors that establish morphogenic gradients for patterning neural tissue, transcriptional regulation of modular patterns of CNS development has been key to differentiating pluripotent stem cells into neural precursors (Zhou et al. 2010), OPCs (Wang et al. 2013), and subsequently differentiating these progenitors and precursors into dopaminergic neurons

(Kriks et al. 2011), cortical neurons (Shi et al. 2012), motor neurons (Chambers et al. 2009; Karumbayaram et al. 2009) as well as retinal cells (Hirami et al. 2009). In addition, identification of synthetic small molecules has also been useful for neural differentiation by activating signaling pathways that promote differentiation into specific cell lineages (Skalova et al. 2015).

A number of studies have successfully directed differentiation of iPSCs into specific neuronal cell types for therapeutic applications (Lindvall 2012; Lindvall et al. 2012). Transplant of iPSC-derived dopaminergic neurons into animal models used for the study of Parkinson's disease have shown functional benefits (Kriks et al. 2011; Wernig et al. 2008). Re-myelination provided by OPCs derived from iPSCs has provided functional improvement in congenital hypomyelination disorder (Wang et al. 2013). Additionally, iPSC-derived neural progenitors transplanted into animal models of multiple sclerosis have ameliorated clinical features (Laterza et al. 2013). In animal models used to study ALS, iPSC-derived neural stem cell transplants has resulted

in improved neuromuscular function and significantly increased life span (Nizzardo et al. 2014). Neural stem cells from iPSCs have also shown benefits for stroke (Yuan et al. 2013). A number of studies employing iPSC-derived neural precursor transplants in models of spinal cord injury have resulted in functional improvements as well (Romanyuk et al. 2014).

The development and differentiation of iPSCs and ESCs into specific neural cell types is often a very complicated, time consuming and expensive process. Furthermore, an inherent attribute of pluripotent stem cells is their ability for teratoma formation. To avoid this complication, a number of studies have used strategies to predifferentiate the stem cells and also selectively remove any remaining pluripotent cells prior to transplantation (Brederlau et al. 2006; Dihne et al. 2006; Doi et al. 2012; Erdo et al. 2003; Wernig et al. 2008).

Another approach is to avoid these developmental complications by direct induction of somatic cells to neural cells, skipping a pluripotent state (Fig. 47.4). Direct conversion has been achieved by forced expression of genes for

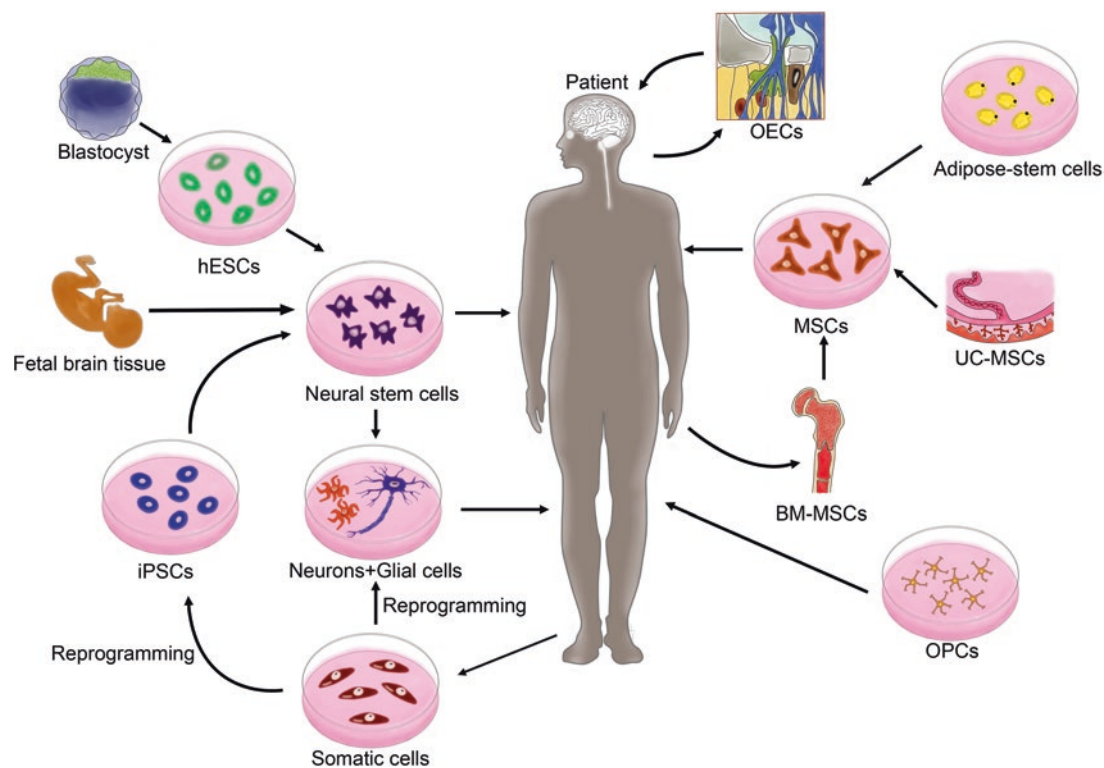


Fig. 47.4 Stem cell sources for CNS transplantation. *Left side of illustration:* Pluripotent hESCs isolated from the inner cell mass of a blastocyst are differentiated towards neural stem cells. Isolation of fetal CNS derived neural stem cells. iPSCs generated by reprogramming of somatic cells isolated from the patient. Direct conversion of somatic cells into neurons and/or glial cells. *Right side of illustration:* Different sources of MSCs for transplantation (BM-MSCs, UC-MSCs, and Adipose stem cells). Examples of other cell sources include OECs and OPCs. Arrows directed away from the patient

indicate potential autologous cell sources. Arrows directed toward the patient indicate cell sources for transplantation to the CNS. *Abbreviations:* hESCs human embryonic stem cells, iPSCs induced pluripotent stem cells, OECs olfactory ensheathing cells, MSCs mesenchymal stem cells, BM-MSCs bone marrow-derived mesenchymal stem cells, hESCs human embryonic stem cells, iPSCs, induced pluripotent stem cells, OECs olfactory ensheathing cells, UC-MSC umbilical cord mesenchymal stem cells, OPCs oligodendrocyte progenitor cells

differentiated neurons and by reprogramming somatic cells to a partially pluripotent state followed by culture with appropriate growth factors associated with neuronal differentiation (Matsui et al. 2014). A number of neuronal subtypes have been generated using these methods and have included, cholinergic neurons (Liu et al. 2013), dopaminergic neurons (Caiazzo et al. 2011; Pfisterer et al. 2011), and motor neurons (Son et al. 2011).

Human iPSCs have been praised as potential replacements for human ESCs. Although ESCs and iPSCs possess many similarities in differentiation potential, some studies suggest there are differences in gene expression and thus questions the equivalence of these two cell types (Ghosh et al. 2010; Narsinh et al. 2011). Comparison between different iPSC lines, as well as between different ESC lines has revealed differences in gene expression profiles, implicating differences in regulatory control (Chin et al. 2009; Wilson et al. 2009).

47.4 Multipotent Stem Cells for CNS Repair Strategies

Multipotent stem cells have the ability of self-renewal and can give rise to other cell types, but with more limited differentiation capacity when compared to pluripotent stem cells. Also referred to as somatic stem cells, multipotent stem cells tend to be tissue specific and are essentially committed to produce a particular set of cell types under normal conditions. Multipotent stem cells have been isolated and characterized from many organs and tissues, and are found in juvenile as well as in adult animals. In general, they appear to function to replenish cells in the various tissues throughout the life of the organism. Because of this cell replacement capacity, adult stem cells are of considerable interest in the field of regenerative medicine. Multipotent stem cells isolated from a number of different tissues have been used for CNS regeneration and repair strategies and include cells isolated from: brain, retina, bone marrow, adipose tissue, teeth, and skin (Fig. 47.4).

An important challenge has been to identify conditions or “factors” that trigger multipotent stem cells to generate specific cell types from that of their original tissue. Furthermore, it appears that at least some multipotent stem cells possess greater “plasticity” than originally thought, as they can be coaxed to go beyond original lineage boundaries for producing other cell types. However, the ability to isolate large quantities of somatic stem cells and to maintain them in a laboratory setting in order to generate therapeutic quantities of cells remains a significant issue. Although a number of challenges must be overcome before multipotent stem cells can be considered for routine use in the CNS, their potential benefits are numerous and they hold promise for treating neurodegenerative diseases and CNS injury (Fig. 47.4).

47.4.1 Neural Stem Cells

Neural stem cells are self-renewing multipotent stem cells that can differentiate into neurons, astrocytes and oligodendrocytes (Fig. 47.2). They have been isolated from the developing and adult CNS and have been produced in vitro through the differentiation of pluripotent stem cells (ESCs and iPSCs). Although significant progress has been made in understanding the biology of neural stem cells, much remains to be elucidated regarding identification of factors and conditions that regulate their fate.

Neural stem cells possess a number of characteristics that make them ideal vectors for CNS rescue and repair. Once isolated, they can be maintained long-term in vitro. In some cases neural stem cells isolated from the neonatal and adult mammalian brain have been maintained in culture as free-floating neurospheres in the presence of EGF and/or FGF-2 (Reynolds and Weiss 1992). Neural stem cells have also been isolated from the adult hippocampus and maintained in monolayer cultures grown on laminin or other ECM substrates in the presence of growth factors (Gage et al. 1998; Palmer et al. 1995). In addition, neural stem cells have also been isolated from the adult spinal cord (Weiss et al. 1996) and adult retinal stem cells from the pigmented ciliary margin (Tropepe et al. 2000).

Novel therapeutic applications are being developed that call for replacement of specific cell types, such as dopaminergic neurons for Parkinson’s disease, oligodendrocytes to treat spinal cord injury, and generating photoreceptors and RPE for blinding ocular disorders. One approach to manipulate neural stem cell fate has been through addition of neurotrophic and growth factors in vitro as well as in vivo. A number of different trophic factors, have been identified that can influence gene expression and ultimately neural stem cell differentiation potential. For example, as mentioned earlier, the mitogenic growth factors EGF and FGF-2 are important for maintenance and proliferation of neural stem cells. In addition, EGF converts transit-amplifying neurogenic precursors in the adult brain into multipotent stem cells (Doetsch 2003). Leukemia Inhibitory Factor (LIF) has been shown to support neural stem cell self-renewal in the adult brain (Bauer 2009; Bauer and Patterson 2006). A number of other studies have also demonstrated that multiple classes of trophic factors and growth factors, individually and especially in combinations, are also required for cell survival, avoidance of programmed cell death and for differentiation of neural stem cells and glial progenitors (Gallo and Deneen 2014; Urban and Guillemot 2014). Epigenetic regulation of transcription as a means of manipulating stem cell fate is a very active area of biomedical research (Fan et al. 2005). Micro-RNAs are an additional means to regulate transcription/translation and to influence neural stem cell fate (Hsieh et al. 2004; Kuwabara et al. 2004). The discovery of neural stem cells in the adult brain as

well as retina has encouraged research into their role during neurogenesis in the normal mature nervous system and following traumatic injury. Gaining a more thorough understanding of adult neurogenesis can contribute greatly to our knowledge of neurodegenerative diseases and to development of novel treatment platforms.

47.4.2 Non-neural Adult Stem Cells

It is well established that endogenous CNS stem cells, as well as those derived through differentiation of ESCs or iPSCs, can generate neurons, astrocytes, and oligodendrocytes. However, since neural stem cells are difficult to harvest from the adult brain, other stem cell sources are being evaluated as alternatives for cell-based therapies for neurological disorders. A number of somatic stem cell sources have been investigated for potential therapeutic applications in the CNS, including mesenchymal stem cells (MSCs), umbilical cord (blood) stem cells, adipose stem cells, and dental pulp stem cells, all sources that normally do not generate neural cell types (Fig. 47.4). In most cases, these non-neural adult stem cells appear to be capable of inducing endogenous neurogenesis. In addition to their neurogenic influence, these cell types may also have trophic effects that exert neuroprotective benefits when used to treat neurodegenerative conditions. However, evidence that they in fact differentiate into specific neural phenotypes is limited (Abraham and Verfaillie 2012; Stewart and Przyborski 2002).

This section will focus on MSCs as they have been isolated from a number of different tissue sources, including bone marrow, adipose tissue, liver, umbilical cord, and dental pulp tissues (Ding et al. 2011). They have the ability to differentiate into osteocytes, chondrocytes, myoblasts and adipocytes (Prockop 1997). Mesenchymal stem cells are emerging as particularly strong candidates for CNS cellular therapies (Fig. 47.4) due to a number of advantages. First, they are readily isolated from a number of tissue sources using well-established procedures. Second, they are easily maintained and expanded in culture and can be engineered to produce neurotrophic growth factors for long-term delivery of neuroprotective substances to the injured CNS (Harper et al. 2011; Levkovitch-Verbin et al. 2010; Sasaki et al. 2009). Third, MSCs can be isolated from the patient and therefore potentially serve as an autologous cell source. In addition, because MSCs express intermediate to low or negligible levels of MHC Class I or MHC Class II antigens, respectively, they are potentially suitable for use in allogeneic transplantation procedures and may thus avoid or minimize the need for aggressive immunosuppressive therapy (Aggarwal and Pittenger 2005; Le Blanc et al. 2003). Fourth, unlike pluripotent stem cells, MSCs have not been reported to form teratomas following transplantation. Another impor-

tant advantage is that there are few if any moral objections associated with the isolation and use of this adult stem cell population.

Mesenchymal stem cells have been used to treat CNS diseases in a number of preclinical models used to study Parkinson's disease, cerebral ischemia, TBI, retinal degeneration, and spinal cord injury. Benefits that have been reported include reduced lesion size, enhanced neuronal survival, axonal regeneration, and improved functional outcomes (Harper et al. 2011; Johnson et al. 2011; Kocsis 2009; Kurozumi et al. 2005; Abraham et al. 2009; Sasaki et al. 2009; Zhao et al. 2002; Lin et al. 2011). Although considerable optimism is associated with the use of MSCs for treatment of CNS neurological conditions, many challenges remain that must be overcome before their widespread clinical application.

In this section we have presented some examples of adult, non-neural stem cells that have been, or are currently, being evaluated for possible neural plasticity and neuroregenerative properties. While it is clear that true neural stem cells have the capacity to generate CNS neurons and glia, there is only limited evidence that non-neural stem cells generate CNS neural progeny. Nevertheless, many sources of non-neural adult stem cells, including bone marrow, adipose tissue, dental pulp and skin, appear to possess unique qualities that make them useful for consideration in cell-based therapies for CNS repair and regeneration. In some cases these cells appear to stimulate endogenous neurogenesis and in many cases can also provide neuroprotection when transplanted into the diseased or damaged brain, spinal cord, or retina.

47.4.3 Cellular Reprogramming Strategies

The biotechnological advancement of the ability to generate iPSCs has brought tremendous excitement to the field of regenerative medicine (Takahashi et al. 2007; Takahashi and Yamanaka 2006). However, the methodology is not without limitations and the reprogramming technology is sometimes inefficient, variable, time intensive and expensive. Furthermore, the pluripotent nature of the iPSCs can result in genetic instability, and when transplanted into animal models, the iPSCs may be tumorigenic. Therefore, it is important to develop alternative (or complementary) approaches for cellular reprogramming.

47.4.3.1 Directed Differentiation Towards Specific Cell Types

Cellular reprogramming is a strategy used to convert fully differentiated somatic cell types with defined functions, into another cell type. As a result, these reprogrammed cells display different characteristics and functions not normally associated with their original phenotype. In developing

reprogramming strategies clues are often collected from normal embryonic or early developmental processes. For example, normal embryonic cellular development is guided by an assortment of specific extracellular soluble factors, gradients of chemical cues and cell-to-cell interactions that ultimately lead to induction and activation of specific combinations of lineage-determining transcription factor pathways. By focusing on key developmental pathways and cell differentiation factors it has been possible to directly convert somatic cells from one fate/lineage to another using a small molecules approach (Zhang et al. 2012). This approach has identified a number of small molecules that serve to maintain self-renewal and to induce and/or facilitate cellular reprogramming (Zhang et al. 2012). Recently, epigenetic reprogramming of skin-derived fibroblasts into neural cells was achieved using a small molecules approach (Thoma et al. 2014). In this approach fibroblasts were initially subjected to conditions that enhance reversion towards a more “primitive” state. Under these conditions the cells were more susceptible to cell-fate changes, a process often referred to as transdifferentiation (Tursun et al. 2011). Potent neural inducing factors were then used to drive the cells towards proliferating neural progenitor cells, and finally the cells were induced towards specific neural cell fates. In this study the selection of small molecules was guided by developmental signaling molecules known to be involved in generation of the particular cell types. This gene-free approach for cellular reprogramming is likely to have applicability beyond generation of peripheral glial cells and may become an important strategy to generate CNS cell types as well (Thoma et al. 2014).

The direct conversion of somatic cells into neural stem/progenitor cells, as well as neurons has been demonstrated using viral delivery of transcription factors. Vierbuchen and colleagues (Vierbuchen et al. 2010; Vierbuchen and Wernig 2012) demonstrated that fibroblasts can be reprogrammed into functioning neurons using combinatorial expression of neural-lineage-specific transcription factors. They identified a combination of three factors, *Ascl1*, *Brn2* (also called *Pou3f2*) and *Myt1l*, which were sufficient to convert mouse embryonic and postnatal fibroblasts into functional neurons in vitro. Recently, the direct conversion of mouse fibroblasts into induced dopaminergic precursors was achieved by ectopic expression of a set of transcription factors that directly reprogram somatic cells into neuronal lineage-restricted progenitors (Tian et al. 2015). Following transplantation into a mouse Parkinson’s disease model (MPTP-lesioned mice), the induced precursors differentiated into dopaminergic neurons and were found to alleviate some motor deficits. Using direct reprogramming strategies to convert somatic cells into neurons or neuronal precursors has important applications for studies of neural development, patient-specific neurological disease modeling and CNS regenerative medicine.

47.4.3.2 Ex Vivo Gene Therapy Approaches for CNS Neuroprotection

Regeneration and repair strategies for the CNS should comprise a multi-factorial approach addressing a number of relevant issues, including optimization of survival and function of remaining CNS elements and modulation of trophic influences to promote neuroregeneration. Neurotrophic/growth factors are important candidates to augment neurorepair. Neurotrophic factors, including NGF, BDNF, and GDNF are essential during neuronal development and plasticity, and also can prevent apoptosis, enhance neuronal survival and facilitate axon regeneration. A number of approaches have been used for delivery of trophic factors to the damaged CNS. These have included direct bolus injections of the factors, incorporation into biodegradable polymer microparticles that slowly release the trophic factors, and gene therapy approaches. While each of these methodologies has advantages, a number of disadvantages necessitate the need to develop alternative strategies. Transplantation of genetically engineered cells can deliver therapeutic factors to the target site in the CNS and produce therapeutic effects at lower doses than are required with other means of delivery. In addition, a cell-based delivery system provides a potentially long-term delivery source.

Ex vivo gene transfer to a variety of different somatic cell types, including neural stem cells, MSCs, Schwann cells, and fibroblasts, prior to transplantation holds promise as cellular platforms for delivery of therapeutic factors. A number of viral vectors are available for *ex vivo* gene delivery, including, adeno-associated viral, adenoviral, retroviral and lentiviral vectors, each with their own advantages and disadvantages (Hendriks et al. 2004). A number of *in vitro* studies have demonstrated proof of concept for use of genetically engineered cells for delivery of trophic factors for neuroprotective strategies. Fibroblasts and MSCs engineered to secrete BDNF (Frim et al. 1994) enhanced the survival of retinal ganglion cells (RGCs) (Castillo et al. 1994) and also provided neuroprotection in a retinal cell line (RGC-5) when exposed to toxic cellular stressors such as glutamate or hydrogen peroxide (Harper et al. 2009), respectively. Mesenchymal stem cells engineered for delivery of BDNF have been transplanted into the striatum of a mouse model of Huntington’s disease and resulted in a decrease in behavioral symptoms typically associated with the disease (Dey et al. 2010). In addition, Harper and colleagues transplanted neurotrophic factor-engineered MSCs to deliver a constant, low level of BDNF and demonstrated that this approach had potential for functional and structural neuroprotection in an experimental rat model used to study glaucoma (Harper et al. 2011). Olfactory ensheathing cells (OECs) genetically modified to secrete GDNF were effective in promoting spinal cord repair. Another study demonstrated that human neural progenitor cells (hNPC) can be genetically modified to release

GDNF using an inducible promoter system (Behrstock et al. 2006). When transplanted into the striatum of mice the engineered cells migrated within the striatum, released physiologically relevant levels of GDNF, and enhanced host dopamine neuron survival and fiber outgrowth. Furthermore, these cells were found to survive and release GDNF for up to 3 months following transplantation into the aged monkey brain. These studies demonstrated the genetically modified hNPCs may be considered a safe and powerful cellular platform for delivering therapeutic factors to specific targets within the CNS for diseases such as Parkinson's.

Stem cells can be used to obtain a more thorough understanding of the complex events occurring during animal and human development. Gaining a more complete understanding of the genetic and molecular controls regulating developmental processes will likely yield information about how neurological diseases arise, and may provide novel strategies and targets for therapy (Fig. 47.3). Furthermore, stem cells (pluripotent and multipotent) and their derivatives are proving to be useful as model cellular systems for drug discovery and for toxicological bioassays (Fig. 47.3). This is particularly important for preclinical testing and verification of drug efficacy. Perhaps an especially significant application is for development and implementation of cell-based transplantation therapies (Fig. 47.3). Stem cells have the ability to generate specific cell types, providing a potentially renewable source of replacement cells that may be used to treat a variety of neurodegenerative conditions. Coupling stem cell biology with biocompatible materials provides a powerful toolbox for development and implementation of experimental strategies for CNS regenerative medicine.

47.5 Stem Cells and Bioengineering for CNS Repair Strategies

Materials fabricated from natural and synthetic polymers have been successfully used for fabrication of biomimetic 3-D scaffolding environments. A central goal of biomimetics in the context of CNS regenerative medicine is to imitate and model the extracellular matrix (ECM) and cellular microenvironment that support the growth and differentiation of native and transplanted cells. When developing materials and constructs for 3-D-scaffolds there are a number of important considerations regarding material selection, including biocompatibility, biodegradability, biological activity, mechanical properties, surface chemistry, cytotoxicity and trophic/growth factor binding capabilities (Dhandayuthapani et al. 2011; Sell et al. 2010; Zhu and Marchant 2011; Marti et al. 2012). Materials that are biodegradable, with chemistries permitting tunable degradation rates, and displaying mechanical and structural properties favoring cell adhesion, growth and proliferation will be important to neural regeneration

applications. This section provides a brief survey of potential stem cell-based biomaterial applications for CNS repair and regeneration. Recent review articles provide greater detail about specific biomaterials and applications in tissue engineering and CNS regeneration (Tam et al. 2014; Mallapragada et al. 2015; Sandquist et al. 2016).

A number of bioactive molecules have been used to enhance plasticity, stimulate neurogenesis, provide neuroprotection and promote CNS regeneration following neurological insults. Neurotrophins are a family of bioactive molecules that play a role in growth and survival of developing as well as maintenance of mature neurons. The neurotrophin family of trophic factors includes NGF, BDNF and Neurotrophin-3 and 4 (NT-3, NT-4). Other neurotrophic factors include CNTF, the GDNF family of ligands (GDNF, Nurturin, artemin and persephin), transforming growth factor β family, interleukin-6-related cytokines, FGF family members, as well as a number of other inductive signaling molecules involved in neural patterning (Reichardt 2006). Development of nano/microparticle systems capable of delivering and releasing such therapeutic molecules has revolutionized the field of drug delivery. Natural and synthetic polymers have been used to fabricate particulate systems for drug release due to their advantages, including improved drug stability, optimal encapsulation capacity, lower toxicity, fewer administration time points, ability to incorporate hydrophobic as well as hydrophilic drugs, sustained drug release capabilities, cellular uptake potential and ability to cross the blood-brain barrier (BBB) (Gelperina et al. 2005; De Jong and Borm 2008; Singh and Lillard 2009). The encapsulation of biologically active agents within biodegradable particulate systems has provided an effective means of overcoming numerous challenges encountered when developing strategies for drug and gene delivery, stem cell differentiation, imaging of live cells and encapsulated cell delivery systems for therapeutic proteins (Mudshinge et al. 2011; Norizadeh-Abbariki et al. 2014; Brustle et al. 2015; Ilie et al. 2012; Lee et al. 2012; Wang et al. 2010).

A number of studies have investigated delivery of neurotrophic growth factors including NGF, BDNF, CNTF and GDNF-encapsulated in poly-lactic co-glycolic acid (PLGA) microparticles as a therapeutic approach in animal models used for study of neurodegenerative conditions, including Alzheimer's, Parkinson's disease and retinal degeneration (Garbayo et al. 2009; Andrieu-Soler et al. 2005; Jollivet et al. 2004a, b; Péan et al. 2000; Grozdanic et al. 2010; Kyhn et al. 2009). Delivery of these trophic factors resulted in functional improvements and tissue regeneration, likely resulting from neuroprotective qualities associated with these factors. As a combination strategy, microparticles have also been incorporated into polymer or hydrogel scaffolds for better localization, sustained release and better clearance when implanted in vivo (Burdick et al. 2006). Other studies have encapsulated cells

along with growth factors within biofunctionalized polymer scaffolds as a promising strategy for neural regeneration (Wang et al. 2012).

A number of synthetic and natural polymers have been used to develop scaffolding platforms for organizing and delivering cells to the CNS. Nano and microfiber systems [such as poly(L-lactic acid) (PLLA), poly(D,L-lactide-co-glycolide) (PLGA), poly(ϵ -caprolactone) (PCL) etc.] have been extensively used to direct neural regeneration. Yang and colleagues produced aligned fibrous scaffolds using an electrospinning technique and found that the aligned nanofibers improved neurite outgrowth and enhanced differentiation of neural stem cells (Yang et al. 2005). Others have also used nano/microfiber systems to direct neurite growth and cellular alignment (Subramanian et al. 2011). In addition to providing physical alignment cues for cell growth, these types of scaffolds have also been functionalized and loaded with neurotrophic factors to promote neural regeneration (Chew et al. 2005).

Different types of scaffolds including films, nanotubes, gels, and porous materials have been used in developing neural regeneration strategies (Spivey et al. 2012; McCreedy and Sakiyama-Elbert 2012). Polymer-based porous films loaded with neurotrophic factors have been used to create gradients and surface modification used to create nano/micropatterns as conduits for neural regeneration (Tang et al. 2013; Kim et al. 2015). Scaffold systems bearing surface patterning have been used to provide topographic guidance cues to create regenerative platforms for a variety of cells, including astrocytes, neural stem cells, MSCs and Schwann cells (Roberts et al. 2014; Ho et al. 2015; McMurtrey 2014; Houchin-Ray et al. 2007; Rutkowski et al. 2004; Recknor et al. 2006; Sharma et al. 2016). Coupling stem cells and cellular reprogramming, along with 3-D scaffolds (i.e., nerve regeneration conduits) is a significant strategy to facilitate nerve regeneration. In many cases these bioengineering approaches have been effective in promoting peripheral nerve regeneration. However, with appropriate modifications these approaches will likely have significant benefits when applied to CNS regeneration as well.

47.6 Summary

The complexity and accessibility of the CNS has been a limitation in development of effective therapeutic interventions promoting neural regeneration. In addition, the environment of the damaged or diseased CNS is generally somewhat hostile and does not support extensive regeneration. The use of stem cells and/or neuroprotective factors coupled with biomaterials may provide a powerful approach to overcome many of these limitations.

This chapter has presented a number of approaches that may be effective in augmenting the limited repair capacity of

the mammalian CNS. In comparison to the CNS, the regenerative capacity of the PNS can be quite robust and a number of investigators have sought to identify differences in the hope of identifying molecular targets that may be exploited to enhance CNS regeneration. Another promising approach has been cell transplantation in order to replace lost cells within the damaged or diseased CNS. The choice of cell type will be critical in developing cell-based regeneration and repair strategies. For example, if stem cells (pluripotent or multipotent) are selected, the cells must be competent to generate specific cell types targeted for each disease. Furthermore, if pluripotent cells are selected, this increases the risk of teratoma formation. Cellular reprogramming or direct conversion of somatic cells into functional “induced” neuronal cell types may also be a feasible approach. However, there may be limitations on the number of cells that can be effectively generated using this methodology.

Once cells have been carefully and rigorously characterized *in vitro* they must be tested *in vivo* in preclinical animal models that best model the disease/condition. *In vivo* the transplanted cells must survive long-term at multiple CNS regions, integrate into existing host circuitry (for neurons), receive appropriate and specific regulatory inputs and elaborating axons that grow and form specific and functional synaptic contacts. Moreover, the transplanted cells must induce a clear long-term functional benefit. While established models may continue to prove beneficial, the generation of new genetic models, toxin-induced lesion models, or surgically induced lesion models should also be considered.

For cell transplantation, not only is choice of cell type important, but also the dose (number of cells) and location of cell transplants will need to be carefully considered. In addition to possible cell replacement, cell transplantation may also mediate repair and recovery through neuroprotection via release of neurotrophic growth factors, as well as the modification of the inflammatory environment. The successful outcome of cell transplants will also depend critically on the timing of the transplant in relation to the optimal stage of the disease at which the patient is likely to benefit the most from the therapy. It is important that preclinical studies also demonstrate protection of remaining neural elements using strategies that might be employed in future patients. Although histological evidence for cellular protection will be important, this must be accompanied with significant functional and behavioral improvements. To be clinically viable as a treatment option for CNS neurological conditions, the therapy must induce substantial amelioration of the neurological deficits without inducing significant deleterious side effects.

The combination of cellular-based therapies along with bioengineering approaches is an extremely powerful approach for regenerative medicine. Stem cell bioengineering provides a means of manipulating the molecular, physical and cellular environment in order to enhance regeneration

and repair of the CNS. Understanding and mimicking the complexity of the local environment, composed of a multitude of soluble and surface-associated signaling molecules, cell-to-cell contacts, cell-to-ECM, and local mechanical/physical cues will be important in regulating not only cell fate, but also cell behavior.

As the field of regenerative medicine in the CNS moves toward the future, it is apparent that systems-level approaches will help guide the field of stem cell bioengineering and the development of effective therapeutics. Understanding regulatory networks as well as the continued elucidation of neural connectomes will be essential for development of novel and effective neurotherapeutics for CNS regeneration and repair.

47.7 Review Questions

1. A prominent difference in the regenerative capacity of the mammalian PNS versus the CNS is associated with the:
 - (a) Neurons
 - (b) *Glial cell types*
 - (c) Stem cells
2. Stem cells are characterized by which of the following features?
 - (a) unlimited capacity for self-renew
 - (b) ability to differentiate into multiple cell types
 - (c) ability to differentiate into neurons only
 - (d) *a and b only*
 - (e) a and c only
3. A CNS neural stem cell can differentiate into which of the following cell types?
 - (a) neurons, muscle, and skin cells
 - (b) *neurons, astrocytes and oligodendrocytes*
 - (c) astrocytes, oligodendrocytes, and Schwann cells
 - (d) Schwann cells, motor neurons and microglia
4. What group of proteins plays a key role in controlling the program of developmental changes?
 - (a) motor proteins
 - (b) transporter proteins
 - (c) *transcription factors*
 - (d) synaptic proteins
 - (e) restriction endonucleases
5. A pluripotent stem cell is capable of:
 - (a) generating skin cells, but not nerve cells
 - (b) generating epidermal cells, but not mesoderm or endodermal cells
 - (c) generating cell types from skin and brain, but not muscle
 - (d) generating muscle and intestinal cells, but not neurons
 - (e) *generating neurons, skin, muscle and lung cells.*
6. Adult neural stem cells have been isolated from:
 - (a) muscle tissue and pancreatic tissue
 - (b) *the hippocampus and the subventricular zone*
 - (c) the pons and the superchiasmatic nucleus of the thalamus
 - (d) lewy bodies and neurofibrillary tangles
7. Which cell type produces factors that actively inhibit CNS regeneration?
 - (a) dorsal root ganglion neurons
 - (b) Schwann cells
 - (c) spinal reticular neurons
 - (d) *oligodendrocytes*
8. Myelin producing cells of the CNS are the:
 - (a) dorsal root ganglion neurons
 - (b) Schwann cells
 - (c) spinal reticular neurons
 - (d) *oligodendrocytes*
9. Myelin producing cells of the PNS are the:
 - (a) dorsal root ganglion neurons
 - (b) *Schwann cells*
 - (c) spinal reticular neurons
 - (d) oligodendrocytes
10. Which of the following are members of the neurotrophin family?
 - (a) CNS, PNS and ESC
 - (b) NCAM, N-cadherin, and laminin
 - (c) *NGF, BDNF and NT-3*
 - (d) IL-6, CNTF and FGF-2
11. Which of the following can be involved in cell-cell or cell-ECM interactions?
 - (a) PLLA, PLGA and PCL
 - (b) *NCAM, N-cadherin, and laminin*
 - (c) NGF, BDNF and NT-3
 - (d) IL-6, CNTF and FGF-2
12. Which of the following are myelin-associated molecules that inhibit axon regrowth?
 - (a) integrin receptors, neural cell adhesion molecule, and N-Cadherin
 - (b) PTEN/mTOR, JAK/STAT, and DLK/JNK
 - (c) fibroblast growth factor-2 and epidermal growth factor
 - (d) *chondroitin sulfate proteoglycans, Nogo-A, myelin-associated glycoprotein, and oligodendrocyte-myelin glycoprotein*
13. Generating dopaminergic neurons would likely be a very important consideration when developing stem cell-based therapies to treat which disease?
 - (a) glaucoma
 - (b) spinal muscular atrophy
 - (c) *Parkinson's disease*
 - (d) macular degeneration

14. Bone marrow-derived mesenchymal stem cells normally differentiate into which of the following cell types?
 - (a) islet cells, Schwann cells, and macrophages
 - (b) *osteocytes, chondrocytes, and adipocytes*
 - (c) neurons, astrocytes, and oligodendrocytes
 - (d) pigment cells, skin cells, and dorsal root ganglion cells
15. In designing an *in vitro* system to isolate and characterize neural stem cells which factors might you select to promote cell proliferation?
 - (a) NGF and/or BDNF
 - (b) CNTF and/or GDNF
 - (c) CSPG and/or LIF
 - (d) *EGF and/or FGF-2*
16. Integrin receptors typically bind ...
 - (a) neurotrophins
 - (b) *ECM molecules*
 - (c) myelin glycoproteins
 - (d) Nogo-A, but not Nogo-B
17. Characteristics of the neuroinflammatory response include invasion of circulating ...
 - (a) immune cells (oligos and astrocytes)
 - (b) MSCs
 - (c) *immune cells (macrophages and lymphocytes)*
 - (d) iPSCs
18. Adult neurogenesis in the SVZ results in production of new ...
 - (a) granule cells
 - (b) photoreceptor cells
 - (c) retinal pigment epithelial cells
 - (d) *olfactory interneurons*
19. Formation of teratomas is a potential risk associated with transplantation of ...
 - (a) MSCs
 - (b) *ESCs*
 - (c) neurons
 - (d) astrocytes
20. In theory, pluripotent cells are able to generate cells from which primary germ layers?
 - (a) *endoderm, mesoderm and ectoderm*
 - (b) periderm, endoderm and meristem
 - (c) mesencephalon, diencephalon and metencephalon

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New Generation of Adjuvants for Protection Against Disease and to Combat Bioterrorism

48

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Abstract

This chapter highlights the characteristics of traditional and non-traditional adjuvants, the advantages and disadvantages of their use in vaccines. It has so served to introduce the concept of molecular and host-derived adjuvants to improve immune outcomes while minimizing toxicities. A particular emphasis was placed on the generation of conformationally-restricted, response-selective agonists of the complement component C5a. These all appear to have therapeutic, manufacturing, and commercial potential. However, the development and use of such molecular adjuvants for vaccines represent only one approach put forward to meet the modern-day threats posed by antibiotic-resistant strains of bacteria and bioterrorism. It is clear that a well-organized and concerted worldwide effort that utilizes multiple approaches to develop novel adjuvant and vaccine designs will be required to overcome these growing threats to human and animal populations.

Keywords

Antigen presenting cells • C5a • Complement • EP67 • Molecular adjuvant • Vaccines

48.1 Introduction

Vaccines remain one of the greatest advances of medical history having led to the eradication of smallpox and the near-eradication of polio. Vaccination is principally a prophylactic approach with the objective of generating long-lived humoral and cellular immune responses that provide protection against invading pathogens. Traditionally, vaccines rely on the use of live-attenuated, killed, or inactivated forms of a whole pathogen as the target antigen (Ag); i.e., that entity against which an immune response is sought. Such whole-pathogen vaccines are effective in generating protective immune responses primarily due to their close resemblance to natural infections. However, these vaccines pose certain

issues including undesirable host reactions, difficulties associated with generating the target Ag in culture, the risk of pathogen reversion to virulence, and logistical challenges in maintaining a cold supply chain for vaccine distribution. These vaccines also pose significant safety issues in immunocompromised and neonate patients.

In contrast to whole-pathogen vaccines that make use of a whole attenuated pathogen as the target Ag, subunit vaccines are short, specific fragments of pathogen-associated proteins, which are safer, noninfectious, and immunologically more defined than traditional, whole pathogen vaccine approaches. Advances in Ag discovery technologies using reverse engineering of protective immune responses from pathogen-infected subjects (disease non-progressors) have heralded a new era in vaccine development for prevention of global diseases.

A prominent feature that underscores the current era of modern vaccinology is the ability to generate a huge variety of Ags by various synthetic chemistry and genetic engineering techniques. As a result, Ags now can be produced rapidly

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and in large quantities with well-defined structures and in highly purified forms—desirable attributes for their use in vaccines intended to generate Ag-specific immune responses in a diverse population. Unfortunately, the routine use of these Ags as integral components of vaccines is encumbered by their inherent lack of immunogenicity. Such vaccines, therefore, require the use of adjuvants in order to potentiate and focus the immune response to the Ag so that optimal immune outcome can be achieved. Thus, the discovery and development of new adjuvants is of growing importance to the design of vaccines capable of meeting the modern threats posed to human and animal populations by new or resurgent infectious and non-infectious diseases.

This chapter highlights recent trends in the development of adjuvants and, their current proposed mechanisms of action with an emphasis on adjuvants derived from components of the host (i.e., host-derived adjuvants) rather than the more commonly-used adjuvants derived from components of pathogens (i.e., pathogen-derived adjuvants).

48.2 Adjuvants: Vehicles and Immunomodulators

An adjuvant is any substance or any formulation of substances that enhances an immune response specific to an Ag. Adjuvants increase the magnitude, longevity and quality of Ag-specific immune responses. Apart from increasing vaccine effectiveness, adjuvants also reduce the amount and number of vaccine doses required to generate protective immune responses and thus contribute to increased patient compliance. As a general rule, adjuvants can be broadly classified into either vaccine delivery vehicles or immunomodulators. Often, both of these can be combined to further increase vaccine effectiveness. The use of adjuvants in vaccines to improve their effectiveness has traditionally been empirical. The necessity for new vaccine adjuvants arises from the shortcomings of currently approved adjuvants, which are incapable of eliciting immune responses that correlate with protection against different target pathogens. Rational design of vaccine adjuvants is essential for polarization of immune responses that correlate with protection against different pathogens and tumors.

48.2.1 Vehicles

Vaccine delivery vehicles are those adjuvants that help carry Ags to and retain them in proximity to lymphocytes and other auxiliary immune cells, particularly antigen presenting cells (APC), within various lymphoid tissues. Indeed, it is

this “depot” effect that is the defining mechanism of vehicle adjuvants. Classic examples of vehicles include liposomes, emulsions, proteosomes, and immunostimulating complexes (ISCOM) (Crouch et al. 2005). Typically, the final vaccine formulation is the Ag contained within the vehicle. Other examples of vaccines that utilize vehicles include formulations in which the Ag is covalently linked to biocompatible and biodegradable microparticles such as poly(D,L-lactide-coglycolide) (PLGA) (Diwan et al. 2004), nanoparticles, or adsorbed on the surface of carriers such as calcium and aluminum salts (phosphate or hydroxide), particularly alum, which is the only adjuvant currently approved for human use (Olive et al. 2001).

48.2.2 Immunomodulators

Immunomodulators are adjuvants defined by their ability to activate APCs and/or lymphocytes. Typically, this activation is characterized by the adjuvant-induced release of cytokines from lymphocytes and other auxiliary immune cells. Examples of immunomodulators include muramyl-dipeptide (MDP), monophosphoryl lipid A (MPL) (Ulrich and Myers 1995), lipopolysaccharide (LPS) (Johnson et al. 1956), bacterial cell membranes (Muhlradt et al. 1998), certain components of the complement system (Dempsey et al. 1996; Jacquier-Sarlin et al. 1995), cytokines (Afonso et al. 1994; Kurzawa et al. 1998), and oligonucleotide mimics of bacterial DNA. The typical vaccine formulation is one in which the Ag is admixed with the immunomodulator. Variations on this theme include those in which the Ag is covalently linked to the immunomodulator with the rationale that elements of the generalized immune response invoked by the adjuvant might be more directed to the Ag. Some examples of this approach include the incorporation of lipids, lipopolysaccharides, and lipoamino acids into peptide Ags by synthetic methods (Defoort et al. 1992; Martinon et al. 1992; Metzger et al. 1991; Olive et al. 2001; Wiesmuller et al. 1992) and the genetic fusion of chemokines/cytokines to protein Ags (Biragyn et al. 1999).

It should be pointed out that this classification of adjuvants is based more on historical observations than on strict mechanisms of action. In fact, there are several examples of adjuvants belonging to the vehicle family that act as immunomodulators. A noteworthy example of this is the saponins, which are extracted from the plant *Quillaja saponaria* and are used to create emulsions into which Ags are added. Over the years, a variety of chemical modifications of saponins have been used as adjuvants and all appear to stimulate the release of various T helper 1 (Th1) and T helper 2 (Th2) cytokines in addition to their vehicle-like mode of action (Shi et al. 2005).

Finally, there are many examples of vaccine formulations in which individual adjuvants are used in combination with others as a way of optimizing immune efficiency (efficacy) and outcome. Despite the current emphasis placed on evaluating the wide variety of adjuvants in experimental use today and the myriad of possible combinations and formulations, no particular adjuvant or adjuvant system has emerged as an ideal product. Also, given the variation within a data set in which adjuvants are employed, it appears that the effectiveness of an adjuvant or adjuvant system is best evaluated on a case-by-case basis.

48.3 Adjuvant Development: General Concepts

As stated above, the principal objective for the use of an adjuvant in a vaccine is to potentiate immune response to an Ag of minimal immunogenicity. How this potentiation is achieved varies from adjuvant-to-adjuvant and in many cases, the precise mechanism of action is unknown. However, as a rule, immune potentiation is accomplished by the ability of the adjuvant to induce a variety of non-specific activities within the innate arm of the immune system. Once activated, the innate branch of immunity, particularly the complement system, orchestrates the various humoral and cell-mediated responses that operate within and between the innate and acquired arms. The result is a generalized activation and potentiation of the immune system in response to the adjuvant with the hope that this generalized immune priming will allow for a more effective processing and recognition of the Ag contained within the vaccine.

48.3.1 Pathogen-Derived Adjuvants

The manner by which an adjuvant induces this immune priming emanates from the exquisite sensitivity of the mammalian immune system to detect the presence of bacteria and bacterial components and respond accordingly in a rapid and vigorous manner. The initial response to these bacterial signals is largely a function of the innate branch of the immune system, which has evolved under continual selective pressure from pathogenic bacteria and other disease-causing microorganisms. It is not surprising, therefore, that the majority of adjuvants in use today are composed of various molecules, components, and structures derived from bacteria. The use of these adjuvants in vaccines, therefore, provides the bacterial signals to which the innate arm of the immune system vigorously reacts, which results in the priming/potentiation of the immune system.

However, this rapid and vigorous innate response to the bacteria-like signals induced by such adjuvants can result in an overly aggressive and misdirected immune response that is accompanied by undesirable side-effects such as anaphylaxis, fever, injection site granulomas or rashes, and local or systemic inflammation. Another drawback is that the adjuvant tends to induce a generalized immune response with little immune specificity directed to the Ag of interest. Also, immunity that might be directed to the Ag can be masked due to the magnitude of the innate response to the adjuvant. In an attempt to overcome these drawbacks, there is a vigorous and concerted research effort to develop modifications of bacterial-derived adjuvants such that they retain their ability to induce innate responses to bacterial signals, but minimize or eliminate the deleterious inflammatory side-effects that can accompany such signals. Notable examples of such efforts include the many chemical and structural modifications developed and tested on MPL (Ulrich and Myers 1995) the saponins (Shi et al. 2005), and cholera toxin (Lycke 2005).

48.3.2 Host-Derived Adjuvants

Against the backdrop of the various issues that accompany the use of adjuvants derived from bacterial components, a new area of adjuvant discovery focuses on the development of adjuvants derived from naturally-occurring components of host innate immunity. Such host-derived adjuvants would provide the required signaling necessary for engagement of the various cellular components of innate immunity, but with a degree of immunologic control, focus, and tolerance that may not be achieved with pathogen-derived components. In this regard, the use of cytokines as immuno-stimulatory adjuvants would qualify as host-derived adjuvants. However, the mode of action of cytokines encompasses a wide range of cellular effects within the innate and acquired arms of immunity and their use is effectively limited by issues of immunologic focus, control, and tolerance. Nonetheless, the idea of developing an adjuvant from a natural component involved in induction of host innate/acquired immunity is of keen interest. As mentioned above, the major issue will be in engineering an element of specificity such that the host-derived adjuvant induces/activates a single (or limited) pathway of immune activation and/or engages a single (or limited) population of immune cells.

48.3.3 Mucosal Adjuvants

Most of the currently approved vaccine adjuvants are intended for use in systemic immunizations. Mucosal sur-

faces, however, are the major portal for the entry of a variety of pathogens and mucosal immunization generates humoral and cellular immune responses at the mucosal surface, which restricts the entry of invading pathogens. Furthermore, generating immune response at one mucosal site is capable of generating similar immune responses at distinct mucosal sites. This phenomenon, known as the “common mucosal immune system”, can be exploited to induce mucosal immune responses at inaccessible mucosal sites like the vagina and rectum, by immunizing through accessible mucosal routes such as the oral and intranasal cavity (Courtney 2010). Mucosal immunization is also the most feasible, non-invasive, and risk-free route for mass-immunization regimens.

In contrast to systemic immunization, which operates in a sterile milieu, mucosal immunization is confronted with the challenge of generating immune responses after being administered at a non-sterile site, which is constantly exposed to large quantities of harmless food and environmental antigens (Tsuji and Kosaka 2008). Mucosal immunizations also need to overcome the immune tolerance mechanisms at the mucosal site, which prevent unnecessary detrimental immune responses against non-pathogens and food antigens. While induction of immune tolerance is essential for preventing immune responses against food and environmental antigens, it necessitates the need for strong immunostimulatory components in the mucosal vaccines to generate immune responses.

Many adjuvant systems used for systemic vaccine applications such as oil-in-water emulsions and mineral salt adjuvants may not be suitable for mucosal applications. Adjuvants delivered at mucosal surfaces need to overcome the inherent tolerogenicity associated with mucosal tissues. The effective mucosal adjuvants currently being used in animal models include CpG, bacterial toxins such as cholera toxin (CT), heat-labile enterotoxins, cytokines/chemokines and VLPs (Courtney 2010; Huang et al. 2008; Lycke 2012; Vajdy and Lycke 1992; van Ginkel et al. 2005). Most of these compounds function as potent adjuvants through activation of antigen-presenting dendritic cells (DCs). Of the existing mucosal adjuvants, bacterial toxin adjuvants are most potent in generating humoral and cellular immune responses, but are very toxic and not fit for human use. CpG and other toll like receptor agonists have shown promise as mucosal adjuvants but are generally associated with inflammation. Whether pathogen-derived or host-derived, safe yet potent adjuvants that overcome mucosal tolerance and generate long-lived protective immune responses in mucosal and systemic immune responses are necessary for vaccination approaches that target pathogens, which infect through mucosal surfaces.

48.3.4 Molecular Adjuvants

The need for an adjuvant capable of inducing a robust *and* Ag-specific immune response accompanied with minimal inflammatory side-effects has given rise to a new class of adjuvants, which have come to be known as molecular adjuvants (Dempsey et al. 1996). The rationale for a molecular adjuvant is based on the notion that a single, well-defined and well-characterized molecular entity might be better in inducing an immune response more directed to the Ag with fewer non-specific side effects, particularly when the Ag is attached directly to the molecular adjuvant. Thus, a molecular adjuvant can be defined as a single molecular entity that targets an Ag to the cells of the immune system responsible for Ag processing and presentation (APCs) and/or activates specific pathways of Ag processing and presentation within these cells.

48.4 Development a Host-Derived, Molecular, and Humoral Adjuvant

As described above, “traditional” adjuvants typically are derived from bacterial components in order to provide the bacteria signals necessary for the activation and potentiation of the innate arm of the immune system. One of the first biological systems of that which becomes activated in response to these bacterial signals is the complement system. Consequently, complement and the various components that comprise the complement system provide a rich source from which a variety of host-derived adjuvants could be developed. This section describes the development of a host-derived adjuvant that was developed from a natural component of human complement known as C5a. This host-derived adjuvant embodies the attributes of a molecular adjuvant as well as a humoral adjuvant, examples of which are discussed below.

48.4.1 Complement

Complement is a plasma system comprised of interrelated proteases arranged in a cascade fashion that becomes activated in response to bacterial signals and Ags associated with bacteria. The role of complement is twofold: (1) to serve as an initial, first line of defense to invading microorganisms and (2) to enhance systemic host defense by activating and orchestrating the various humoral and cell-mediated responses within the acquired and innate arms of the immune system necessary for the effective elimination of the microorganism.

The “first line of defense” is accomplished through the generation of the membrane attack complex (MAC), which is assembled at the end of the complement cascade from the components generated by the proteolytic steps along the cascade. The MAC is directly involved in lysing the membranes of foreign microorganisms.

Secondly, the various humoral and cell-mediated responses operating at the interface of the innate and acquired arms of the immune system are activated and coordinated by pharmacologically active components that are released at certain steps along the complement cascade. These proteolytic byproducts induce the various humoral and cell-mediated aspects of the innate and acquired arms of immunity, all of which are necessary for the concerted elimination of the microorganism.

Principally because of this latter reason, these pharmacologically active components of complement are attractive as molecular adjuvants. This is because their use has the potential of enhancing specific pathways of the innate and acquired immune processing and subsequent recognition of an Ag as opposed to the broader and less Ag-focused activation of the innate system in response to bacterial signals that come from adjuvants derived from bacterial components.

48.4.2 Adjuvants Derived from Complement Components: Host-Derived Adjuvants

Components of complement have been used as molecular adjuvants to enhance Ag-specific immune responses to model Ags, specifically C3b, C4b (Arvieux et al. 1988; Jacquier-Sarlin et al. 1995), and C3d (Dempsey et al. 1996). These components were chosen primarily for their opsonic properties; i.e., their ability to bind to and coat the surface of a microorganism rendering it more susceptible to immune cell uptake via phagocytosis. The rationale, therefore, was that an Ag covalently attached to these complement fragments similarly would be rendered more susceptible for uptake by APCs and, consequently, would be more effectively processed and presented by the APC. In all cases, the immunogenicity of the model Ag in the Ag-complement vaccine complex was markedly enhanced relative to the Ag alone, suggesting that the complement fragment acted as a molecular adjuvant by enhancing Ag uptake by APCs and, in turn, the ability of the APC to process and present the Ag. These studies clearly demonstrate the immunologic potential of using complement components as molecular adjuvants in the design of vaccines.

48.4.3 The Anaphylatoxins

An important group of pharmacologically active byproducts of complement activation are the anaphylatoxins C3a, C4a, and C5a, which are small (74–76 residue) fragments cleaved from the larger, parent complement components C3, C4, and C5, respectively. The principal roles of the anaphylatoxins are to recruit inflammatory cells and lymphocytes to sites of tissue injury and infection and to then activate these cells’ various effector responses once recruited (Hugli 1981). However, the anaphylatoxins play important roles in the activation and regulation of humoral and cell-mediated responses to Ags due to their ability to modulate various humoral and cell-mediated activities between the innate and acquired arms of the immune system (Dempsey et al. 1996; Mastellos et al. 2005; Morgan 1986). Consequently, the anaphylatoxins are attractive as molecular adjuvants, which may be capable of invoking Ag-specific humoral and/or cell-mediated immune responses.

Unfortunately, the anaphylatoxins are also potent inflammatory mediators and their use as molecular adjuvants would surely be accompanied by local and/or systemic inflammatory side effects. Also, under certain conditions, the anaphylatoxins appear to downregulate immune function (Kawamoto et al. 2004; Morgan 1986). Thus, while the anaphylatoxins have desirable immune stimulatory activities that make them attractive for use as molecular adjuvants, they carry with them the potential for adverse inflammatory side effects and immune downregulation. Therefore, immune stimulatory activities must be enhanced at the expense of its inflammatory activities and any tendency to downregulate immune response.

48.4.4 Immunostimulatory and Inflammatory Properties of C5a

C5a is a 74-residue polypeptide with pleiotropic biological functions (Fig. 48.1) including smooth muscle contraction, vascular permeability, mast cell degranulation, and chemotaxis. C5a potentiates antibody and Ag-induced T cell proliferative responses in vitro possibly through activation of T helper cells (Morgan et al. 1983). Direct stimulation of mouse and human dendritic cells with C5a enhances the expression of MHC class II, co-stimulatory molecules CD80, CD86, CD40 and CD54, and induces secretion of cytokines of Th1 phenotype (Zaal et al. 2013; Rudilla et al. 2012; Li et al. 2012). Also, C5a induces the release of a variety of immunoregulatory cytokines from APCs including IL-1, IL-6, IL-8, IL-12, TNF α , and IFN γ (Buchner et al. 1995;

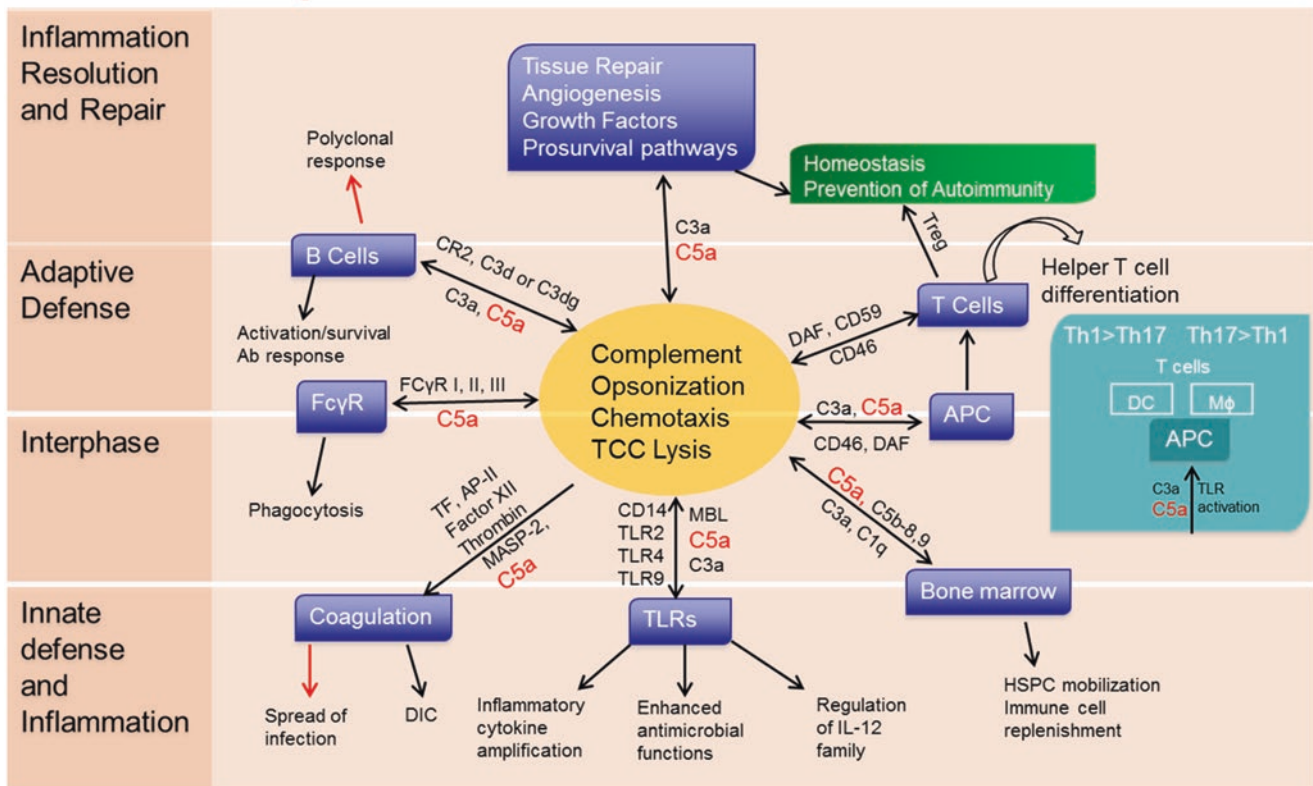


Fig. 48.1 Role of complement C5a in host defense and homeostasis. Adapted from Ricklin et al. (2010)

Goodman et al. 1982; Okusawa et al. 1987; Wetsel 1995b) (Gasque et al. 1995; Scholz et al. 1990); (Ember et al. 1994); (Floreni et al. 2007). C5a-mediated stimulation of DCs also decreased intracellular production of cAMP, which is a negative regulator of DC activation and function (Li et al. 2012). Furthermore, C5a interaction with C5aR activates PI3K, ERK1/2, NF-κB signaling pathways, which positively regulates Ag uptake, presentation, and secretion of proinflammatory cytokines by DCs (Li et al. 2012; Zaal et al. 2013). C5a was also shown to exhibit both synergistic as well as antagonistic crosstalk with toll like receptors (TLRs) in functional modulation of DCs (Li et al. 2012; Zaal et al. 2013). C5a decreased TLR4-mediated IL-12 secretion by DCs, but significantly increased secretion of other Th1 cytokines and CXCL16 involved in NK cell activation. C5aR ligation with C5a was shown to be essential for generation of protective anti-viral CD8⁺ T cells (Kim et al. 2004). In contrast, genetic ablation or pharmacologic blocking of C5aR signaling predominantly generated Th2 and regulatory T cell responses (Kim et al. 2004). Furthermore, previous studies found that blockade of the C5aR impaired the ability of immune system to generate memory CD4⁺ T cells (Moulton et al. 2007). Also, DCs stimulated with C5a showed increased expression of multiple co-stimulatory molecules, migration to the drain-

ing lymph nodes, and strong interaction with antigen-specific naïve T cells (Li et al. 2012).

Besides immunostimulatory properties that promote Ag presentation and functional immune responses, C5a also possesses highly proinflammatory properties. These include its potent chemotactic activities for the recruitment of inflammatory cells to sites of tissue injury and infection, its ability to induce smooth muscle contraction (Hugli et al. 1987; Shin et al. 1968), increase vascular permeability (Hugli 1990; Hugli et al. 1981; Hugli and Muller-Eberhard 1978), and induce the release of a variety of secondary inflammatory mediators such as histamine, lysosomal enzymes, and vasoactive eicosanoids from responsive cells such as mast cells, neutrophils, eosinophils, and macrophages (Drapeau et al. 1993; Goldstein et al. 1974; Johnson et al. 1975; Lundberg et al. 1987; Schorlemmer et al. 1976).

These biologic responses (inflammation and immunomodulation) are induced by the ligation of C5a to its specific, high affinity receptor (C5aR/CD88) that is expressed on the surface of the C5a-responsive cell(s). Traditionally, C5aR expression has been viewed as being limited to cells of myeloid origin such as neutrophils, monocytes, mast cells, and eosinophils (Wetsel 1995a). However, it is now known that a variety of cells of non-

myeloid origin express C5aRs. These include hepatocytes (Buchner et al. 1995), astrocytes (Gasque et al. 1995), bronchial epithelial cells (Floreani et al. 1998), epithelial cells of the gut and kidney (Wetsel 1995a), and osteoblasts (Pobanz et al. 2000).

As the inflammatory properties of C5a have been the motivation behind the development of C5a antagonists, so the immunostimulatory properties of C5a been the motivating factor for the development of agonists of C5a that can be used as surrogates of the natural factor for enhancing humoral and cell-mediated immune responses; i.e., as a molecular adjuvant. Using such agonists of C5a as a molecular adjuvant, however, requires developing response-selective agonists that are capable of invoking C5a-like immunostimulatory properties at the expense of C5a-like inflammatory properties.

48.4.5 C5a: Structure-Function Considerations

Human C5a is a 74-residue glycopolypeptide that is comprised of two important structural and functional domains. The first is the well-ordered N-terminal core domain comprised of residues 1–63, which is primarily involved in the recognition and binding of C5aRs (Mollison et al. 1989; Zuiderweg et al. 1989). The second is the C-terminal domain, residues 64–74, which extends from the N-terminal core as a finger-like projection. C5a_{65–74} (ISHKDMQLGR) is a region of considerable backbone flexibility and poorly-defined structure, yet the inflammatory *and* immunostimulatory activities characteristic of natural C5a reside in this small C-terminal stretch (Ember et al. 1992, 1994; Morgan et al. 1992).

Flexibility in the C-terminal region of C5a (C5a_{65–74} or ISHKDMQLGR) is a dominant feature of natural C5a. It may be that this flexibility allows the C5aR the ability to induce a unique conformation in this region of the C5a ligand that is conducive to the expression of biologic activity characteristic of that particular C5aR-bearing cell. It may be, in fact, that a biologically active conformation in this effector region of C5a responsible for activity in one type of C5aR-bearing cell may not be the ideal conformation for the expression of activity in another. This suggests that by employing a synthetic strategy in which the flexibility in this effector region of C5a is restricted, one might bias certain conformational features that are important for the expression of certain types of C5aR-mediated activities; i.e., a response-selective agonist. Such a specific and stabilized conformation, when presented to the C5aR, would be more likely to interact with those C5aRs that are capable of accommodating this particular conformation.

48.4.6 Conformationally Restricted Analogues of C5a_{65–74}

Over the years, our laboratory has generated a library of analogues of C5a_{65–74} in which backbone flexibility was restricted by specific amino acid substitutions. This was done with the goal of biasing certain conformational features that might be helpful in the search for biologically relevant conformations responsible for the induction of C5a-like immunostimulatory activities versus those responsible for C5a-like inflammation.

Backbone flexibility was restricted by introducing three principal types of residue substitutions within C5a_{65–74}: (1) Pro substitutions to restrict ϕ angle flexibility and to narrow the range of sterically allowed backbone conformations in the pre-proline residue (Misicka et al. 1991), (2) Ala substitutions to evaluate the biological importance of the side-chains in the peptide, and (3) D-residue substitutions to assess the contribution of stereoisomeric arrangements. The use of such peptide modifications has an additional advantage in that Pro, Ala, and D-residues occupy well-defined regions of sterically-allowed Ramachandran space (Hruby et al. 1991). Thus, an evaluation of the changes made in the biological activity of these conformationally restricted analogues of C5a_{65–74} can provide information about the specific types of backbone conformation features that are important to specific types of biological activity.

Using this approach, we showed that one peptide from this library, C5a_{65–74} Y65,F67,P69,P71,D-Ala73 or YSFKPMPLaR (EP54) exhibited about 10 % of the potency of natural C5a for its ability to induce the release of spasmogenic eicosanoids from human macrophages, but only about 0.1 % of C5a activity in its ability to induce the release of β -glucuronidase from human neutrophils (Finch et al. 1997) and was reflected by a corresponding difference in binding affinity to the C5aRs expressed on these cells (Finch et al. 1997; Vogen et al. 1999).

Structural analysis of YSFKPMPLaR indicated the presence of unique conformational features that appear to be responsible for the selective accommodation by C5aRs expressed on antigen presenting cells (APC) relative to C5aRs expressed on inflammatory neutrophils (Vogen et al. 1999). These backbone conformational features identified in EP54 were then used to guide the generation of other analogues designed to enhance their biologic importance. Prominent among these new analogues is EP67 (YSFKDMP(MeL)aR), which contains an N-methylated Leu residue to enhance backbone elongation in a crucial region of the peptide, which was deemed biologically important from the NMR structure of EP54. Indeed, EP67 was shown to exhibit more potency and macrophage/dendritic cell selectivity than EP54 (Vogen et al. 2001; Taylor et al. 2001).

48.4.7 Use of EP54 and EP67 as Host-Derived, Molecular and Humoral Adjuvants

48.4.7.1 Generation of Ag-Specific Humoral Immune Responses

In one study (Tempero et al. 1997), EP54 was used to induce Ag-specific antibody (Ab) responses to a B cell epitope derived from the human mucin type 1 glycoprotein (MUC1). A vaccine was generated by covalently attaching the MUC1 epitope (YKQGGFLGL) to the N-terminus of EP54 (YKQGGFLGLYSFKPMPLaR) and mice immunized with this vaccine generated high Ab titers specific for the MUC1 epitope. These anti-epitope Abs cross-reacted with MUC1 protein expressed on the surface of a transfected pancreatic cell line, indicating that the anti-YKQGGFLGL Abs recognized the epitope within intact whole MUC1 protein. Also, Ab isotypes generated by this EP54-containing vaccine were IgM, IgG2a, and IgG2b, contrasted with those generated by an analogous KLH construct, which were IgM and IgG1. This suggests that the EP54-containing vaccine induced an Ab class switch characteristic of a Th1-like response and, consequently, generated Ab with isotypes distinct from the traditional KLH construct. Over the years, we have used epitope-EP54 vaccine constructs to immunize rats, hamsters, rabbits, and cattle to a wide variety of epitopes of various lengths (8–35 residues) and small molecules such as nicotine (Sanderson et al. 2003) and methamphetamine (Duryee et al. 2009).

In similar fashion, EP67-containing vaccines have been used to generate Ab responses to peptide epitopes and proteins in mouse models. Mice immunized with these EP67-containing vaccines generated significant levels of Ag-specific IgM, IgG2a, and IgG2b responses (Morgan et al. 2009). Also, mice immunized with intact fungal spores from an attenuated strain of *Coccidioides posadasii* conjugated to EP67 increased the protective efficacy by a corresponding increase in Ag-specific IgG1 and IgG2a Ab responses (Hung et al. 2012).

48.4.7.2 Generation of Ag-Specific Cell-Mediated Immune Responses

Cytotoxic T cell responses (CTL) (CD4⁺ and CD8⁺) in murine models have been generated with EP54- and EP67-containing vaccines designed by their covalent attachment to peptide epitopes from the hepatitis B surface antigen (Ulrich et al. 2000), a tandem repeat region of MUC1 expressed on pancreatic cancer cells (Pisarev et al. 2005), and the gp70 glycoprotein from RAW 117-H10 lymphoma cells (Kollessery et al. 2011). Also, EP67-containing vaccines have been generated in which the target Ag is an entire protein using straightforward conjugation methods (Phillips et al. 2009). Using this approach, we have shown protective Th1 and Th17 CTL responses to *Coccidioides posadasii* in mice immunized with vaccines in which EP67 was conju-

gated to intact fungal spores from an attenuated strain of *C. posadasii* (Hung et al. 2012).

48.4.7.3 Generation of Ag-Specific Mucosal Immune Responses

Simple vaccines were made by the covalent conjugation of CD8⁺ T cell epitopes from proteins expressed on murine cytomegalovirus (MCMV) to EP67. Intranasal immunization generated functional mucosal and systemic epitope-specific CD8⁺ T cells that increased protection against primary mucosal infection with MCMV. Moreover, a large proportion of these CD8⁺ T cells formed a pool of long-lived memory CD8⁺ T cells that respond strongly upon reinfection even in the absence of CD4⁺ Th1 help. This is supported by findings that EP67-based CTL peptides generated a higher proportion of epitope-specific mucosal (lungs) and systemic (spleen) CD8a⁺/CD44⁺ cells with cell surface phenotype (CD127⁺/KLRG1[−]) associated long-lived memory (Karuturi 2014). In addition to CD127 and KLRG1, we found that EP67-based CTL peptides generated epitope-specific CD8⁺ T cells with an increased expression of CD27, which is a strong predictor of proliferative recall responses upon Ag encounter (Ochsenbein et al. 2004; Xiao et al. 2008).

The immunization results in mice demonstrate the unique ability of complement C5a-derived/host-derived adjuvants for use in systemic as well as mucosal vaccine applications. In particular, EP67 because of its significantly reduced inflammatory properties may be acceptable as a universal adjuvant for various vaccine applications. More recent studies have shown the ability of EP67 in protecting against influenza and MRSA infections via induction of innate immunity from its selective engagement of C5aR-bearing APCs (Sanderson et al. 2012; Hanke et al. 2013). Taken together, these studies indicate that EP67 has significant immunostimulatory properties that are conducive to desirable innate and acquired immune outcomes (Table 48.1).

48.5 Mechanism of Action

The immune outcomes obtained from EP54- and EP67-induced humoral and cell-mediated responses suggest that these adjuvants deliver both Ag and stimulatory signals to C5aR-bearing APCs—events that are consistent with the mechanism shown in Fig. 48.2.

This proposed mechanism is supported by confocal microscopy where fluorescent-labeled EP54 and fluorescent-labeled B and T cell epitopes attached to EP54 were rapidly internalized by human DCs (Hegde et al. 2008). Twenty-four hours later, the internalized epitopes were presented in the context of HLA-I and HLA-II determinants on the DC surface. Thus, EP54 (and EP67) both targets and activates APCs suggesting properties of both a vehicle and an immunomodulatory adjuvant.

Table 48.1 Vaccine adjuvant properties of EP54 and EP67

Vaccine construct	Species	Route	Immune response	Ref
Peptide epitope from MUC1 glycoprotein conjugated to EP54	C57BL6 and BALB/c mice	i.p.	Higher titers of IgG2b, IgG2c and IgM Abs in sera reactive against recombinant MUC1 and MUC1 expressing cell line	Tempero et al. (1997)
CTL peptide epitope derived from Hepatitis B surface Antigen (HBsAg) conjugated to EP54	BALB/c mice	s.c	Ag-specific CD8+ CTL responses against murine P815S target cells expressing an H-2L ^d restricted CTL epitope of the HBsAg	Ulrich et al. (2000)
Nicotine hapten conjugated to EP54	Sprague–Dawley rats	i.p.	Nicotine-specific Abs capable of attenuating nicotine-induced behavioral effects	Sanderson et al. (2003)
Methamphetamine (meth) hapten conjugated to EP54	Rats	s.c/i.p.	Meth-specific Abs in sera capable of altering meth self-administration	Duryee et al. (2009)
Ovalbumin (OVA) conjugated to EP67	C57BL6 BALB/c mice	i.p.	OVA-specific Th1-like Ab class switch and OVA-specific proliferative responses in splenocytes	Morgan et al. (2009)
OVA conjugated to EP67	Young/aged mice	i.p.	Higher Ag-specific humoral responses compared to alum adjuvanted OVA	Morgan et al. (2010)
rPrp1, a protein from cell wall of <i>coccidioides</i> conjugated to EP67	Young/aged mice	i.p.	Higher Ag-specific humoral responses compared alum and CpG adjuvanted rPrp1	Morgan et al. (2010)
Peptide epitope derived from gp70 glycoprotein conjugated to EP54 and EP67	BALB/c mice	s.c	Ag-specific CTL responses and protection against metastatic RAW117-H10 lymphoma lethal challenge	Kollessery et al. (2011)
Live spores of attenuated vaccine strain of <i>Coccidioides posadasii</i> conjugated to EP67	BALB/c mice	s.c	Increased protective efficacy of live vaccine by increasing Ag-IgG1, and IgG2a, and Th1, and Th17 immune responses	Hung et al. (2012)

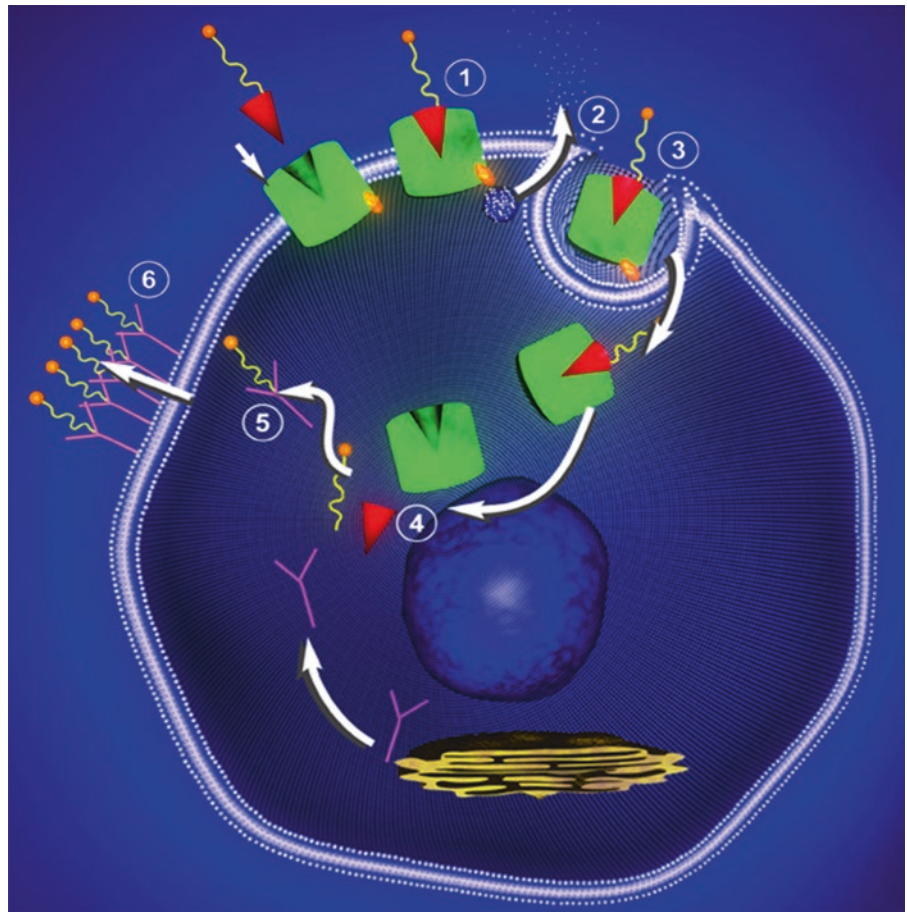
48.6 Review Questions

1. Describe the principal means by which a molecular adjuvant such as EP67 induces innate and/or acquired immune responses.
2. As we age, our immune response profile gradually shifts toward T helper type II (Th2) dominated responses. With this in mind, describe how a molecular adjuvant like EP67 could be advantageous in the generation of vaccines for the growing elderly population.
3. Describe how a molecular adjuvant like EP67 could accommodate the need for the rapid generation of vaccines to counter new and emerging diseases and bioterrorism attacks.

48.7 Answers

1. By the selective engagement and activation of C5aR-bearing antigen presenting cells.
2. This is because EP67, unlike other conventional adjuvants, drives a T helper type I (Th1) dominated immune response, which could restore an immunologically advantageous Th1/Th2 balance that is lost during the aging process.
3. EP67 can be rapidly generated in large quantities by standard peptide synthesis. Vaccines can be rapidly made in large quantities by the simple covalent conjugation of the target antigen to the N-terminus of EP67. Manufacturing of such vaccines is rapid and inexpensive, requires no

Fig. 48.2 Mechanism of action for EP54 and EP67 molecular adjuvants



additives or preservatives, and vaccination can be accomplished on a large scale by the administration of the EP67-based vaccine dissolved merely in water.

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James Hilaire and Howard E. Gendelman

Abstract

Recent advances in molecular neuroscience and neuropharmacology have improved our knowledge of the intricate mechanisms that define and treat infectious, immune and degenerative disorders of the brain and spinal cord. As our knowledge expands, treatment of central nervous system diseases still remains a challenge. One obstacle is drug delivery. Indeed, designed to protect the brain from toxic molecules, the blood–brain barrier also limits therapeutic drug entry into the brain. The dynamic balance between the exclusion and therapeutic delivery remains a key component to improve disease outcomes.

Keywords

Blood–brain barrier • Carrier-mediated transport • Central nervous system • GLUT1 • LAT1 • Lipidization • Prodrugs

49.1 Introduction

A range of disease combating strategies has emerged to circumvent the blood–brain barrier's (BBB) restrictive nature. This includes neurosurgery to permit direct intra-cerebroventricular (ICV) infusion, convection-enhanced delivery (CED), and the use of intra-cerebral drug implants (Pardridge 2005). While each are designed to improve drug delivery to sites of central nervous system (CNS) injury and infection, their applications remain limited (Pardridge 2005; Gabathuler 2010). Osmotic or biochemical disruptions cause aberration in endothelial cell tight junctions (Patel et al. 2009). Less invasive platforms such as chemical or biologically modified drugs and nanoparticle delivery systems show, perhaps, the most significant promise (Juillerat-Jeanneret 2008). This chapter will focus on chemical modifications and prodrugs, and their ability to provide enhanced BBB penetrance for improved treatment of CNS disorders.

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49.2 Prodrugs

49.2.1 Background

The development of new treatments for CNS disorders has paralleled the discovery of molecular mechanisms for disease. This information has enabled medicinal chemists to rationally design drugs at the molecular level. In all, drug(s) developed for CNS disease have been broad based and not limited by design or functionality. A drug's major factor in success rests in its ability to cross the BBB and retain function in the brain's microenvironment. It is estimated that 98 % of marketed small molecules do not penetrate the brain and as such severely limit the effectiveness and therapeutic potential (Pardridge 2003). In order to improve the drug's therapeutic benefit, medicinal chemists commonly depend on prodrugs. A prodrug, while often not itself efficacious, is a bioreversible chemical modification to an active compound (Albert 1958). Therefore, chemical or enzymatic cleavage is required in vivo to yield an active molecule. Reversible chemical modification provides the flexibility of adding chemical moieties specifically designed to improve barrier penetrance without negatively affecting the drug's action. Presently, between 7 and 10 % of marketed drugs can be

identified as prodrugs (Stella 2004; Zawilska et al. 2013). Prodrugs are designed to improve upon suboptimal characteristics of existing pharmaceuticals. A compound's solubility, absorption, metabolism, tissue targeting, as well as pharmacokinetics and biodistribution, can be improved (Rautio et al. 2008). Prodrug synthesis is dependent on the presence of reactive functional groups such as hydroxyl, amine, carboxylic, or phosphate moieties, which can generate a range of bonds including ester, amide, carbamates, ethers, and phosphates (Rautio et al. 2008). Bond stability prominently affects systemic release kinetics; therefore, the design of prodrugs requires knowledge of the biological system and desired pharmacological outcome. For example, an esterified prodrug designed to target the CNS may cleave in systemic circulation before reaching the brain; therefore, alternative, more stable bonds such as an amide may be considered to affect brain penetrance.

49.3 CNS Drug Delivery

49.3.1 Blood–Brain Barrier

The restrictive nature of the BBB protects potentially harmful molecules from accumulating within the CNS. Consisting of a continuous layer of endothelial cells connected by tight junctions, the BBB separates circulating blood from the extracellular fluid within the brain (Brightman 1977). The BBB is not limited to molecular restriction, but police the entry of essential molecules, thereby regulating the homeostatic, nutritive, and immune environments of the brain (Quan and Banks 2007; Banks 2009). However, the BBB limits the delivery of drugs aimed to function within the brain, thus providing challenges for drug delivery. Low molecular weight molecules with high lipophilicity commonly cross the BBB by transmembrane diffusion, while other molecules can access the brain by using saturable transport systems (Oldendorf 1974; Banks 2009). Furthermore, drugs that enter the brain are subject to be released out by drug efflux transporters belonging to the ATP-binding cassette (ABC), such as P-glycoprotein (P-gp) and multidrug resistance protein 2 (MRP2) (Fig. 49.1) (Juliano and Ling 1976; Miller et al. 2000; Loscher and Potschka 2005). Thus, using an intricate knowledge of BBB physiology, researchers have developed a number of prodrug strategies designed to specifically enhance brain penetrance by transmembrane diffusion, efflux pump inhibition, or exploiting endogenous transport systems on the surface of the BBB.

49.3.2 CNS Targeted Prodrugs

Multiple prodrug techniques can increase CNS drug penetrance. Specifically, prodrugs containing cleavable chemical moieties improve BBB permeability. Lipidization

approaches, as well as carrier and receptor mediated targeting, can improve the translocation across the blood–brain interface.

49.3.2.1 Lipidization

CNS drug access by transmembrane diffusion is directly linked to its molecular weight, lipophilicity, and the presence of ionizable functional groups at physiological pH (Patel et al. 2009). Specifically, for compounds under 400 Da, passive diffusion across the BBB linearly correlates with an increase in log P (Levin 1980). To improve penetrance of water-soluble molecules, an approach termed lipidization is employed. Here, hydrophobic moieties block hydrogen bonding functional groups on the existing molecule, thus increasing lipophilicity and potentiating brain entry. A prime example of lipidization is morphine and its subsequent prodrugs, codeine and heroin. Morphine, an opioid analgesic, has limited CNS accumulation upon systemic administration (Letrent et al. 1999). Methyl etherification of morphine's phenol group generates codeine. Addition of the methyl ether imparts increased hydrophobicity when compared to morphine, largely due to reduced hydrogen bonding capability (Fig. 49.2). Additionally, heroin, which is even more lipophilic than codeine, results from the diesterification of morphine. Oldendorf et al. studied the ability of morphine and its subsequent prodrugs, codeine and heroin, to enter the CNS after direct injection into the rat common carotid artery. Compared with morphine, codeine and heroin displayed enhanced BBB penetrance, exemplified by 12 and 34 fold increases in CNS uptake respectively (Oldendorf et al. 1972). Therefore, heroin more efficiently enters the CNS, is quickly metabolized in the brain to 6-acetyl morphine, and then subsequently converted to morphine where it can engage opioid receptors (Begley 2004).

Research focusing on chlorambucil, an alkylating agent used clinically for the treatment of chronic lymphocytic leukemia, as a new treatment for brain cancer was hindered by its lack of CNS uptake impart due to its ionization at physiological pH (Greig et al. 1988, 1990). In order to increase CNS permeability, seven lipophilic chloromethyl esters were synthesized. Following intravenous administration, no significant differences in brain uptake were observed between the ester prodrugs and chlorambucil (Greig et al. 1990). The lack of significant egress into the brain is due to ester bond cleavage during systemic circulation before the prodrug is able to reach the brain (Greig et al. 1990). To improve prodrug stability, a chlorambucil tertiary butyl ester prodrug was synthesized. Studies confirmed the tertiary butyl ester prodrug of chlorambucil produced peak brain levels three times that of chlorambucil, while extending its CNS half-life two times (Greig et al. 1990).

Theoretically, lipidization should improve passive diffusion across the BBB and promote CNS accumulation. Unfortunately, the complexity of CNS delivery exemplified

Systemic Circulation

Brain

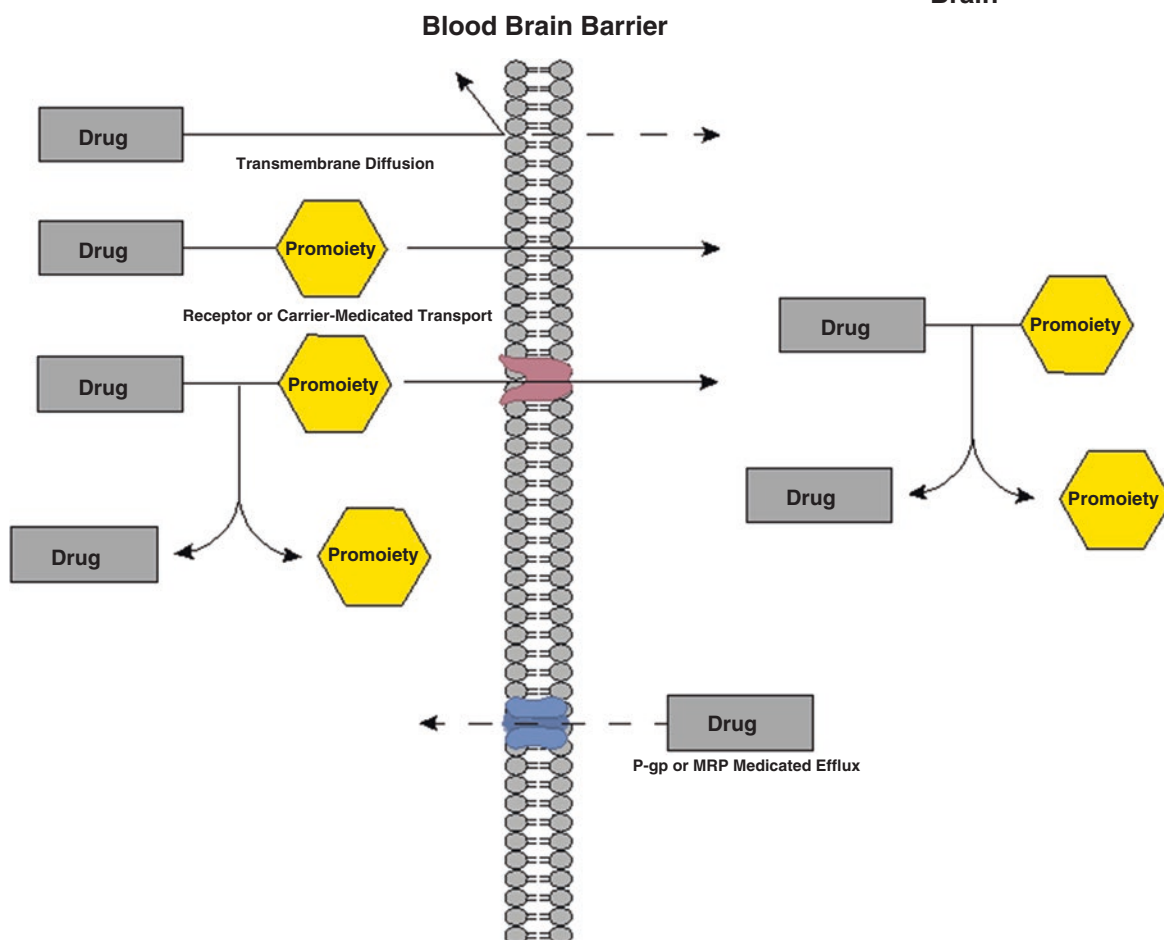


Fig. 49.1 Prodrug Brain Delivery. Drugs entering systemic circulation may gain access to the brain by transmembrane diffusion. However, most compounds do not possess the necessary physiochemical properties to readily transverse the BBB. Therefore, prodrugs are synthesized to improve brain penetrance. They consist of a drug bioreversibly conjugated to a promoiety, transiently changing the compounds physicochemical properties. Lipidization is used to promote transmembrane diffusion across the BBB. Additionally, when the promoiety is a ligand

for a receptor or carrier on the BBB surface, the prodrug can enter the CNS via receptor or carrier-mediated transport. Prodrugs that lack stability may be cleaved in circulation before reaching the brain. Once in the brain, prodrugs are cleaved to yield the active drug and promoiety. Based upon prodrug design, the promoiety can be inert or have a pharmacological effect of its own. Newly released drugs can now act within the brain for pharmaceutical effect or be pumped out of the CNS by efflux transporters such as P-gp or MRPs

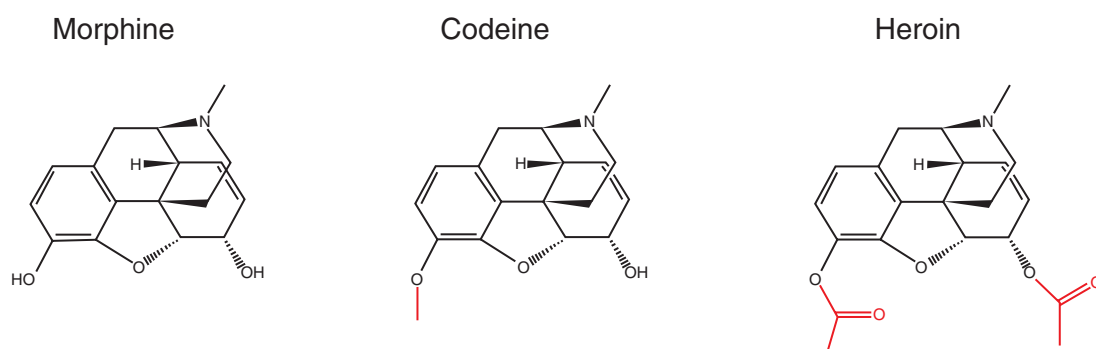


Fig. 49.2 Lipidization of Morphine. Methyl etherification of morphine yields codeine, while diesterification of results in heroin. Both heroin and codeine are more hydrophobic than morphine. Each can readily

cross the BBB and subsequently release morphine for physiological effects within the brain.

in the development of chlorambucil esters is more involved. Indeed, the lack of site-specific delivery limits the effectiveness of lipidization. Simply increasing hydrophobicity will enhance diffusion across all biological membranes. Therefore, increased CNS uptake may be offset by suboptimal pharmacokinetics due to increased plasma clearance, reductions in the area under the curve (AUC), and enhancement on protein binding (Pardridge 2007). Furthermore, lipidization requires the addition of hydrophobic moieties. Molecular weight increases may compromise CNS entry, as for molecules >400 Da, BBB permeability no longer linearly correlates with lipid solubility (Fischer et al. 1998; Pardridge 2007). Therefore, other methods to achieve BBB penetration and appreciable CNS accumulation were explored.

49.3.2.2 Biological Systems of CNS Entry

Certainly, notwithstanding the improved CNS delivery of morphine by its subsequent prodrugs, codeine and heroin, lipidization has limited utility in terms of CNS drug delivery. In addition to passive diffusion of lipophilic small molecules across the BBB, endogenous transport systems located within the brain capillary endothelium allow specific molecules such as amino acids, nutrients, glucose, and vitamins to transverse the BBB (Peura et al. 2013). Thus, bioreversible conjugation of ligands targeting specific endogenous transport systems along the BBB presents a prodrug strategy for CNS delivery (Pavan and Dalpiaz 2011). Research focused on targeting a multitude of receptors or transporters on the surface of the BBB will be expanded in more detail.

49.3.2.3 LAT1

CNS-specific drug delivery is designed to improve drug accumulation within the brain, as well as potentially lower side effects resulting from non-specific distribution. Harnessing endogenous receptors and transporters at the surface of the BBB to enhance prodrug delivery may significantly improve CNS drug delivery. Neutral L-amino acids enter the brain via the large neutral amino acid transporter (LAT1) expressed on capillary endothelial cells of the BBB (Pardridge 1983; Peura et al. 2011). In fact, LAT1 expressed at the BBB has a higher affinity for amino acid substrates than LAT subtypes found in peripheral tissues. This makes LAT1 an ideal target for prodrug delivery to the CNS (Pardridge 1983; Boado et al. 1999). Prodrugs consisting of bioreversible ligands capable of specifically binding LAT1, facilitates entry into the CNS that subsequently metabolize in the brain yielding the active drug is a model investigated by numerous research groups as presented below.

Dopamine prodrugs were studied extensively for Parkinson's disease (PD). CNS delivery of dopamine is difficult due to its ionization at physiological pH, as well as its metabolism after oral administration (Chemuturi and Donovan 2007). Dopamine needs to accumulate in the brain

to exert its therapeutic effect. Currently, levodopa (L-DOPA), which uses LAT1 transporters at the BBB, is a clinically relevant prodrug of dopamine (Gomes and Soares-da-Silva 1999; Peura et al. 2013). L-DOPA is a metabolic precursor converted to dopamine in the brain by aromatic L-amino acid decarboxylase (AAAD). In actuality, a significant portion of administered L-DOPA is converted to dopamine in peripheral circulation; therefore, carbidopa (CD), an inhibitor of AAAD is frequently administered in conjunction with L-DOPA (Lewitt 2008; Huttunen and Rautio 2011). Prodrugs that can more effectively deliver dopamine to the brain than L-DOPA are needed and are currently under development.

To this end, attaching a meta-substituted carboxylic acid analogue of phenylalanine to dopamine a LAT1-targeted prodrug was developed. In situ brain perfusion studies resulted in significant prodrug brain uptake with evidence of carrier-mediated passage of the BBB (Peura et al. 2013). Unfortunately, intravenous administration of the prodrug did not result in improved accumulation of dopamine within the brain; in fact it performed unfavorably in comparison to L-dopa treatment. These results indicate that while LAT1 may be a promising target for CNS drug delivery, more research is needed to translate this targeting into a therapeutic benefit.

L-tyrosine, a natural ligand for LAT1, has been used to generate prodrugs designed for CNS delivery. Particularly, a prodrug of the nonsteroidal anti-inflammatory drug Ketoprofen was synthesized using L-tyrosine as a bioreversible targeting ligand for LAT1 (Gynther et al. 2008). The carboxylic acid functional group of Ketoprofen was derivatized to form a labile ester bond with the phenolic hydroxyl group of L-tyrosine (Gynther et al. 2008). In situ perfusion resulted in enhanced brain uptake of the prodrug, which could be reduced with the co-administration of a 2-aminobicyclo (2,2,1) heptane-2-carboxylic acid (BCH), a specific LAT1 substrate (Gynther et al. 2008). Systemic administration yielded inadequate CNS uptake, potentially due to the instability of the ester bond linkage, exemplifying the challenges associated with CNS drug delivery. An optimal CNS-targeted prodrug must exhibit stability in systemic circulation, impart BBB receptor specificity and binding affinity, gain entrance into the brain parenchyma, and release the active compound, while avoiding efflux pumps to achieve appreciable brain drug concentrations. Directed at improving systemic stability, L-lysine targeted ketoprofen prodrug was synthesized using an amide bond. Amide bonds are inherently more stable than esters due to their reduced susceptibility to chemical or enzymatic cleavage (Vig et al. 2013). Intravenous injection yielded CNS uptake although without a significant difference compared with Ketoprofen (Gynther et al. 2010). Future research may focus on using this targeting strategy with compounds that exhibit less endogenous uptake in order to achieve desired differences in CNS uptake.

49.3.2.4 GLUT1

Physiological brain function is dependent on energy provided by blood borne glucose traversing the BBB via the glucose transporter (GLUT1), which is located on the luminal and abluminal side of BBB endothelial cells (Mueckler et al. 1985; Farrell and Pardridge 1991; Maher et al. 1994). GLUT1 allows for movement of glucose down its concentration gradient and in terms of the CNS glucose is predominantly carried from the blood, across the BBB, to the extracellular fluid of the brain (Carruthers 1990; Regina et al. 1997). Therefore, GLUT1 is an excellent candidate for prodrug CNS delivery.

The idea of conjugating d-glucose to a hydrophilic drug in order to improve its CNS drug delivery was done through GLUT1-mediated mechanisms. Models of d-glucose bound to GLUT1 suggest the hydroxyl group bonded to carbon 6 of glucose do not form any hydrogen bonds that would affect binding affinity (Mueckler and Makepeace 2008). Therefore, ketoprofen and indomethacin prodrugs were synthesized conjugating d-glucose via the hydroxyl group through carbon 6 and forming an ester linkage (Gynther et al. 2009). Rat perfusion models determined both prodrugs were able to cross the BBB and enter brain tissue in a GLUT1 dependent manner (Gynther et al. 2009). The relative weakness of the ester bond may limit effectiveness once administered into systemic circulation, as cleavage may occur before entering the CNS. An L-ascorbic acid (vitamin C) targeted prodrug of ibuprofen was made aimed at improving CNS targeting. GLUT1 is able to transport dehydroascorbic acid, the oxidized form of ascorbic acid, into the brain where it is converted to vitamin C (Rumsey et al. 1997). In addition; L-ascorbic acid also enters the brain by Na⁺-dependent vitamin C transporter (SVCT₂) at the BBB surface (Tsukaguchi et al. 1999). Given that GLUT1 and SVCT₂ are bidirectional transporters, thereby permitting movement of the prodrug out of the brain, a prodrug was developed that could be “locked in” via thiamine disulfide system until ibuprofen release (Zhao and Keating 2007; Portugal et al. 2009; Zhao et al. 2014). Intravenous injections yielded significantly higher CNS concentrations of ibuprofen in prodrug-treated animals compared with those treated with ibuprofen (Zhao et al. 2014). These results suggest CNS targeting combined with a “lock-in” system has potential to improve CNS delivery of BBB impermeable compounds.

49.3.3 Alzheimer's Disease

Alzheimer's disease (AD), which affects more than 24 million people worldwide, is characterized by progressive decline in memory and cognitive function leading to the onset of dementia (Whitehouse et al. 1982; Ferri et al. 2005). Currently, there is no AD cure. Post-mortem studies have

identified two prominent pathological hallmarks of AD. Extracellular senile plaques comprised of aggregated amyloid β -peptide, and intracellular neurofibrillary tangles constituted of hyperphosphorylated tau that contribute to neuronal cell death (Braak and Braak 1991; Lee et al. 2001). Numerous postulations exist regarding AD etiology, such as the cholinergic, genetic, amyloid, and tau hypotheses (Hardy and Allsop 1991; Francis et al. 1999; Mudher and Lovestone 2002). In an attempt to improve the patients overall quality of life, traditional AD therapy aspires to improve the cognitive and behavioral symptoms associated with AD. Clinical drug treatments for AD act within the CNS and, therefore, need to cross the BBB. Numerous prodrugs are under development to provide specific CNS delivery for AD therapeutics, reducing peripheral toxicity, the cause of numerous unwanted side effects.

Currently, moderate AD patients are often prescribed an acetylcholinesterase (AChE) inhibitor (donepezil) in combination with *N*-methyl-D-aspartic acid (NMDA) receptor antagonist memantine (Savonenko et al. 2012). This treatment aims to ameliorate the pathological reduction of acetylcholine caused by loss of cholinergic neurons in the basal forebrain, which is in part responsible for characteristic cognitive deficits in AD (Terry and Buccafusco 2003). Dose escalation during advanced stages of AD cause an increase in peripheral toxicities associated with AChE inhibitors, thereby deeming CNS selective drug development paramount (Bohn et al. 2015). To this end, Bohn et al. developed a “bio-oxidizable prodrug” of a cyclic 1,4-dihydroquinoline carbamate derivative of AChE inhibitor rivastigmine. Once in the CNS, this prodrug converts to a quinolinium salt, which then acts as a pseudoirreversible AChE inhibitor (Bohn et al. 2015). Consequently, the AChE inhibitor becomes “locked in” within the CNS due to the presence of a permanent positive charge (Bohn et al. 2015). Additionally, the positive charge on the active drug promotes rapid systemic elimination, thereby reducing peripheral toxicity. Thus, by analyzing markers of peripheral (salivation) and central (hypothermia and shivering) cholinergic activity, it was deemed the prodrugs improve the balance between peripheral stability and central activation (Bohn et al. 2015). Therefore, this strategy of “bio-oxidizable” prodrug may be used to develop new AChE inhibitors with fewer side effects in the treatment of (AD).

Huperzine A (HUP A), a novel and selective AChE inhibitor, has been found to inhibit AChE more effectively than donepezil and rivastigmine (Cheng et al. 1996; Jia et al. 2013). Jia et al. developed a HUP A prodrug termed ZT-1 in order to improve CNS delivery and reduce its peripheral side effects (diarrhea, vomiting, and nausea) associated with BuChE inhibition. ZT-1, when tested in healthy male volunteers in a Phase I study, was well tolerated and converted to HUP A (Jia et al. 2013). Further clinical investigation is needed to determine the therapeutic viability of ZT-1.

In addition to modifying acetylcholine levels in the CNS, nonsteroidal anti-inflammatory drugs (NSAID) have been investigated as a treatment for AD. NSAIDs may prove beneficial due to their anti-inflammatory effects, which could counteract inflammatory responses (microglial, complement, and cytokine activation) associated with AD (Akiyama et al. 2000). In fact, long-term use of NSAIDs, which inhibit cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), may have a beneficial effect on the risk of AD (in t' Veld et al. 2001). COX-2 inhibition provides therapeutic effects, while COX-1 antagonism can cause unwanted side effects (MacDonald 2000). Therefore, DP-155, a prodrug consisting of indomethacin covalently attached to lecithin via a 5-carbon linker, which acts specifically as a COX-2 inhibitor, was investigated as a potential AD therapeutic (Dvir et al. 2006). Furthermore, the PK profile of indomethacin cleaved from DP-155 differs from the commercially available compound. Specifically, oral administration of DP-155 in rats resulted in a 2.5 fold increase in brain/plasma concentration ratio compared with native indomethacin (Dvir et al. 2007). DP-155 and indomethacin similarly reduced soluble A β 42 when studied in the tg2576 mouse model of AD (Dvir et al. 2006). Therefore, the potential benefit of DP-155 over traditional indomethacin stems from its selective inhibition of COX-2 in the periphery, thereby limiting side effects, without compromising its therapeutic benefit.

Gastrointestinal (GI) toxicity associated with chronic NSAID administration is concerning in regards to its potential use for a treatment of AD (Gasparini et al. 2004). To this end, a prodrug linking naproxen with dihydropyridine (DHP) via an ester spacer was designed to increase brain delivery and subsequently reduce GI discomfort (Sheha 2012). Oral administration of the DHP-Naproxen increased brain concentrations twofold, while reducing GI side effects compared to naproxen (Sheha 2012). Prodrug strategies aimed at improving the CNS specific targeting of NSAIDs, while subsequently reducing peripheral toxicities, are integral in developing their therapeutic use as a treatment for AD.

49.3.4 Parkinson's Disease

Parkinson's Disease (PD) is a neurodegenerative disorder with an unknown etiology, in which patients present with motor deficits such as slowed movement, rigidity, resting tremor, and decreased dexterity (Lewitt 2008). Loss of dopaminergic neurons projecting from the substantia nigra pars compacta to the striatum is responsible for the motor deficits associated with PD (Fearnley and Lees 1991). The loss of more than 50% of these specific dopaminergic neurons results in Parkinsonian symptoms (Davie 2008). Hence, dopamine replacement therapy is currently the standard

therapeutic option in PD. Dopamine is completely ionized at physiological pH and is unable to cross the BBB (Chemturi and Donovan 2007). Therefore, levodopa (L-DOPA) or 3,4-dihydroxy-L-phenylalanine, a naturally occurring amino acid precursor of dopamine, is used for PD treatment (Cotzias et al. 1967). Essentially L-DOPA acts as a natural prodrug for dopamine. L-DOPA can actively transverse the BBB using LAT1 and is enzymatically converted to dopamine in the CNS via AAAD (Nutt et al. 1984; Lewitt 2008). In practice, a substantial amount of administered L-DOPA is metabolized in peripheral circulation, limiting the amount that actually reaches the CNS and available for conversion to dopamine (Huttunen and Rautio 2011). To circumvent these inherent limitations, L-DOPA is generally co-administered with AAAD inhibitor Carbidopa (Lewitt 2008). Unfortunately, the development of motor complications (dyskinesia, "wearing-off," and on/off fluctuations) often results from the chronic long-term use of L-DOPA (Obeso et al. 2000). Reducing fluctuations in L-DOPA plasma concentrations by improving absorption consistency may reduce associated motor complications, as the clinical response tends to correlate with L-DOPA blood levels (Chan et al. 2005). To this end, LeWitt et al. designed an L-DOPA prodrug termed XP21279 to improve upon the pharmacological limitations associated with immediate-release L-DOPA (IR-LD) therapy. XP21279 utilizes high-capacity nutritional transporters in the GI tract, thereby improving absorption, thus potentially reducing fluctuations (Lewitt et al. 2012). In a phase II, randomized, double blinded study, XP21279 was co-administered with CD (XP21279-CD) and compared with immediate-release CD-L-DOPA (IR-CD-L-DOPA). XP21279-CD reduced variability and increased mean L-DOPA concentrations compared with IR-CD-L-DOPA (LeWitt et al. 2014). Studies of XP21279-CD provide encouraging results towards potentially reducing the motor complications associated with long-term L-DOPA therapy, and its future progress will continue to be monitored.

L-DOPA prodrugs are not limited to those designed to improve absorption and pharmacokinetics. In fact, research suggests oxidative stress may contribute to PD pathogenesis (Olanow 1992). Therefore, multifunctional prodrugs, devised to deliver L-DOPA in conjunction with relevant antioxidants, are under investigation. To this end, Pinnen et al. designed a multifunctional prodrug of acetylated L-DOPA conjugated to methionine (LD-MET) to reduce DA-induced cell death exacerbated by oxidative stress. LD-MET exhibited radical scavenging activity on par with acetylcysteine in invitro assays (Pinnen et al. 2009). Additionally, LD-MET displayed higher and sustained levels of dopamine in the rat striatum compared with L-DOPA administration (Pinnen et al. 2009). Furthermore, a hybrid glutathione-methionine peptidomimetic prodrug of L-DOPA methyl ester was synthesized to improve CNS uptake of L-DOPA methyl ester

and reduce oxidative stress associated with PD (Pinnen et al. 2012). Glutathione (GSH), in which cysteine was replaced with methionine and L-DOPA methylester, was linked through an amide bond. In addition to *in vitro* radical scavenging activity, oral administration in rats yielded sustained and increased striatal DA levels, as well as enhanced plasma concentrations compared to animals treated with L-DOPA (Pinnen et al. 2012). Further investigation is necessary; but conceptually, multifunctional L-DOPA prodrugs provide intriguing options for the development of future PD therapeutics.

Improving motor complications (“wearing off,” dyskinesia, and on/off phenomena) that persist during long-term L-DOPA treatment remains a significant goal in PD therapy. Interestingly, A_{2A} adenosine receptor antagonists have shown promising preclinical results improving the effects of L-DOPA treatment, while reducing associated motor complications (Simola et al. 2008). To this end, Dalpiaz et al. synthesized a dopamine prodrug linking A_{2A} receptor antagonist (7-amino-5-(aminomethyl)-cyclohexylmethyl-amino-2-(2-furyl)-1,2,4-triazolo [1,5-a]-1,3,4-triazine trifluoroacetate) to dopamine by a succinic linker. The prodrug exhibited A_{2A} receptor antagonism, as well as increase dopamine affinity toward striatal D_2 receptors (Dalpiaz et al. 2012). Further pharmacokinetic analysis is necessary, but the progress of A_{2A} adenosine receptor antagonists as a PD therapeutic bears monitoring.

49.3.5 Schizophrenia

Schizophrenia is a complex neuropsychological disorder that is characterized by positive (e.g., hallucinations, delusions) or negative (e.g., poverty of speech, inability to experience pleasure) symptoms (Andreasen and Olsen 1982). Clinical treatment is complicated by the prospect that each type of symptomatic manifestation is derived from a different neurological receptor (Kim et al. 2009). This concept has led to the development of pharmaceuticals that deviate from the traditional first and second-generation anti-psychotics, predominantly dopamine (D_2) and serotonin ($5HT_{2A}$) receptor antagonists (Conn et al. 2008). In fact, it has been hypothesized that while presynaptic dopamine is responsible for positive symptoms, negative and cognitive symptoms may center on glutamate (Howes et al. 2015). To this end, new therapeutic prodrugs derived from the latest advancements in schizophrenia research continue to emerge and will be discussed in detail.

γ -aminobutyric acid (GABA) combined with dopamine antagonists has been reported to improve the cognitive deficits as well as reduce extrapyramidal symptoms (EPS) associated with schizophrenia (Soares et al. 2001; Nudelman

et al. 2008). In practice, this therapeutic paradigm is limited due to GABAs lack of BBB penetrance (Kuriyama and Sze 1971). In order to improve CNS delivery and reduce EPS, an ester prodrug consisting of GABA and typical antipsychotic perphenazine (BL-1020) was designed (Nudelman et al. 2008). BL-1020 exhibited the ability to cross the BBB and release GABA in the brain of orally administered rats. Additionally, the prodrug was effective reducing amphetamine induced hyperactivity and locomotion similar to perphenazine (Geffen et al. 2009). In a randomized, double blind study, BL-1020 exhibited similar improvement in the Positive and Negative Syndrome Scale (PANSS) compared with risperidone. Most importantly, BL-1020 was able to improve patients’ cognitive performance as accessed by the Brief Assessment of Cognition in Schizophrenia (Geffen et al. 2012). Further tests are necessary, but BL-1020 provides a unique therapeutic platform for schizophrenia aiming to treat psychotic symptoms while concurrently reducing ESP and improving cognition.

Treatment strategies for schizophrenia continue to evolve as researchers search for answers outside the realm of dopamine modulation. In fact, much of the focus in the development of new anti-psychotics centers on the modulation of glutamate receptors and its potential benefits for patients with schizophrenia (Weinberger 2007). To this end, LY404039, a novel and selective mGlu2/3 receptor agonist, was developed (Rorick-Kehn et al. 2007). Low oral bioavailability prompted the development and synthesis of LY2140023, a methionine prodrug of LY404039. In a phase II clinical trial, LY2140023 given twice daily (40 mg) was able to significantly improve symptoms of schizophrenia compared with placebo, as assessed by the PANSS (Kay et al. 1987; Patil et al. 2007). Investigation continued with a 24-week phase II trial in which LY2140023 was compared with olanzapine, risperidone, or aripiprazole in patients suffering from schizophrenia. LH2130023 exhibited similar improvements in PANSS compared with traditional antipsychotic treatment during the initial 6–8 weeks, although at week 24, the effect was significantly less for LH2130023 (Adams et al. 2013).

Standard schizophrenia treatment with atypical antipsychotics is contingent on successful CNS entry and accumulation. Even compounds that overcome the impediment of the BBB can have limited efficacy due to efflux pumps such as P-glycoprotein (P-gp). Located on the luminal surface of brain endothelial cells, P-gp can serve as an efflux pump for its substrates, effectively reducing drug concentrations in the brain substantially (Loscher and Potschka 2005). In fact, atypical antipsychotics such as quetiapine (QT) and risperidone act as P-gp substrates, thereby limiting their CNS accumulation and efficacy (Boulton et al. 2002). To this end, Emmert et al. synthesized a dimeric ester QT prodrug designed to act as a P-gp inhibitor as well as enhance QT

accumulation in the CNS. Specifically, dimerization converts a substrate (QT) for P-gp into an inhibitor and upon entry into endothelial cells of the brain can be cleaved by esterases to deliver the QT therapy (Emmert et al. 2014). Certainly, *in vivo* characterization of systemic administration is necessary, but the concept of P-gp inhibition through therapeutic dimerization has potential.

49.3.6 HIV-1

Current HIV-1 treatment, known as highly active antiretroviral therapy (HAART) consists of numerous antiretroviral drugs targeting various steps in the viral life cycle (entry, fusion, reverse transcription, integration, maturation), used in combination to reduce virus below clinically detectable levels and subsequently diminish HIV-1 associated morbidity and mortality (Perelson et al. 1997; Palella et al. 1998; Arts and Hazuda 2012). Despite potent HAART, HIV-1 infection still persists in latently infected CD4+ T-cell populations harboring integrated copies of HIV-1 proviral DNA (Chun et al. 1995). Additionally, virus can persist in anatomical sanctuaries such as the CNS and gut-associated lymphoid tissue (GALT) due to inadequate antiretroviral drug accumulation (Eisele and Siliciano 2012). Particularly, HIV-1 enters the CNS days after infection and can be identified within perivascular macrophages (Wiley et al. 1986; Davis et al. 1992). Subsequently, HIV-1 infection within the brain can result in HIV-associated neurocognitive disorders (HAND), which can be further defined as HIV-associated asymptomatic neurocognitive impairment (ANI), HIV-1-associated mild neurocognitive disorder (MND), or HIV-1-associated dementia (HAD) (Antinori et al. 2007; Spudich and Gonzalez-Scarano 2012). Antiretrovirals used in HAART each penetrate the CNS at various levels; thus, numerous research groups have designed prodrugs to facilitate CNS ART accumulation (Letendre et al. 2008).

Prodrugs designed to improve antiretroviral therapy (ART), such as tenofovir and fosamprenavir, have mainly focused on improving oral bioavailability (Palombo et al. 2009). For example, tenofovir disoproxil fumarate, which is a tenofovir prodrug possessing two additional alkyl methyl carbonate esters, enhances intestinal absorption and subsequent bioavailability (Shaw et al. 1997; Kearney et al. 2004). In addition, fosamprenavir, the phosphate ester prodrug of protease inhibitor amprenavir exhibits increased aqueous solubility, thereby improving oral bioavailability and limiting pill burden (Furfin et al. 2004; Palombo et al. 2009). While these prodrugs are aimed at improving systemic absorption, others have investigated prodrug designs specifically tailored towards improved ART delivery to the CNS as discussed next.

Saquinavir (Saq), a protease inhibitor used in the treatment of HIV-1, is a P-gp substrate, thus limiting its CNS penetration (Kim et al. 1998). Jain et al. synthesized peptide prodrugs of Saq designed to reduce P-gp-mediated drug efflux. Specifically, the hydroxyl group on Saq was modified with amino acids to form Saq conjugated with two valine molecules (Saq-Val-Val) or glycine-valine (Saq-Gly-Val) (Jain et al. 2005). In cell culture models, these modified prodrugs elude P-gp and MRP-2 efflux, thereby increasing their potential tissue distribution to the CNS (Jain et al. 2005, 2008). Additionally, an ascorbic acid (AA) prodrug consisting of vitamin C conjugated to Saq via a succinic acid linker, demonstrated SVCT selective uptake in MDCK-MDR1 cells (Luo et al. 2011). SVCT is also expressed at the BBB and could, therefore, provide an additional mechanism to improve CNS uptake of Saq.

The limited CNS bioavailability exhibited by numerous antiretroviral drugs designed to treat HIV-1 is not simply due to suboptimal physicochemical properties. Transporters located on the surface of the BBB such as P-gp and MDRs serve as efflux pumps, removing drug substrates from the CNS, significantly reducing its accumulation within the brain. To this end, Namanja et al. designed prodrug dimers of abacavir, a reverse transcriptase inhibitor used in HIV-1 treatment. Specifically, the hydroxyl group of each abacavir molecule was linked via an ester bond to a sulfide linker (Namanja et al. 2012). Therefore, dimerization occurs by forming a disulfide bond between the two modified abacavir molecules. This abacavir prodrug dimer effect is twofold; it has the ability to act as a P-gp inhibitor, while releasing abacavir monomers in the reducing environment of the cytosol (Namanja et al. 2012). Further investigation into the pharmacokinetic and biodistribution of this prodrug is necessary, but the multifunctional prodrug dimer approach bears monitoring.

49.3.7 Chemotherapy

Treatment strategies for aggressive brain tumors such as glioblastomas often follow a multimodal approach in which a combination of surgery, radiation, and chemotherapy is used (Castro et al. 2003). Chemical therapy is often limited by the lack of CNS accumulation of marketed chemotherapeutics. Even potent anti-cancer agents such as paclitaxel, which is used to treat breast and ovarian cancers, has limited *in vivo* efficacy against cancers of the brain due to poor BBB penetrance and P-gp efflux (Wani et al. 1971; Heimans et al. 1994; Crown and O'Leary 2000; Taub et al. 2005). Specifically, despite its lipophilicity; lack of BBB permeability may be due to its high protein binding (90 %) and large molecular weight (853.9) (Heimans et al. 1994).

Regina et al. synthesized a paclitaxel-angiopep-2 conjugate (GRN1005) designed to improve CNS delivery and therapeutic efficacy of paclitaxel in brain cancer treatment. Angiopep-2 is a peptide that targets low-density lipoprotein (LDL) receptors at the BBB and is used as a delivery vehicle to enhance brain uptake of paclitaxel (Demeule et al. 2008). Specifically, GRN1005 consists of three molecules of paclitaxel conjugated to angiopep-2 through cleavable ester bonds (Regina et al. 2008). GRN1005 significantly increased brain uptake compared with paclitaxel, independent of P-gp receptor expression (Regina et al. 2008; Thomas et al. 2009). In a Phase I clinical trial involving patients with advanced solid tumors, 25 % of individuals treated with GRN1005 achieved a partial response and reduction in brain lesions (Kurzrock et al. 2012). Further studies in patients with recurrent malignant glioma, resulted in GRN1005 delivering paclitaxel across the BBB in concentrations greater than previously reported for free paclitaxel, although therapeutic efficacy was limited (Drappatz et al. 2013). Research into this angiopep-2 conjugate model, known specifically as engineered peptide compound (EPiC) platform, is ongoing with other cancer agents such as doxorubicin and etoposide (Che et al. 2010).

Temozolamide (TMZ), a DNA alkylating prodrug of methyl-triazenyl imidazole carboxamide (MTIC), is currently used in conjunction with radiation therapy for the treatment of glioblastoma (Stevens et al. 1984; Omuro and DeAngelis 2013). Specifically, TMZ is readily converted to active DNA methylating agent MTIC at physiological pH (Stevens et al. 1987; Friedman et al. 2000). In fact, the ability of TMZ to cross the BBB made it a prime candidate for CNS cancer treatment and today remains a first-line treatment for glioblastoma (Patel et al. 2003; Omuro and DeAngelis 2013). TMZ provides an excellent example of the substantial impact prodrugs can have in the clinical setting.

49.4 Summary and Future Perspectives

Delivery of active pharmaceuticals to the CNS is a monumental task. First, a compound must possess the required physicochemical properties (lipophilicity, molecular weight) to passively diffuse across the BBB (Oldendorf 1974). In specific cases, larger, more polar molecules gain entrance into the CNS via carrier or receptor-mediated transport (Banks 2009). Compounds that successfully transverse the BBB may be subject to efflux by P-gp and MDRs. The chore of a medicinal chemist to design a drug with the appropriate physicochemical properties to enter and remain in the brain is quite daunting. In fact, prodrugs are utilized to overcome such impediments. Furthermore, prodrugs can be designed and synthesized to improve a drug's oral bioavailability, absorption, solubility, and/or tissue distribution (Rautio et al. 2008). In particular, lipidization can be used to enhance pas-

sive diffusion across the BBB but is often limited by its non-specific nature. Therefore, targeting receptors or carrier-mediated transporters on the BBB surface using a ligand-prodrug approach provides the specificity necessary to mediate CNS uptake. Systemic metabolism is another hurdle that must be overcome to successfully target a prodrug to the CNS. Each prodrug consists of a unique bioreversible bond (ester, amide, ether, or carbamate) with inherently different bond strengths. Upon systemic administration, this bioreversible bond needs to be strong enough to endure circulation and reach the CNS as an intact prodrug. Furthermore, once in the brain, the bond must be labile enough to release the active compound. Designing a prodrug with the correct physicochemical properties, with optimized cleavage kinetics, and with CNS-specific targeting is certainly difficult. As CNS drug delivery research progresses, merging of multiple disciplines will be imperative towards the development of improved brain-targeted therapeutics. Just as the disciplines of medical chemistry and biology work together in prodrug development, inclusion of the burgeoning field of nanomedicine is the next natural progression. Nanomaterials for medical applications such as liposomes, polymeric micelles, dendrimers, nanosuspensions, and polymer-drug conjugates allow for the packaging and delivery of drugs in myriad of ways (Moghimi et al. 2005). The power of nanomedicine lies in its versatility and its ability to enhance the function of pharmaceuticals, such as prodrugs (Wagner et al. 2006). In addition to enhancing a drug's pharmacokinetics and pharmacodynamics, nanoparticles can be surface functionalized with targeting ligands designed to improve specific tissue distribution, such as the CNS (Debbage 2009). Nanoparticle designs encapsulating diverse drugs for the treatment of a multitude of diseases are currently being developed (Wong et al. 2012). Nanoformulation of hydrophilic compounds is often challenging due to a lack of stable interaction between the drug and polymer. Therefore, prodrug synthesis by lipidization can increase a drug's formulation potential. The generation of hydrophobic prodrugs of hydrophilic compounds increases the scope and potential of nanomedicine. Hence, the combined efforts of medicinal chemistry, biology, and nanomedicine have the potential to considerably improve CNS drug delivery. A listing of relevant prodrug structures for CNS disease treatments with linked references is seen in Fig. 49.3.

49.5 Review Questions

49.5.1 True/False

1. Prodrug synthesis generates a new compound, which is a derivative of the parent organic compound.
2. Prodrug synthesis by lipidization selectively increases drug penetration across of the blood-brain barrier (BBB).

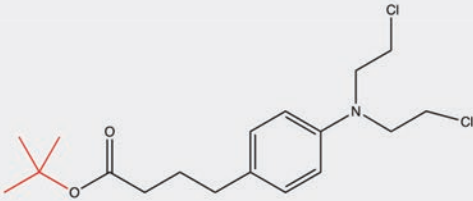
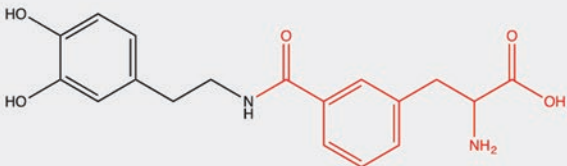
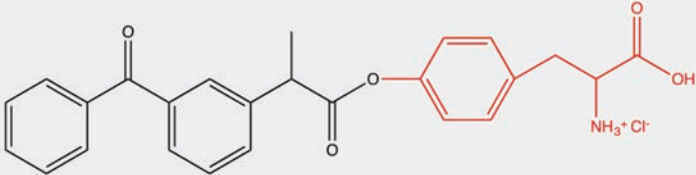
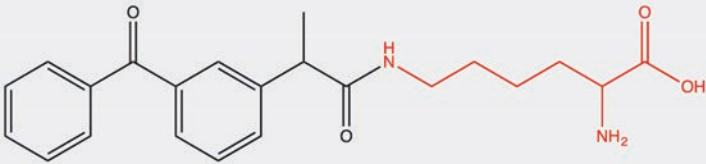
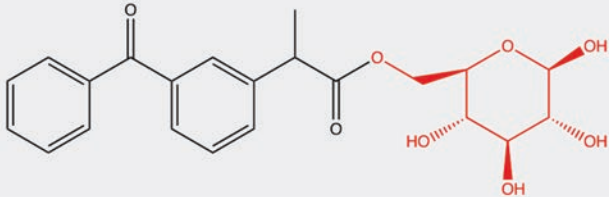
Structure	Literature
	<p>Greig, N. H., Daly, E. M., Sweeney, D. J., & Rapoport, S. I. (1990). Pharmacokinetics of chlorambucil-tertiary butyl ester, a lipophilic chlorambucil derivative that achieves and maintains high concentrations in brain. <i>Cancer chemotherapy and pharmacology</i>, 25(5), 320-325.</p>
	<p>Peura, L., Malmioja, K., Huttunen, K., Leppänen, J., Hämäläinen, M., Forsberg, M. M., ... & Laine, K. (2013). Design, synthesis and brain uptake of LAT1-targeted amino acid prodrugs of dopamine. <i>Pharmaceutical research</i>, 30(10), 2523-2537.</p>
	<p>Gynther, M., Laine, K., Ropponen, J., Leppänen, J., Mannila, A., Nevalainen, T., ... & Rautio, J. (2008). Large neutral amino acid transporter enables brain drug delivery via prodrugs. <i>Journal of medicinal chemistry</i>, 51(4), 932-936.</p>
	<p>Gynther, M., Jalkanen, A., Lehtonen, M., Forsberg, M., Laine, K., Ropponen, J., ... & Rautio, J. (2010). Brain uptake of ketoprofen-lysine prodrug in rats. <i>International journal of pharmaceuticals</i>, 399(1), 121-128.</p>
	<p>Gynther, M., Ropponen, J., Laine, K., Leppänen, J., Haapakoski, P., Peura, L., ... & Rautio, J. (2009). Glucose moiety enables glucose transporter mediated brain uptake of ketoprofen and indomethacin prodrugs in rats. <i>Journal of medicinal chemistry</i>, 52(10), 3348-3353.</p>

Fig. 49.3 Prodrug Structures

3. Efflux pumps on the inner surface of the BBB are capable of removing drugs that have already successfully entered the brain.
4. Prodrug entry into the CNS can be mediated by receptors on the surface of brain endothelium.
5. Bond strength and cleavage kinetics are key determinants to the stability and subsequent functionality of prodrugs.

49.5.2 Multiple Choice

6. Factors that contribute to any drug's success to combat brain disease include.
 - (A) Its ability to cross the BBB
 - (B) Can retain function in the brain's microenvironment.
 - (C) Limited CNS drug penetrance for most drugs does not limit the effectiveness and therapeutic potential of any drug
 - (D) *A and B*
 - (E) *A, B and C*
7. Properties of the BBB include all except?
 - (A) Protection against potentially harmful molecules from accumulating within the CNS.
 - (B) Consists of a continuous layer of endothelial cells connected by tight junctions
 - (C) Serves to separate circulating blood from the extracellular fluid within the brain
 - (D) Police the entry of essential molecules, thereby regulating the homeostatic, nutritive, and immune environments of the brain
 - (E) *Prevents entry only of activated macrophages and T cells*
8. What properties of prodrugs serve to facilitate its passage across the blood brain barrier?
 - (A) Contain cleavable chemical moieties that improve BBB permeability.
 - (B) Lipidization, carrier and receptor mediated targeting commonly improve drug's translocation across the blood-brain interface.
 - (C) Resistance to metabolism
 - (D) *A and B*
 - (E) *A, B and C*
9. What consequences of lipidization may potentially reduce CNS penetrance?
 - (A) Increased molecular weight
 - (B) Blocking of functional groups capable of forming hydrogen bonds
 - (C) Enhanced protein binding
 - (D) *B and C*
 - (E) *A and C*
10. L-DOPA prodrugs aim to ameliorate treatment limitations such as _____.
 - (A) Dyskinesias
 - (B) On-Off phenomenon

- (C) Peripheral metabolism
- (D) Absorption constancy
- (E) *All of the above*

49.6 Answers

1. T
2. F
3. T
4. T
5. T

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Abstract

Tremendous efforts in the last several decades have resulted in numerous inventions for central nervous system (CNS) drug delivery systems. Many of these innovative systems have a significant potential for the development of new biomedical applications. The wide variety of strategies reflects the inherent difficulty in transport of therapeutic and imaging agents across the blood brain barrier (BBB). In fact, the effective combination of several approaches, such as encapsulation of drugs into nanoparticles (NPs) conjugated with vector moieties or using micelles of Pluronic® block copolymers along with Pluronic® “unimers” that will inhibit drug efflux transporters in the brain microvessel endothelial cells BMVEC, may give the most promising CNS therapeutic outcomes.

Keywords

Block copolymer • Block ionomer complex • Cell-mediated delivery • Drug delivery • Hydro-philicity • Lipophilicity • Liposome • MDR inhibitor • Micelle • Multidrug resistance • Nanofiber • Nanogel • Nanoparticle • Nanosphere • Nanosuspension • Nanotube • Polyion block

50.1 Introduction

The blood brain barrier (BBB) is one of the most challenging barriers for drug delivery in the body. It significantly restricts the entry of low molecular weight compounds and biomacromolecules to the brain from the periphery. Inefficient delivery of the drugs, DNA and proteins to the brain is a major bottleneck in development of more efficacious and safe modalities for diagnostics and treatment of neurological diseases, especially at early stages of the disease when the BBB remains intact. The low permeability of the BBB is attributed, in large part, to the brain microvessel endothelial cells (BMVEC), which form tight extracellular junctions and have low pinocytotic activity (Pardridge 2005a; Mayhan 2001). Some relatively lipophilic and low molecular weight

substances can transport across the BMVEC by passive diffusion. However, a large number of lipophilic compounds are rapidly effluxed from the brain into the blood by extremely effective drug efflux systems expressed in the BBB (Begley 1996; Tamai and Tsuji 2000; Fromm 2000; Loscher and Potschka 2005). These efflux systems include P-glycoprotein (Pgp), Multidrug Resistance Proteins (MRPs), breast cancer resistance protein (BCRP), and the multi-specific organic anion transporter (MOAT). There is also an enzymatic barrier to drug transport in the BMVEC. Activity of many enzymes that participate in the metabolism and inactivation of endogenous compounds, such as γ -glutamyl transpeptidase, alkaline phosphatase, and aromatic acid decarboxylase is elevated in cerebral microvessels (Minn et al. 1991; Abbott and Romero 1996). These features of the BBB require discovery of new modalities allowing for effective drug delivery to the central nervous system (CNS), which is of great need and importance for treatment of neurodegenerative disorders.

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A number of earlier publications and extensive reviews on delivery of small drug molecules and biomacromolecules to the brain are available in the literature (Spector 2000; Alyaudtin et al. 2001; Pardridge 2002; Cornford and Cornford 2002; Begley 2004; Kas 2004; Kabanov and Batrakova 2008b; Banks and Lebel 2002; Roney et al. 2005; Liu and Chen 2005; Gaillard et al. 2005; Liu et al. 2005; Garcia-Garcia et al. 2005; Masserini 2013; Guo et al. 2012; Upadhyay 2014; Patel 2014; Gidwani and Singh 2014; Xu et al. 2014; Krupa et al. 2015). The present chapter describes recent findings in the development of a new generation of polymer nanomaterials for drug delivery to the CNS.

50.2 History and Principles of Drug Delivery Using Polymers

The original discovery of a polymer drug delivery system was published in 1964 by Folkman and Long (Folkman and Long 1964). This work reported that hydrophobic low molecular weight drugs can diffuse through the wall of silicone tubing at a controlled rate. Since then, polymers have occupied a central status in controlled drug release as well as in the fabrication of drug delivery systems (Langer 2001; Duncan 2003). During the past decades a large number of drug delivery systems, mostly in the form of microspheres, films, tablets, or implantation devices, have been designed to achieve sustained drug release by taking advantage of the unique properties of polymers.

There are several fundamental properties of polymers that are useful in solving drug delivery problems. First, polymers can be designed to be intrinsically multifunctional and can, for example, be combined either covalently or non-covalently with drugs to overcome multiple problems such as solubility, stability, permeability, etc. Second, polymers can be easily modified with various targeting vectors to direct drugs to specific sites in the body. Third, polymers can be designed to be environmentally responsive materials, allowing for the controlled and sustained release of a drug at its site of action. Finally, polymers themselves can be biologically active, and this property can be exploited in order to modify the activity of various endogenous drug transport systems within the body to improve delivery and, therefore, drug performance.

Numerous polymer-based therapeutics are on the market or undergoing clinical evaluation to treat cancer and other diseases. Many of them are low molecular weight drug molecules or therapeutic proteins that are chemically linked to water-soluble polymers to increase drug solubility, drug stability, or enable site-specific transport of drugs to target tissues affected by the disease.

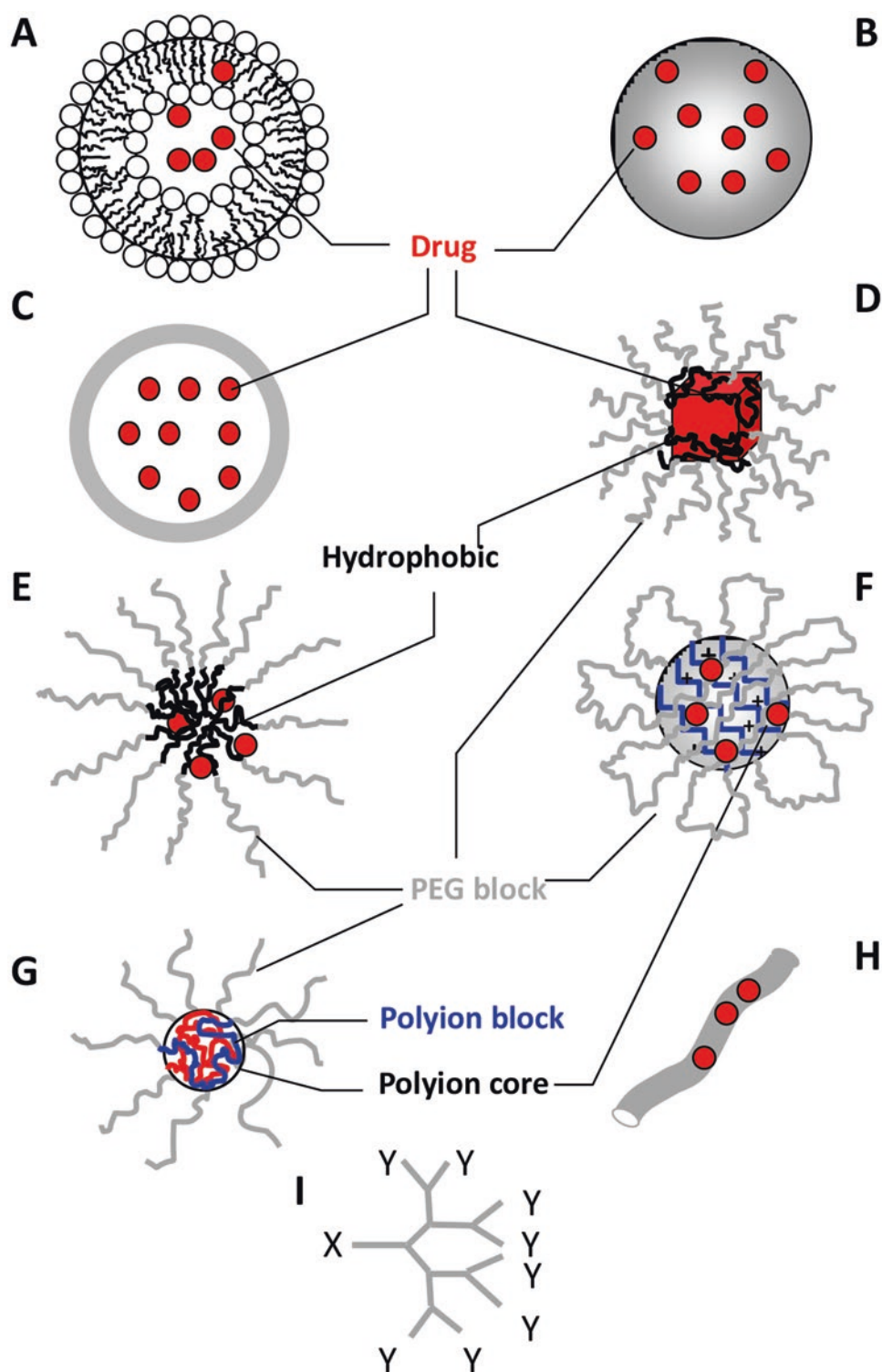
Recently, as a result of the rapid development of novel nanotechnology-derived materials, a new generation of polymer therapeutics has emerged which uses materials and

devices of nanoscale size for the delivery of drugs, genes, and imaging molecules (Kabanov et al. 1995, 2004; Savic et al. 2003; Trentin et al. 2005; Missirlis et al. 2005; Salem et al. 2003; Nayak and Lyon 2005; Gilmore et al. 2008; De Jong and Borm 2008; Oberoi et al. 2013; Musyanovych and Landfester 2014; Spencer et al. 2015; Jain et al. 2015). These materials include liposomes, dendrimers, polymer micelles, polymer-DNA complexes ("polyplexes"), and other nanostructured materials for medical use, that are collectively called nanomedicines. Compared to the first generation of polymer therapeutics, the new generation of nanomedicines is more advanced. They entrap small drugs or biopharmaceutical agents, such as therapeutic proteins and DNA, and can be designed to trigger the release of these agents at the target site. Moreover, they can be targeted not only to the particular organ or tissue, but to a particular cell or even an intracellular compartment. The essence of nanotechnology is the ability to work at the molecular level to create large structures with fundamentally new molecular organization. Materials with features on the scale of nanometers often have properties different from their macroscale counterparts. Many nanomedicines are constructed using self-assembly principles, such as spontaneous formation of micelles or interpolyelectrolyte complexes driven by diverse molecular interactions (hydrophobic, electrostatic, etc.). Nanotechnology focuses not only on formulating therapeutic agents in biocompatible nanocomposites but also on exploiting distinct advantages associated with a reduced dimensional scale within 1–100 nm. Some examples of nanoscaled polymeric carriers involve polymer conjugates, polymeric micelles, and polymersomes (Panyam and Labhasetwar 2003). Because these systems often exhibit similarity in their size and structure to natural carriers such as viruses and serum lipoproteins, they offer multifaceted specific properties in drug delivery applications (Lavasanifar et al. 2002). There is still a lack of understanding in terms of why certain arrangements of small molecules can cause dramatic changes in their behavior. With this in mind, the goal of this chapter is to explore current research in polymeric nanoparticles, where the specific arrangement of the polymeric matter at the nanoscale imparts new properties that are not attainable from simple polymer solutions, and to summarize their applications for drug delivery systems to the CNS.

50.3 Nanocarriers for Drug Delivery

The need for a protective drug carrier for CNS drugs is highlighted by the fact that many drugs have a low hydrolytic stability and are subject to degradation by blood proteins or by enzymes encountered in the BBB. Furthermore, a drug carrier can be targeted via receptor-mediated transport using a brain-specific vector moiety. A single unit of a given drug

Fig. 50.1 Types of nanocarriers for drug delivery. (a) liposomes; (b) nanoparticles; (c) nanospheres; (d) nanosuspensions; (e) polymer micelles; (f) nanogel; (g) block ionomer complexes; (h) nanofibers and nanotubes; (i) dendrimers



carrier can incorporate many drug molecules, resulting in high “payloads” per one targeting moiety. By increasing the payload of the carrier, one might improve the efficacy of the delivery while maintaining a relatively low level of involvement of numbers of targeted moieties and receptors. There

are various types of vehicles proposed for transport of neuropharmaceuticals across the BBB: liposomes, degradable nanoparticles, nanospheres, nanosuspensions, polymeric micelles, nanogels, block ionomer complexes, nanofibers and nanotubes, and dendrimers (Fig. 50.1).

50.3.1 Types of Nanocarriers for Drug Delivery to the Brain

Liposomes are vesicular structures, which are usually composed of unilamellar or multilamellar lipid bilayers (similar to biological membranes) that surround internal aqueous compartments (Fig. 50.1a). The size of the vesicles varies from several nanometers to several microns. Relatively large amounts of drug molecules can be incorporated into either the aqueous compartment (water soluble compounds) or the lipid bilayers (lipophilic compounds) providing the possibility to use liposomes as carriers for drug delivery. The use of liposomes for drug delivery across the brain capillaries has been reported extensively (Umezawa and Eto 1988; Huwyler et al. 2002; Rousseau et al. 1999; Shi et al. 2001; Mora et al. 2002; Thole et al. 2002; Wu et al. 2002; Zhang et al. 2003; Schmidt et al. 2003; Omori et al. 2003; Aoki et al. 2004; Gosk et al. 2004; Chekhonin et al. 2005; Pardridge 2005a; Kundu et al. 2014; Cozar-Bernal et al. 2015; Helm and Fricker 2015). In general, encapsulation of a drug into liposomes prolongs the circulation time of the drug in the blood stream, reduces adverse side effects, and in selected cases enhances therapeutic effects of CNS agents. Conventional liposomes containing hydrocortisone were demonstrated to penetrate the BBB in experimental autoimmune encephalomyelitis (Rousseau et al. 1999). In addition, liposomes prepared using lecithin, cholesterol, and *p*-aminophenyl- α -mannoside were efficiently transported across the BBB into mouse via mannose receptor-mediated transcytosis (Umezawa and Eto 1988). Liposomes conjugated with cationized bovine serum albumin (cBSA) as transport vectors were readily taken up and appeared to be concentrated in intracellular vesicles in porcine brain capillary endothelial cells (PBCEC) (Helm and Fricker 2015). After intravenous application of cBSA-liposomes with a diameter between 120 and 150 nm, confocal fluorescence microscopy showed fluorescently-labeled liposomes in brain capillary surrounding tissue from male Wistar rats, suggesting their successful brain delivery. An interesting approach using thermosensitive liposomes loaded with an antineoplastic agent, doxorubicin (Dox), for treatment of malignant gliomas was reported (Aoki et al. 2004). These liposomes released their content when the tumor core was heated to 40 °C by a brain heating system. Elevated accumulation of the drug in the brain of the heated animals resulted in a significantly longer overall survival time compared to the non-heated animals.

Conventional liposomes normally are cleared rapidly from the circulation by the reticuloendothelial system. Extended circulation time of these carriers can be accomplished with small-sized liposomes (<100 nm) composed of neutral, saturated phospholipids and cholesterol. Further, it was demonstrated that water-soluble polymers such as polyethylene glycol (PEG) attached to the surface of liposomes

reduce adhesion of opsonic plasma proteins, and decrease recognition and rapid removal of liposomes from the circulation by the mononuclear phagocyte system in liver and spleen (Huwyler et al. 2002; Kozubek et al. 2000; Voinea and Simionescu 2002). Using this approach, PEG-coated ("PEGylated") long-circulating liposomes (or "stealth liposomes") were shown to remain in the circulation with a half-life as long as 50 h in humans (Gabizon et al. 1994). Particularly, commercial PEGylated liposome-encapsulated Dox, Doxil®, was already approved for use in the treatment of recurrent ovarian cancer and AIDS-related Kaposi's sarcoma (Gabizon et al. 2003) and was shown to be effective in patients with metastatic breast cancer (Papaldo et al. 2006). Long-circulating PEGylated liposomes were also used for the delivery of high doses of glucocorticosteroids to the CNS to treat multiple sclerosis (Schmidt et al. 2003). The PEGylated liposomes encapsulating prednisolone provided selective targeting to the inflamed CNS in rats (up to 4.5-fold higher accumulation compared to the healthy animals). The mechanism of preferential accumulation of these liposomes in the brain is not fully understood. It appears to be crucial that the liposomes exhibit long-circulating behavior. Their effect is related to either (1) the change in the pharmacokinetics of the encapsulated drug resulting in a greater drug exposure to the BBB or (2) to liposome capture by monocytes/macrophages (in liver, spleen, or blood) followed by cell-mediated transport to the brain. In another study, a novel β -sheet breaker peptide, H102, was delivered across BBB in liposomes following intranasal administration (Zheng et al. 2015). H102 was encapsulated into liposomes to reduce peptide degradation and increase brain penetration for the treatment of Alzheimer's disease (AD). The AUC of H102-loaded liposomes in the hippocampus was 2.92-fold larger than that of the free peptide group. H102 liposomal formulation ameliorated spatial memory impairment of AD model rats, increased the activities of choline acetyltransferase (ChAT) and insulin degrading enzyme (IDE), and inhibited plaque deposition. Noteworthy, H102 liposomes showed no toxicity on nasal mucosa (Zheng et al. 2015).

Attachment of targeting moieties to PEGylated liposomes can direct them to the BBB. The efficient delivery of PEG-liposomes conjugated with transferrin (Tf) to the post-ischemic cerebral endothelium was achieved in rats (Omori et al. 2003). The expression of Tf receptor in the cerebral endothelium was reported to increase with a peak at the first day after induced transient middle cerebral occlusion, which can be employed for drug delivery to the brain after stroke. PEGylated immunoliposomes were also successfully employed to target and transfect brain tissues (Shi et al. 2001). A plasmid DNA was encapsulated within the liposome. Packaging of the DNA in the interior of the liposome prevented degradation of the therapeutic gene by the ubiquitous endonucleases *in vivo*. These liposomes were

directed to Tf receptor-rich tissues, such as brain, liver, and spleen by monoclonal antibodies, OX26, that were linked to the free termini of the PEG chains. When a reporter gene was also coupled with a brain-specific glialfibrillary acidic protein promoter, its expression was achieved predominantly in the brain (Shi and Pardridge 2000).

In addition, considerable work was reported on antibodies to transferrin receptor, OX26, that were linked to the surface of PEGylated liposomes via PEG spacers. Such immunoliposome constructs were used to deliver small drugs, Daunomycin (Huwylar et al. 2002), and Digoxin (Huwylar et al. 2002) as well as plasmid DNA (Shi et al. 2001) to the brain. Notably, OX26-conjugated liposomes selectively distributed to BMVEC but avoided choroid plexus epithelium, neurons, and glia (Gosk et al. 2004). In related work OX26 antibodies directly linked to oligonucleotides (Huwylar et al. 1996) or fibroblast growth factor (Song et al. 2002) enhanced delivery of these molecules to the brain.

Another example of a brain targeting vector with a remarkable species-specificity is reported by Coloma et al. (2000). This vector was genetically engineered from monoclonal antibody to the human insulin receptor, 83-14 MAb, where most of the immunogenic murine sequences were replaced by a human antibody sequence. The intravenously administered antibody showed robust transport to the brain in a rhesus monkey but not in a rat. Furthermore, this vector was also used for targeting liposomes with reporter genes to the brain (Zhang et al. 2003). As a result, the level of gene expression in the brain was 50-fold higher in the rhesus monkey as compared to the rat. In this regard, the new covalent method of coupling IgG to liposomes for the drug delivery to the brain was reported recently (Cozar-Bernal et al. 2015). Functionalized liposomes were prepared with maleimide derivative linked to pegylated phospholipids containing free amino groups, PEG2000-DSPE/PE. The homogeneous population of vesicles prepared by extrusion process with a nanometric size was suitable to further anchor the antibody.

Monoclonal antibody directed insulin or Tf receptors were also utilized to target PEGylated immunoliposomes for transvascular gene therapy of Parkinson's disease (PD) (Pardridge 2005b). In the 6-hydroxydopamine rat model of experimental PD, striatal tyrosine hydroxylase (TH) activity was completely normalized after an intravenous administration of TfmAb-targeted liposomes carrying a TH expression plasmid. Treatment of PD using this approach may be possible with dual gene therapy that seeks both to replace striatal TH gene expression with TH gene therapy, and to halt or reverse neurodegeneration of the nigro-striatal tract with neurotrophin gene therapy.

The mechanism by which immunoliposomes penetrate across the BBB is not fully understood. It was hypothesized that the process involves the binding of immunoliposomes to multiple capillary luminal membrane receptors, fusion of the

liposomes with several vesicular pits into a large vesicle and transcytosis of this vesicle to the abluminal membrane border (Cornford and Cornford 2002). Studies by electron microscopy support this hypothesis (Faustmann and Dermietzel 1985).

In addition to approaches pursuing delivery across the intact BBB, the methods are developed to target sites in the brain under conditions when the integrity of the BBB is compromised by the progressing disease. For example, PEGylated immunoliposomes were coupled with the monoclonal anti-GFAP antibodies (Chekhonin et al. 2005). Experiments with cell cultures demonstrated specific and competitive binding of these immunoliposomes to embryonic rat brain astrocytes. Administered intravenously into rats, the immunoliposomes displayed typical kinetics with elimination half-lives of 8–15 h. Being incapable of penetrating the unimpaired BBB, these immunoliposomes, can be used to deliver drugs to glial brain tumors that express GFAP or to other pathological loci in the brain with a partially disintegrated BBB (Chekhonin et al. 2005).

Another approach involving temporal opening of the BBB using liposomes was proposed by Zhang et al. (2004). An endogenetic bioactive peptide, RMP-7 ("cereport") that is known to open tight junctions in the BBB by affecting bradykinin 2 receptors, was linked to conventional liposomes. It was demonstrated that transport of vectorized liposomes loaded with HRP was increased three times in an *in vitro* model of the BBB compared to the non-vectorized vehicles (Zhang et al. 2004). The opening of the tight junctions in the BBB following traumatic brain injury (TBI) was utilized to enable accumulation of stealth liposomes in the brain (Boyd et al. 2015). Dual-radiolabeled PEGylated liposomes were administered either immediately after induction of TBI or at increasing times post-TBI to mimic the likely clinical scenario. Significantly enhanced accumulation of liposomes was occurred in mice subjected to TBI compared to anaesthetized controls, and the accumulation was greater in the injured versus the contralateral side of the brain. Nevertheless, potential toxicological implications of such approach still need to be addressed.

Overall, liposomes have been extensively studied for CNS drug delivery showing increased drug efficacy and reduced drug toxicity. At present, the translation potential of targeted to BBB liposomes is well-recognized (Park 2002; Patel 2014). This drug delivery system represents a platform based on a proven technology, and already entered clinical trials (Immordino et al. 2006). While the examples presented suggest that the liposome technology provides a promising approach for CNS drug delivery, a considerable limitation of liposomes is their relatively low loading capacity for water-insoluble drugs and biomacromolecules. In addition, despite improvements in the targeting efficacy, major proportion of liposomes is found accumulated in the liver as a consequence of inadequate time for the communication between the target and targeted liposomes (Kundu et al. 2014).

Nanoparticles (NPs) are solid species of nanoscale size usually composed of an insoluble and biodegradable polymer or polymer blend (Fig. 50.1b). Generally, the methods of preparation of such NPs are simple and easy to scale-up. The drug is captured within the particle upon its preparation and is released upon degradation of the polymer in the body. The use of NPs as carriers for drug and gene delivery has been an area of intensive research and development for over a decade (Moghimi et al. 1990; Moghimi and Szebeni 2003; Gref et al. 1994; Peracchia et al. 1998; Torchilin 1998; Calvo et al. 2002; Vinogradov et al. 1999, 2004b; Lemieux et al. 2000; Alyaudtin et al. 2001; Gupta et al. 2003; Kreuter 2004; Liu et al. 2005; Roney et al. 2005; Liu and Chen 2005; Cui et al. 2005). The surface of such carriers is often modified by a PEG brush to increase the stability of NPs in dispersion and extend circulation time of NPs in the body (Peracchia et al. 1998; Torchilin 1998; Calvo et al. 2002). To allow for efficient transcytosis across the BMVEC the particle size must be kept small and not exceed ca. 100–200 nm.

For example, poly(butyl)cianoacrylate NPs were successfully used to deliver a wide range of drugs to the CNS, which were either incorporated into the particle structure or absorbed onto the particle surface (Calvo et al. 2001, 2002; Alyaudtin et al. 2001; Kreuter et al. 2003; Kreuter 2004; Steiniger et al. 2004). Prior to administration in the body, the NPs were coated with a PEG-containing surfactant, Tween 80. After the administration, they also absorbed apolipoprotein E available in the blood. The resulting coated NPs were transported across the cerebral endothelial cells by endocytosis via the low density lipoprotein (LDL) receptor. They localized in the ependymal cells of the choroid plexuses, the epithelial cells of ventricles, and, to a lower extent, in the capillary endothelial cells. The list of the drugs delivered to the CNS in these constructs include, analgesics (dalargin, loperamide), anti-cancer agents (Dox), anticonvulsants (NMDA receptor antagonist, MRZ 2/576), and peptides (dalargin and kytorphin) (Steiniger et al. 2004; Kreuter et al. 2003). Particularly, incorporation of MRZ 2/576, into such NPs prolonged the anticonvulsive activity of the drug almost twofold compared to the drug alone (Friesen et al. 2000). Dox bound to the NPs was successfully used for treatment of an aggressive human cancer, glioblastoma, resulting in increased survival times in rats (Steiniger et al. 2004). Enhanced transport of Dox in the NPs to the brain involved bypassing the P-glycoprotein (Pgp) and MRP efflux systems in the BBB that impede the delivery of the drug alone. Moreover, an ability of NPs and microspheres (MPs) with incorporated Celecoxib (CXB), a selective COX-2 inhibitor, was tested in rats with malignant gliomas (Vera et al. 2014). Uncoated and polysorbate 80-coated CXB-NPs are prepared by nanoprecipitation. Cerebral cortex images showed a marked increase of fluorescence when the surfactant-coated NPs were administered to rats. These results suggest that

both CXB formulations (MPs and NPs) are adequate systems to enhance the effects of chemotherapy in the treatment of malignant brain tumor. In another example the NPs were coupled with chelators (desferioxamine or D-penicillamine) and proposed for treatment of AD and other CNS diseases by reducing oxidative stress in the brain (Liu et al. 2005); (Cui et al. 2005). Another nanoformulation based on poly (D, L-lactide-co-glycolide) NPs loaded with cerebrolysin, a neuroprotective agent, was reported to have a superior neuroprotective efficacy than free drug in a rat model of concussive head injury (Ruozi et al. 2014). These findings have potential clinical relevance with regard to nanodelivery of different therapeutic agents across the BBB. In order to increase targeted CNS delivery of polymer-based NPs, MR-guided focused ultrasound with intravascular microbubbles (MBs) was suggested to locally and reversibly disrupt the BBB with submillimeter spatial accuracy (Nance et al. 2014).

However, a concern regarding the toxicity associated with this delivery approach has somewhat hampered further development for specific therapeutic applications (Olivier et al. 1999). The methods for preparation of NPs commonly employ the use of organic solvents that may result in degradation of immobilized drug agents, especially biomacromolecules. Another key issue is that it is virtually impossible to define the in vivo fate of polymers, especially in the brain, which is a regulatory requirement (Patel 2014). To this end, using a biodegradable nanoparticle platform is of great interest (Hunter et al. 2014). The development of such platforms carries broad implications for the treatment of a variety of CNS diseases.

Nanospheres are hollow nanosized particles (Fig. 50.1c) that can be prepared by microemulsion polymerization or covering the surfaces of colloidal templates with thin layers of the desired material followed by selective removal of the templates (Hyuk Im et al. 2005). The carboxylated polystyrene nanospheres (20 nm) were evaluated for drug delivery to CNS (Yang et al. 2004). It was demonstrated that these carriers accumulated in the brain in vivo following cerebral ischemia and reperfusion. The intravenously injected nanospheres remained in the vasculature under normal conditions. However, during cerebral ischemia-induced stress that leads to the opening of tight junctions between endothelial cells the extravasation of the nanospheres to the disease site was increased (Kreuter 2001). Therefore, polystyrene nanospheres may have potential clinical applications for CNS delivery of drugs and imaging agents during ischemia and stroke.

Nanosuspensions of hydrophobic drugs have also attracted attention as drug delivery systems (Rabinow 2004). These systems represent crystalline particles of a solid drug, which are often stabilized by nonionic PEG-containing surfactants (Fig. 50.1d) (Jacobs et al. 2000). They can be manufactured by a variety of techniques such as media milling, high-pressure homogenization or by employing emulsions

and microemulsions as templates (Friedrich and Muller-Goymann 2003; Friedrich et al. 2005). The major advantages of the nanosuspension technology include its simplicity and general applicability to most drugs including very hydrophobic compounds (Muller et al. 2001; Friedrich et al. 2005). It was suggested that similar to regular NPs (described above), the surface modification of nanosuspensions with Tween 80 may increase the delivery of these particles to the brain (Muller et al. 2001). Currently nanosuspensions of anti-retroviral drugs are evaluated in animal models for CNS delivery and treatment of HIV infection in the brain using cell-mediated delivery approach described below (H. Gendelman, University of Nebraska Medical Center) as well as by Dr. Sarmento's group in Portugal (Gomes et al. 2014). It was reported that nanosuspension-based approaches may help providing solutions for antiretroviral drug delivery to the CNS by potentially prolonging systemic drug circulation, increasing the crossing and reducing the efflux of active compounds at the blood–brain barrier, and providing cell/tissue-targeting and intracellular drug delivery.

Polymeric micelles represent core-shell structures that spontaneously form in aqueous solutions of amphiphilic block copolymers (Fig. 50.1e). Block copolymers are polymers containing at least two chains of different chemical nature, for example, a hydrophilic and a hydrophobic chain. In aqueous solutions above a threshold concentration, called a “critical micelle concentration” (CMC) the individual block copolymer molecules termed “unimers” form micelles composed from dozen to a couple of hundred of molecules. The size of the micelles usually varies in the range of from 10 to 100 nm. The hydrophobic chains of the block copolymers segregate forming a hydrophobic core of the micelles while the hydrophilic chains (often PEG) form a hydrophilic protective shell. Polymer micelles can incorporate considerable amounts (up to 20–30 % wt.) of hydrophobic compounds. The resulting nanomaterials can serve as carriers for drug delivery (“*micellar nanocontainers*”) (Kataoka et al. 2001; Kwon 2003; Torchilin 2004, 2005; Allen and Cullis 2004; Moghimi and Agrawal 2005; Tao and Uhrich 2006; Aliabadi and Lavasanifar 2006). The core-shell architecture of polymeric micelles is essential for their pharmaceutical application. The core is a water-incompatible compartment that is hidden from the aqueous exterior by the hydrophilic chains of the corona, preventing premature drug release and degradation. The hydrophilic shell maintains the micelles in a dispersed state and decreases undesirable drug interactions with cells and proteins through steric-stabilization effects. Upon reaching the target the incorporated drug is released from the micelle via diffusion mechanisms. Polymeric micelles have been extensively evaluated in multiple pharmaceutical applications as drug delivery systems (Kabanov et al. 1989b; Kabanov and Alakhov 2002; Jones et al. 2003; Torchilin et al. 2003; Lee et al. 2005b; Aliabadi et al. 2005; Uchino et al. 2005; Bronich et al. 2005; Yan and

Tsujii 2005; Gaucher et al. 2005; Miyata et al. 2005), and carriers for diagnostic imaging agents (Torchilin 2002). Several clinical trials have been completed or are underway to evaluate polymer micelles for the delivery of anti-cancer drugs for chemotherapy of tumors (Danson et al. 2004; Valle et al. 2004); (Nishiyama et al. 2003). Polymeric micelles formed by Pluronic®, PEG-phospholipid conjugates, PEG-b-polyesters, or PEG-b-poly(L-amino acid)s were proposed for drug delivery of poorly water-soluble compounds, such as amphotericin B, propofol, paclitaxel, and photosensitizers (Lavasanifar et al. 2002; Kwon 2003; Adams and Kwon 2004; Vakil and Kwon 2005). It was also emphasized that using polymeric micelles one can significantly increase the drug transport into the brain.

One of the early studies of targeted drug delivery to the brain used micelles of Pluronic® block copolymers as carriers for CNS drugs (Kabanov et al. 1989b, 1992c). These micelles were conjugated with either polyclonal antibodies against brain α_2 -glycoprotein or insulin to target the receptors at the luminal side of BMVEC. Both the antibody-conjugated and insulin-conjugated micelles were shown to effectively deliver a drug or a fluorescent probe incorporated into the micelles to the brain tissue in vivo (Kabanov et al. 1992a). Studies that monitored animal behavior as a means to determine the pharmacological activity of dopamenergic compounds, such as mobility and grooming were performed. It was demonstrated that incorporation of a neuroleptic, haloperidol, into the Pluronic® micelles vectorized with insulin resulted in 25-fold enhancement of the neuroleptic effects compared with the free drug. Vectorization of the drug-loaded micelles with antibodies lead to an even more pronounced (up to 500-fold) enhancement of the neuroleptic effects. Subsequent studies demonstrated that the insulin vectorized micelles undergo receptor-mediated transcytosis in BMVEC from luminal (blood) to abluminal (brain) side (Batrakova et al. 1998).

A new type of functional polymer micelles with cross-linked ionic cores was recently developed (Bronich et al. 2005). Instead of using amphiphilic block copolymers these micelles are prepared using double hydrophilic block copolymers with nonionic (PEG) and ionic blocks. The micelles are self-assembled by reacting the ionic block with a condensing agent of an opposite charge. The ionic chains incorporated into the core of the micelles were chemically cross-linked and then the condensing agent was removed. The resulting cross-linked micelles contain a hydrophilic ionic core, which is swollen in water, and a hydrophilic PEO shell. The core can incorporate various hydrophilic drugs and imaging agents, which can be then delivered to the target site in the body. Overall, this strategy has potential for the development of novel modalities for delivery of various drugs to the brain, including selected anti-cancer agents to treat metastatic brain tumors as well as HIV protease inhibitors to eradicate HIV virus in the CNS.

Nanogels are a new family of hydrophilic carriers that were recently developed for targeted delivery of biomacromolecules and other drug molecules to the brain (Vinogradov et al. 1999, 2004b, 2005a, b). Nanogels represent a nanoscale size polymer network of cross-linked ionic, e.g., polyethyleneimine (PEI), and nonionic PEG chains (Fig. 50.1f). It swells in an aqueous solution and collapses upon binding of a drug through ionic interactions. Because of the effect of PEG chains, the collapsed nanogel forms stable dispersions with the particles size of ca. 80 nm. Nanogels can spontaneously absorb biomacromolecules including oligonucleotides (ODNs), plasmid DNA and proteins as well as small charged molecules. A key advantage is that the nanogels display very high loading capacities, 40–60 % “payload” by weight, which is not achieved with conventional nanocarrier systems. The transport of ODNs incorporated in the nanogel particles across an in vitro model of the BBB was recently reported (Vinogradov et al. 2004a). To enhance delivery across the BBB the surface of the nanogels were modified by either Tf or insulin (Vinogradov et al. 2004b). Both peptides were shown to increase transcellular permeability of the nanogel and enhance delivery of ODNs across BMVEC monolayers. Overall, the nanogels were shown to be a promising carrier for CNS drug delivery, although only in the early stages of development. *Block ionomer complexes* are formed as a result of the reaction of double hydrophilic block copolymers containing ionic and nonionic blocks with biomacromolecules of opposite charge including oligonucleotides, plasmid DNA and proteins (Fig. 50.1g) (Kabanov and Kabanov 1995; Nguyen et al. 2000; Zhang et al. 2003; Jaturanpinyo et al. 2004; Harada and Kataoka 1999, 2003). For example, block ionomer complexes were prepared by reacting trypsin or lysozyme (that are positively charged under physiological conditions) with an anionic block copolymer, PEG-poly(R, α -aspartic acid) (Jaturanpinyo et al. 2004; Harada and Kataoka 1999). Such complexes spontaneously assemble into nanosized particles having core-shell architecture. The core contains polyion complexes of the biomacromolecules and ionic block of the copolymer. The shell is formed by the nonionic block. These nanomaterials were shown to efficiently deliver DNA molecules in vitro and in vivo into a cell and release them at the site of action (Roy et al. 1999; Nguyen et al. 2000; Harada-Shiba et al. 2002; Junghans et al. 2005). To improve stability of the complexes in the body the biopolymer and block ionomer can be additionally cross-linked with each other (Jaturanpinyo et al. 2004; Harada and Kataoka 2003) or the polyion chains of the block ionomer within the core can be cross-linked using degradable links. The advantages of such systems in drug delivery applications include simplicity and versatility of the design allowing the incorporation of considerable amounts of different biomacromolecules. Furthermore, block ionomer complexes are environmentally

responsive nanomaterials allowing for biomacromolecule release in response to an external stimulus such as change of pH (acidification), concentration and chemical structure of elementary salt, etc.

Nanofibers and nanotubes (Fig. 50.1h) have considerable promise in futuristic biomedical applications, for example, for sustained drug release from implants, or as channels for tiny volumes of chemicals in nanofluidic reactor devices, or as the “world’s smallest hypodermic needles” for injecting molecules one at a time. These nanomaterials have a prototype in nature. In an organism, microtubules and their assembled structures are critical components for a broad range of cellular functions—from providing tracks for the transport of cargo to forming the spindle structure in cell division. There are various types of synthetic nanofibers and nanotubes manufactured from carbon, silicon, or diverse natural or man-made polymers. They can be vapor-grown (Che et al. 1998), self-assembled from peptide amphiphiles (Guler et al. 2005; Bull et al. 2005) or electrospun manufactured from virtually any polymer material (Dzenis 2004; Gilmore et al. 2008). Carbon nanotubes and nanofibers have lately attracted great attention in nanomedicine including their potential use as drug carriers, although there are also considerable concerns associated with their safety (Muller et al. 2006; Lange et al. 2003). Self-assembled nanofibers can be designed to incorporate diverse chemical functionalities and thus may have a variety of applications for delivery of small drugs, biomacromolecules or imaging contrast agents (Bull et al. 2005). Electrospun continuous nanofibers are unique since they represent nanostructures in two dimensions and macroscopic structures in another dimension (Fong et al. 1999; Dzenis 2004). They are safer to manufacture than the carbon nanotubes since there is less risk of air pollution.

At present a few studies of nanofibers and nanotubes are focused on CNS drug delivery. One study evaluated electrospun nanofibers of a degradable polymer, PLGA, loaded with anti-inflammatory agent, dexamethasone for neural prosthetic applications (Abidian and Martin 2005). A conducting polymer, poly(3,4-ethylenedioxythiophene), was deposited to the nanofiber surface and the coated nanofibers were then mounted on the microfabricated neural microelectrodes, which were implanted into brain. The drug was released by electrical stimulation that induced a local dilation of the coat and increased permeability.

In the future, nanotubes and nanofibers can be administered systemically, if the problem of their toxicity is addressed, for example, by appropriate polymer coating. In this respect, the continuous nanofibers are more likely to be used in implants or tissue engineering applications.

Dendrimers exhibit a highly branched, 3D architecture and comprise an initiator core, several interior layers composed of repeating units, and multiple active surface terminal groups (Lee et al. 2005a). The branches and surface groups of dendrimers

increase exponentially in number with the generation (G) of the dendrimers, whereas the diameter of dendrimers increases by about 1 nm with the generation. The most remarkable property of dendrimers is their low polydispersity and high functionality (Xu et al. 2014). Dendrimers with amphiphilic structure are particularly interested as drug carriers. The loading of dendrimers with drug molecules is achieved by hydrophobic/hydrophilic interactions. Modification of the degree of branching may allow the encapsulation of different molecules within this structure (Lee et al. 2005a).

In vivo delivery of siRNA to the brain by carbosilane dendrimer was reported by Serramia et al. (2015). Functional validation was performed by using specific siRNA against HIV-1 Nef to interfere to HIV-1 infectivity. The second generation of carbosilane dendrimer transported efficiently siRNA into the brain in BALB/c mice. Moreover, this dendrimer successfully delivered and transfected siRNA to HIV-infected human primary astrocytes and achieved gene silencing without causing cytotoxicity (Serramia et al. 2015). The internalization properties and diffusion of G4 and G4-C12 modified polyamidoamine (PAMAM) dendrimers in the CNS of live animals was described in (Albertazzi et al. 2013). 100 nM G4-C12 PAMAM dendrimer induced dramatic apoptotic cell death of neurons in vitro. On the contrary, G4 PAMAM does not induce apoptotic cell death of neural cells in the sub-micromolar range of concentration and induces low microglia activation in brain tissue after a week. Noteworthy, different PAMAM dendrimer surface functionalization resulted in dramatic effects on their ability to diffuse in brain parenchyma after intracerebroventricular administration, and improved the neural cell internalization.

Overall, dendrimer-based drug formulations provided efficient drug delivery in the brain tissues. In addition, targeting to specific organs and a reduction in the toxicity of the free drug to non-target organs, and an increase of therapeutic index could be achieved. These results highlight the potential of dendrimer-based nanoformulations in the treatment of neurological disorders. The main drawbacks of dendrimers as drug carriers are a significant entrapment in the mononuclear phagocytic system in the liver and spleen, and a relatively high toxicity due to their high density of positively charged functional groups.

50.3.2 Cell-Mediated Delivery of Nanocarriers to the Brain

A distinct case of the vehicle-mediated CNS drug delivery employs specific cells carriers that can incorporate micro- and nano-containers (such as liposomes, micelles, and block-ionomer complexes) loaded with drugs and act as perfect Trojan horses by migrating across the BBB and carrying drugs to the site of action (Daleke et al. 1990; Fujiwara et al. 1996;

Jain et al. 2003; Khan et al. 2005; Batrakova et al. 2011; Haney et al. 2011, 2012; Zhao et al. 2011a, b; Batrakova and Kabanov 2013). It is documented that many neurological diseases, such as PD, AD, Prion disease, meningitis, encephalitis and HIV-associated dementia, have in common an inflammatory component (Perry et al. 1995). The process of inflammation is characterized by extensive leukocytes (neutrophils and monocytes) recruitment (Anthony et al. 1997, 2001; Blamire et al. 2000; Persidsky et al. 1999). These cells have a unique property of migrating toward the site of inflammation via the processes known as diapedesis and chemotaxis (Kuby 1994). Interestingly, prior reports indicate that monocytes traffic primarily between adjacent endothelial cells, i.e., paracellularly through the junctional complexes (Pawlowski et al. 1988; Lossinsky and Shivers 2004). Their combat arsenal consists of uptake of the foreign particle, producing toxic compounds, and liberation of substances stored in intracellular vesicles via exocytosis. Therefore, brain inflammation that develops in patients with neurodegenerative disorders may support targeted cell-mediated drug delivery to the brain.

It has been shown that cells capable of phagocytosis, such as macrophages or monocytes/neutrophils, can endocytose colloidal nanomaterials, for example, liposomes, and subsequently release them into the external media (Daleke et al. 1990; Jain et al. 2003). To accelerate the transport of monocytes/neutrophils to the brain site, the drug-containing liposomes were additionally loaded with magnetic particles (Jain et al. 2003). Magnetic liposomes demonstrated about a tenfold increase in brain levels compared to non-magnetic liposomes when local magnetic field was applied. It is noteworthy that both cell types showed preferential uptake of liposomes containing negatively-charged lipids (such as phosphatidylserine), or liposomes modified with polyanion than liposomes containing only neutral lipids (such as phosphatidylcholine) (Fujiwara et al. 1996). This suggests that by engineering the surface of the nanomaterials one may modulate their uptake into and release from the cell carriers to optimize the therapeutic regimen. The phosphatidylserine-containing negatively charged liposomes were shown to increase therapeutic activity of an encapsulated antifungal agent, chloroquine, against *C. neoformans* infection in the mouse brain (Khan et al. 2005). The chloroquine-loaded liposomes accumulated inside macrophage phagolysosomes and resulted in a remarkable reduction in fungal load in the brain even at low doses compared to the free drug at high doses, thus increasing the antifungal activity of macrophages. T lymphocytes were also proposed as a potential therapeutic drug carriers for cancer treatment (Steinfeld et al. 2006). The kinetics of loading and release of NPs coated with cytotoxic antibiotic Dox into the cells were examined. It was suggested that the immune cells can accomplish target-specific and sheltered transport to the diseased site.

In our research, we developed and optimized a cell-based delivery system for a potent anti-inflammatory polypeptide, catalase (Batrakova et al. 2007, 2011; Kabanov and Batrakova 2008a; Brynskikh et al. 2010; Haney et al. 2011, 2012, 2013; Zhao et al. 2011a, b; Klyachko et al. 2012, 2014) for the treatment of PD. A packaging of catalase into a polyion complex micelle (“nanozyme”) with a synthetic polyelectrolyte block copolymer protected the enzyme against degradation in cell-carriers, and improved therapeutic outcomes. We demonstrated that macrophages (1) efficiently accumulate catalase NPs and slowly release them *ex vivo* (Batrakova et al. 2007; Zhao et al. 2011a; Klyachko et al. 2014), and in PD mice providing sustained catalase blood levels (Brynskikh et al. 2010); (2) cross the BBB and deliver catalase to the inflamed brain (Zhao et al. 2011b; Haney et al. 2013; Klyachko et al. 2014); and (3) discharge catalase to neural cells, decreasing inflammation and ultimately producing potent neuroprotection in PD mouse models (Brynskikh et al. 2010; Haney et al. 2013; Klyachko et al. 2014). Noteworthy, the optimization of the drug nanoformulation is crucial for the successful delivery in living cells. Thus, the macrophage-based approach offers a paradigm switch for CNS delivery of nanotherapeutics. A formulation design for drug carriers typically works to avoid entrapment in monocytes and macrophages focusing on small size NPs with a PEG corona (perpetuate a stealth effect). In contrast, the best nanozymes for delivery in macrophages reported by us (Klyachko et al. 2014) have a relatively large size (*c.a.* 200 nm) that resulted in improved loading capacity and release from macrophages. Furthermore, the cross-linking of nanozymes with the excess of a non-biodegradable linker ensured their low cytotoxicity, and efficient catalase protection in cell-carriers.

The ability of host cells to home diseased sites after *ex vivo* manipulations is a fundamental requirement and a major problem for their use as vehicles to target locally acting gene therapy to specific diseased sites. It was demonstrated that in the short term, up to 2 h after re-implantation, macrophages accumulate primarily in the lungs and, to a lesser extent, in the liver and spleen rather than in the target diseased tissue. A small proportion of manipulated macrophages (ranging from 0.2 to 28.8%) homes to the diseased site of interest following systemic administration. However, the presence of labeled macrophages in these sites was found to persist for at least 6–7 days in both of these mouse studies (Audran et al. 1995). Therefore, increasing the amount of cell carriers reaching the target site appears to be a crucial point in this type of drug delivery system. We demonstrated that macrophages can serve as a “depot” for drug nanoformulations increasing area under the curve (AUC), half-life, and mean residence time in blood circulation, when compared to the NPs administered alone (Zhao et al. 2011b). Accordingly, bioavailability of the nanozyme for the brain,

spleen, kidney, and liver was substantially increased. Importantly, inflammation induced in the brain (or peripheral organs) attracted macrophages loaded with nanozyme resulting in the increased drug accumulation in these tissues. In contrast, effect of inflammation on cell-free nanozyme accumulation levels was negligible, except brain. Thus, nanozyme-loaded macrophages targeted diseased sites and improved transport across the blood brain barrier. The engaging natural immune cells such as monocyte-macrophages as drug carriers provides a new perspective for therapeutic delivery for PD and also likely a range of other inflammatory and degenerative diseases (Zhao et al. 2011b).

50.3.3 Permeability Enhancers for CNS Drug Delivery

There are a number of efflux mechanisms within the CNS that influence drug concentrations in the brain. Some of them are passive while others are active. Recently much attention has been focused on the so-called multi-drug efflux transporters: Pgp, MRP1, and BCRP, MOAT that belong to the ABC cassette (ATP-binding cassette) family (Pardridge 1998; Tamai and Tsuji 2000; Begley 2004). Cerebral capillary endothelium expresses a number of efflux transport proteins, which actively remove a broad range of drug molecules before they cross into the brain parenchyma. Pgp is the most thoroughly investigated brain efflux transport protein with broad affinity for dissimilar lipophilic and amphiphilic substrates (Tsuji and Tamai 1997). As a consequence, the therapeutic value of many promising drugs, such as protease inhibitors for HIV-1 encephalitis (ritonavir, nelfinavir, and indinavir) (Kim et al. 1998), anti-inflammatory drugs (prednesolone, dexamethasone and indomethacin) for treatment of microglial inflammation during idiopathic PD and AD (Tsuji and Tamai 1997; Perloff et al. 2004), neuroleptic agents (amitriptyline and haloperidol) (Uhr et al. 2000), analgesic drugs (morphine, beta-endorphin, and asimadoline) (Moriki et al. 2004), as well as anti-fungal agents (itraconazole and ketoconazole) (Miyama et al. 1998), is diminished, and cerebral diseases have proven to be most refractory to therapeutic interventions. Moreover, delivery of many anticancer agents (Dox, vinblastine, taxol, etc.) to the brain for treatment of brain tumors (Tsuji 1998), and drugs for treatment of epilepsy (carbamazepine, phenobarbital, phenytoin, and lamotrigine) (Potschka et al. 2002) is also restricted by the Pgp drug efflux transporter.

An emerging strategy for enhanced BBB penetration of drugs is co-administration of competitive or noncompetitive inhibitors of the efflux transporter together with the desired CNS drug. First generation low molecular weight Pgp inhibitors (cyclosporine A, verapamil, PSC833, etc.) are substrates of the drug efflux transporter, which compete for the active site with the therapeutic agent (Kemper et al. 2003).

Second generation inhibitors (LY335979, XR9576 and GF120918) are non-competitive inhibitors, which allosterically bind to Pgp, inactivating it and increasing drug transport to the brain (Kemper et al. 2004). Despite their high efficiency in cell culture models, the small therapeutic range of these inhibitors, high in vivo toxicity, and fast clearance are the main obstacles for their therapeutic application.

Recently, a new class of inhibitors (nonionic polymer surfactants) was identified as a promising component of drug formulations. These compounds are two- or three-block copolymers arranged in a linear AB or ABA structure. The A block is a hydrophilic PEG chain. The B block can be a hydrophobic lipid (BRIJs, MYRJs, Tritons, Tweens, and Chremophor), or a poly(propylene glycol) (PPG) chain (Pluronics®) (Fig. 50.2).

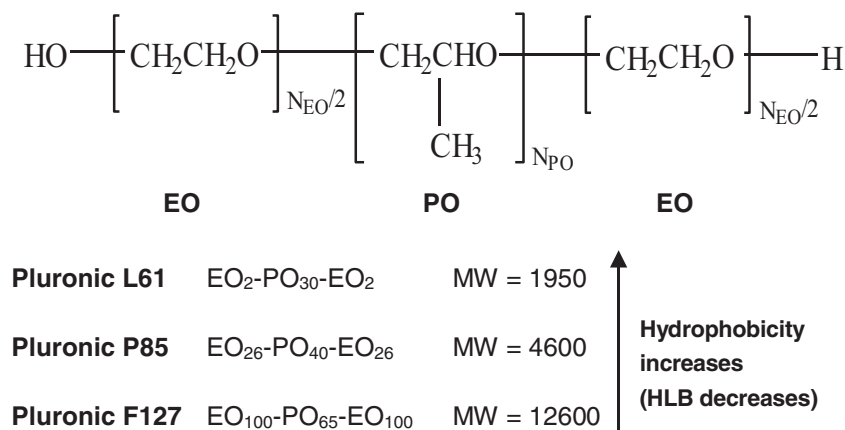
Studies in multidrug resistant (MDR) cancer cells, polarized intestinal epithelial cells, Caco-2, and polarized BMVEC monolayers provided compelling evidence that selected Pluronic® block copolymers can inhibit drug efflux transport systems (Miller et al. 1997; Batrakova et al. 1998, 1999, 2001a, b, 2003a). Specifically, in primary cultured BMVEC monolayers, used as an in vitro model of BBB, the inhibition of drug efflux systems, Pgp and MRP was associated with an increased accumulation and permeability of a broad spectrum of drugs in the BBB, including low molecular drugs (Batrakova et al. 1998, 1999) and peptides (Witt et al. 2002). These effects were most apparent at concentrations below the CMC (Miller et al. 1997; Batrakova et al. 1998). It was suggested that the unimers, are responsible for the inhibition of Pgp and MRPs efflux transport system. Incorporation of the probe into the micelles formed at high concentrations of the block copolymer decreases its availability to the cells and reduces the transport of this probe in BMVEC.

Recent findings suggest that effects of Pluronic® on drug efflux transport proteins involve interactions of the block copolymers with the cell membranes (Batrakova et al. 2001d, 2003a). It was demonstrated that a fine balance is needed between hydrophilic (PEG) and lipophilic (PPG) components

in the Pluronic® molecule to enable inhibition of the drug efflux systems (Batrakova et al. 2003b). Overall, the most efficacious block copolymers are those with intermediate lengths of PPG block and relatively hydrophobic structure (HLB < 20), such as Pluronic® P85 or L61 (Batrakova et al. 1999). The hydrophobic PPG chains of Pluronic® immerse into the membrane hydrophobic areas, resulting in alterations of the membrane structure and decreases in its microviscosity ("membrane fluidization"). Pluronic® at relatively low concentrations (e.g., 0.01 %) inhibits the Pgp ATPase activity, possibly due to conformational changes in the transport protein induced by the immersed copolymer chains in the Pgp-expressing membranes (Batrakova et al. 2001a). In particular, Pluronic® P85 displayed the effects characteristic of a mixed type enzyme inhibitor—decreasing maximal reaction rate (V_{\max}) and increasing Michaelis constant (K_m) for ATP as well as Pgp-specific substrates such as vinblastine (Batrakova et al. 2004b). The magnitude of these effects for vinblastine was as high as over 200-fold V_{\max}/K_m change (interestingly, MRP1 ATPase activity was affected less, which could explain somewhat smaller effects of Pluronic® on this transporter). In contrast, at high concentrations (e.g., 1 %), binding of Pluronic® to the membrane actually resulted in restoration of Pgp ATPase activity. This could be due to the segregation of the block copolymer molecules in the 2D clusters in the membrane, which diminishes its interactions with the transport proteins.

Various drug resistance mechanisms, including drug transport and detoxification systems, require consumption of energy to sustain their function in the barrier cells. Because of this fact, mechanistic studies have focused on the effects of Pluronic® block copolymers on metabolism and energy conservation in BMVEC (Batrakova et al. 2001a). The basis for such studies was the earlier reports that Pluronic® block copolymers can affect mitochondria function and energy conservation in the cells (Kirillova et al. 1993). Recent studies have demonstrated that exposure to Pluronic® P85 induced significant decrease in ATP levels in BMVEC monolayers (Batrakova et al. 2001d). The observed energy depletion was due to inhibition of the

Fig. 50.2 Pluronic® block copolymers with various numbers of hydrophilic EO (n) and hydrophobic PO (m) units are characterized by distinct hydrophilic-lipophilic balance (HLB). Due to their amphiphilic character these copolymers display surfactant properties including ability to interact with hydrophobic surfaces and biological membranes. In aqueous solutions at concentrations above critical micelle concentration (CMC) these copolymers self-assemble into micelles



cellular metabolism rather than a loss of ATP in the environment. The study of Rapoport et al. suggested that Pluronic® P85 can be transported into the cells and decrease the activity of electron transport chains in the mitochondria (Rapoport et al. 2000). Remarkably, the ATP depletion induced by Pluronic® appears to be tightly linked to the specific cell phenotype, since this effect is observed selectively in the cells that overexpress Pgp (as well as MRPs) (Batrakova et al. 2001c, 2004a; Kabanov et al. 2003a, b). We suggested that inhibition of ATP production in high energy-consuming cells, such as cells overexpressing Pgp, results in the rapid exhaustion of intracellular ATP, i.e., ATP depletion (Batrakova et al. 2001a). Further, our recent studies indicate that these differences were related to the differences in the major fuel sources used by MDR or sensitive cells (fatty acids vs. glucose respectively). It was shown that Pluronics affected the stage of the electron entrance in the mitochondrial electron transport chain, presumably due to the interaction of the hydrophobic block of the copolymer with fatty acids, which decreased fuel transport into the mitochondria matrix. In contrast, Pluronics exerted only mild effect on the glucose-based respiration in drug-sensitive cells. Overall, the energy depletion (decreasing ATP pool available for drug transport proteins) and membrane interactions (inhibiting of ATPase activity of drug transport proteins) are critical factors collectively contributing to a potent inhibition of the drug efflux systems by Pluronic® (Batrakova et al. 2001a) (Fig. 50.3).

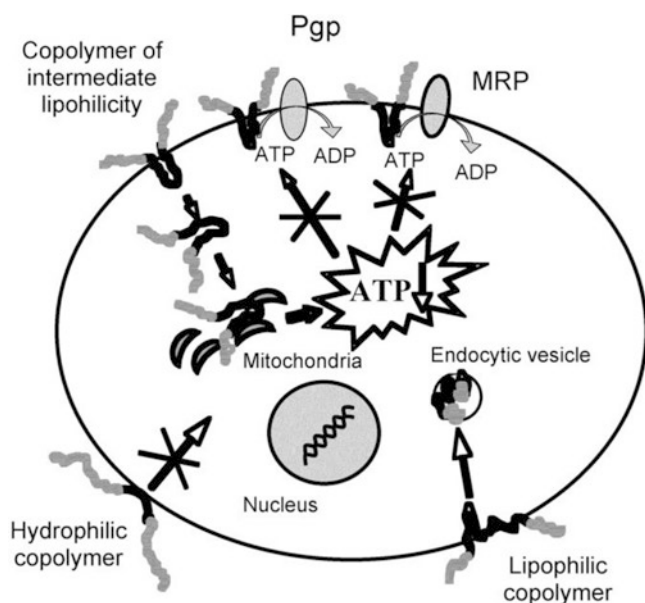


Fig. 50.3 Schematic illustrating twofold effects of Pluronic® block copolymers with intermediate lipophilicity on Pgp and MRPs drug efflux system. These effects include (a) decrease in membrane viscosity (“fluidization”) resulting in inhibition of Pgp and MRPs ATPase activity, and (b) ATP depletion in BMVEC. Extremely lipophilic or hydrophilic Pluronic® block copolymers do not cross the cellular membranes and do not cause energy depletion in the cells

The effect of Pluronic® P85 on drug transport to the brain was evaluated in animal experiments (Batrakova et al. 2001b). Brain delivery of a Pgp substrate, digoxin, administered intravenously in the wild-type mice expressing functional Pgp, was greatly enhanced in the presence of Pluronic® P85. It was found that the digoxin brain/plasma ratios in the Pluronic® treated animals were practically the same as those in the knockout mice, an animal model that is deficient in both *mdr1a* and *mdr1b* isoforms of Pgp. This suggests that co-administration of Pluronic® with the drug in mice resulted in inhibition of Pgp in the BBB of the wild-type animals (Batrakova et al. 2001b).

One possible concern in these studies is that by virtue of inhibiting the ATP in BMVEC, the copolymer may display toxic effects on the BBB. However, the ATP depletion was found to be transient; following removal of the block copolymers from BMVEC monolayers the initial ATP levels were restored (Batrakova et al. 2001a). Although there were significant decreases in cellular ATP following Pluronic® treatment, even during peak depletion of ATP by Pluronic® there was no evidence of loss of barrier functions of BBB as demonstrated using ³H-mannitol as a permeability marker both in vitro and in vivo (Batrakova et al. 1998, 2001a). Moreover, Pluronic® does not affect the glucose transporter, GLUT1, and only slightly inhibits the lactate transporter, MCT1, the two transporters playing an important role in the brain metabolism (Batrakova et al. 2004a). Pluronic® also does not inhibit the amino acid transporters, LAT1, CAT1, and SAT1, in the BBB (Zhang et al. 2008). A histochemical examination of the tissue sections obtained from animals treated with Pluronic® revealed no pathological changes in the BBB. Importantly, no cerebral toxicity of any kind has been observed in the human Phase I and Phase II studies of SP1049C, a Pluronic®-based formulation of Dox to treat MDR tumors (Danson et al. 2004; Valle et al. 2004). It is possible, that this formulation, evaluated in human trials, can be adopted for use with CNS drugs to enhance drug delivery to the brain.

50.3.4 Chemical Modification of Polypeptides with Fatty Acids and Amphiphilic Block Copolymers

Delivery of potentially therapeutic polypeptides and proteins to the brain is significantly hampered by the BBB. The hydrophilicity, the lack of stability due to enzymatic or chemical degradation, and the lack of transport carriers capable of shuttling polypeptides across cell membranes all play a part in precluding most polypeptides from transport into the brain (Lee 1991). Several approaches to modify polypeptides to alter their BBB permeability have been attempted. First, conjugation of proteins with wheatgerm agglutinin (Raub and Audus 1990; Banks and Broadwell 1994) or cationic groups

("cationization") (Kumagai et al. 1987) (Triguero et al. 1989, 1991) was shown to enhance delivery of polypeptides to the brain through adsorptive endocytosis. The cationized polypeptides demonstrated enhanced permeability and greater accumulation in the brain *in vitro* and *in vivo*. However, toxicity and antigenicity of cationized polypeptides could be an issue for their medical use (Bickel et al. 2001). Further, vectors targeting polypeptides to insulin and Tf receptors (monoclonal antibodies) have been considered to enhance passage of these compounds to the brain through receptor-mediated endocytosis (Bickel et al. 2001; Frank et al. 1986; Duffy and Pardridge 1987; Friden et al. 1991). Thus, potential therapeutic polypeptides, basic fibroblast growth factor and brain-derived neurotrophic factor, were conjugated to OX26, which resulted in increased entry of these polypeptides to the brain and increased drug neuroprotective effects (Zhang and Pardridge 2001; Song et al. 2002).

Another approach, artificial hydrophobization of polypeptides with a small number of fatty acid residues (e.g., stearate or palmitate) has been shown to enhance cellular uptake (Kabanov et al. 1989a). Specifically, this technique involves point modification of lysine or N-terminal amino groups with one or two fatty acid residues per protein molecule. As a result of such modification, the protein molecule remains water-soluble but also acquires hydrophobic anchors that can target even very hydrophilic proteins to cell surfaces (Slepnev et al. 1995). To obtain low and controlled degrees of modification, a system of reverse micelles of a surfactant, sodium bis-(2-ethylhexyl)sulfosuccinate (Aerosol OT) in octane was used as a reaction medium (Kabanov et al. 1987b) (Fig. 50.4). Over a dozen water-soluble polypeptides (enzymes, antibodies, toxins, cytokines) have been modified by this method (Kabanov et al. 1987a, 1989a; Alakhov et al. 1990; Chekhonin et al. 1991; Robert et al. 1993, 1995; Slepnev et al. 1995). Further studies of interactions of the fatty acylated polypeptides with cells were also conducted (Kabanov et al. 1989a; Hashimoto et al. 1989; Alakhov et al. 1990; Colsky and

Peacock 1991; Melik-Nubarov et al. 1993; Slepnev et al. 1995; Ekrami et al. 1995; Chopineau et al. 1998; Kozlova et al. 1999). Modification of water-soluble polypeptides, such as HRP, resulted in enhanced polypeptide binding with the cell membranes and internalization in many cell types (Slepnev et al. 1995). The point modification does not inhibit the specific activity of the polypeptides in the cells. To the contrary, in selected cases, when polypeptides are known to exhibit their effects in cells through binding with a cell surface receptor (e.g., *Staphylococcus aureus* enterotoxin A and recombinant α -interferon) the modification resulted in significant (10 to 100-times) enhancement of these effects (Alakhov et al. 1990; Kabanov et al. 1992b). The increased activity of selected modified polypeptides in cells can possibly be explained by their concentration at the cell membrane, which promotes their binding with receptors (Kabanov et al. 1992b). Furthermore, insulin modified with one palmitic acid residue produced a prolonged hypoglycemic effect compared to the native insulin after intravenous injection and was shown to be less immunoreactive than the native insulin (Hashimoto et al. 1989).

The relevance of this technology to CNS delivery emerged from the studies by Chekhonin et al. (1991, 1995). Those studies showed that modification of the Fab fragments of antibodies against gliofibrillar acid protein (GFAP) and brain specific alpha 2-glycoprotein (alpha 2GP) with stearate led to an increased accumulation of the modified Fab fragments in the brain in a rat. Furthermore, a neuroleptic drug conjugated with the stearylated antibody Fab fragments was much more potent compared to the free drug. In comparison, fatty acylated Fab fragments of non-specific antibodies did not accumulate in the brain but instead accumulated in the liver, while stearylated Fab fragments of brain-specific antibodies displayed preferential accumulation in the brain (Chekhonin et al. 1991). The mechanism by which the stearylated Fab-fragments were directed to the brain was not elucidated at that time. It was not clear also whether the Fab

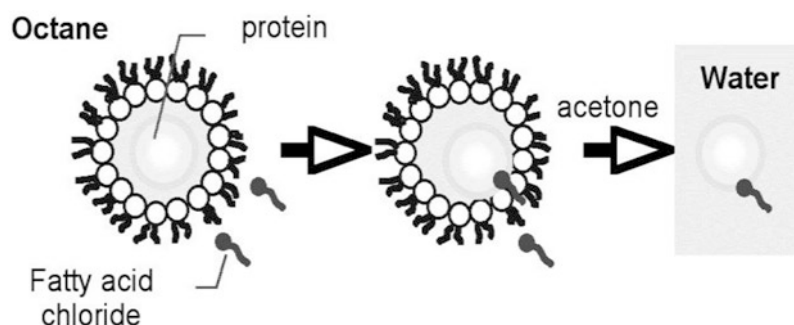


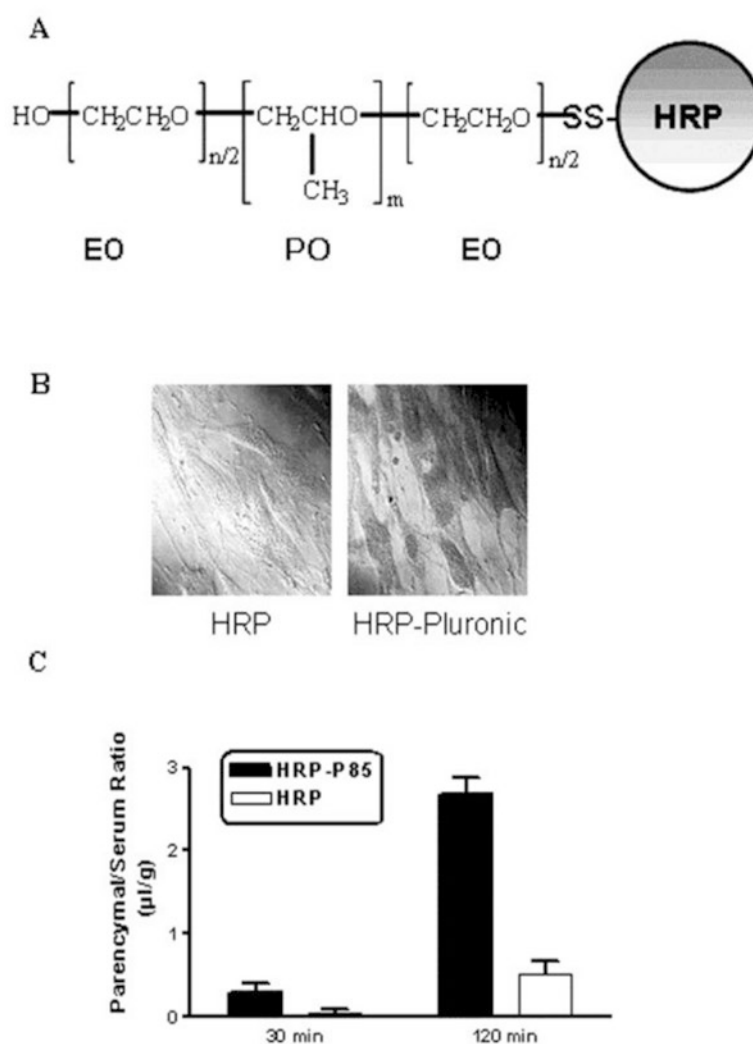
Fig. 50.4 Chemical modification of the protein with a water-insoluble reagent in the reverse micelles of Aerosol OT in octane. (1) Protein molecule incorporates into the inner water pool of the reverse micelle, acquiring a monolayer cover of the hydrated surfactant molecules. (2) The modifying reagent incorporates into the surfactant layer of the

micelle coming into contact with the modified group of the protein. (3) Following the completion of the reaction the modified protein is precipitated and the surfactant and excess of the reagent are removed by adding cold acetone. Proteins modified with fatty acid residues with controlled and low degree of modification are obtained

fragments actually crossed the BBB or remained associated with the luminal surface of the brain capillaries. Subsequent studies using bovine brain microvessel endothelial cells (BBMEC) as an *in vitro* model of BBB (Chopineau et al. 1998) demonstrated that stearylation of ribonuclease A (approx. 13.6 kDa) increases the passage of this enzyme across the BBB by almost tenfold. Of the three fatty acid derivatives analyzed—myristic, palmitic and stearic, the latter was the most active. A possible mechanism for the entry of the fatty acylated polypeptides to the brain is adsorptive endocytosis. Given the characteristics of the BBB transport for nonessential free fatty acids as reviewed elsewhere, (Banks et al. 1997) it is unlikely that the modified polypeptides are able to use those transporters. Thus, use of free fatty acid receptor mediated transport by the modified polypeptides is not likely. However, if such a pathway is feasible, it should be more pronounced when the essential fatty acids, such as linoleic, are used to modify polypeptides (Edmond 2001; Edmond et al. 1998).

A similar principle was used in the case of a protein modification with Pluronic® block copolymers (Fig. 50.5); the designed conjugates had different balance between hydrophilic and lipophilic properties (Batrakova et al. 2005). The cell binding and transport of Pluronic® P85 and L121 modified HRP were increased up to 6 and 10 times, respectively, compared with the native HRP. A method of conjugation using biodegradable and non-biodegradable links was also varied. As expected, the modification of the protein via a biodegradable link (Fig. 50.5a) resulted in the most effective transport of the protein across the brain microvessel endothelial cell monolayers. It is likely, that being highly hydrophilic the HRP molecule requires a highly lipophilic moiety to obtain the optimal hydrophilic-lipophilic balance of the conjugate. Such a lipophilic group has a tendency to remain associated with the cellular membrane as an anchor. Therefore, a biodegradable link allowed the polypeptide molecule to separate from its lipophilic moiety and enter the brain after the conjugate passed membranes of the barrier cells. Data obtained by confocal

Fig. 50.5 Effect of HRP modification with Pluronic block copolymer on transport across the BBB in an *in vitro* and *in vivo* models. (a) HRP conjugated with Pluronic P85 via the biodegradable bond; (b) confocal microphotograph of BBMEC monolayers treated with rhodamine-labeled HRP and Pluronic-HRP for 2 h; (c) blood-to-brain transport of HRP and Pluronic-HRP in mice



microscopy visualizes this effect. Modification of the protein with Pluronic® P85 via biodegradable link drastically enhanced the transport of HRP into the cells and its accumulation in the cytoplasm, nuclei, and other cellular organelles (Fig. 50.5b). It was suggested that the protein conjugate was initially bound to the cellular membrane through the Pluronic® moiety incorporated into the lipid bilayer followed by cleavage of the HRP molecule from the Pluronic® anchors. In vivo studies demonstrated that (1) hydrophobization with stearyl group or (2) amphiphilic modification with a Pluronic® block copolymer had increased the rate of HRP penetration across the BBB and increased accumulation of HRP by the brain (Fig. 50.5c). Overall modification with Pluronic® block copolymers appeared to be more promising for HPR delivery to the brain. In this case the permeability of modified protein in the BBB in vivo was increased almost fourfold with no statistically significant effects on the protein peripheral pharmacokinetics or entry into the parenchymal space.

50.4 Review Questions

- What are the main features of the BBB that restrict drug transport to the brain?
- Describe the properties of polymers that are useful for drug delivery systems.
- What are the advantages of nanoscaled polymeric carriers?
- List types of nanocarriers for drug delivery. Describe their structure, principal differences, advantages and limitations.
- Describe cell-mediated drug delivery to the CNS.
- Explain how the drug efflux transporters affect drug transport to the brain? List examples of drug efflux transporters expressed in the BBB.
- Describe three generations of inhibitors of drug efflux transporters in the BBB.
- Describe the effects of Pluronic block copolymers on drug efflux transporters in the BBB.
- How is the chemical modification of polypeptides with fatty acids and amphiphilic block copolymers applied to increase delivery of polypeptides to CNS?
- Which of the following molecules can penetrate across the BBB through passive diffusion?
 - Lipophilic low molecular weight compounds*
 - Hydrophilic low molecular weight compounds
 - Lipophilic high molecular weight compounds
 - Hydrophilic high molecular weight compounds
- What size of polymer nanocarriers is the most appropriate for drug delivery?
 - 0.1–1 nm
 - 1–100 nm
 - 100–1000 nm
 - 1–100 μm
- Which types of drugs can be incorporated into polymeric micelles?
 - lipophilic compounds*
 - hydrophilic compounds
 - charged compounds
 - proteins compounds
- What does CMC mean?
 - colloidal microemulsion complex
 - cell monocarrier
 - critical micelle concentration*
 - contrast multi-compound
- Which polymer is used to stabilize nanoparticles in an aqueous dispersion?
 - polystyrene
 - polymethacrylic acid
 - DNA
 - PEG
 - polycyanoacrylate
- Which type of cells can be used for cell-mediated delivery?
 - brain microvessel endothelial cells
 - neurons
 - macrophages*
 - astrocytes
 - erythrocytes

50.5 Answers

- The low permeability of the BBB is attributed, in large part, to the brain microvessel endothelial cells (BMVEC), which form tight extracellular junctions and have low pinocytotic activity. There is also an enzymatic barrier to drug transport in the BMVEC and overexpression of drug efflux transporters on the luminal side of BMVEC.
- There are several fundamental properties of polymers that are useful in solving drug delivery problems. First, polymers can be designed to be intrinsically multifunctional and can, for example, be combined either covalently or non-covalently with drugs to overcome multiple problems such as solubility, stability, permeability, etc. Second, polymers can be easily modified with various targeting vectors to direct drugs to specific sites in the body. Third, polymers can be designed to be environmentally responsive materials, allowing for the controlled and sustained release of a drug at its site of action. Finally, polymers themselves can be biologically active, and this property can be exploited in order to modify the activity of various endogenous drug transport systems within the body to improve delivery and, therefore, drug performance.
- Nanoscaled polymeric carriers have several advantages. Because these systems often exhibit similarity in their size

and structure to natural carries such as viruses and serum lipoproteins, they offer multifaceted specific properties in drug delivery applications. They entrap small drugs or biopharmaceutical agents, such as therapeutic proteins and DNA, and can be designed to trigger the release of these agents at the target site. Moreover, they can be targeted not only to the particular organ or tissue, but to a particular cell or even an intracellular compartment.

4. There are various types of vehicles proposed for transport of neuropharmaceuticals across the BBB: liposomes, degradable nanoparticles, nanospheres, nanosuspensions, polymeric micelles, nanogels, block ionomer complexes, nanofibers and nanotubes, and dendrimers. Each of them has advantages and limitations. The overall advantages of polymer-based nanocarriers are:
 - a. Increase drug solubility
 - b. Improve drug pharmacokinetics and biodistribution
 - c. Increased protection of the drug in the blood stream and long circulation
 - d. Target solid tumors by enhanced permeability and retention (EPR) effect
 - e. Decrease side effects (diminish drug extravasation into normal tissues)
 - f. Ability to vectorize the particle and capability of receptor-mediated transport across the BBB
 - g. Drug resistance
 - h. Triggered release

The limitations may include: (a) disassembly upon dilution in the blood stream for non-crosslinked aggregates; (b) low loading capacity; (c) entrapment in the mononuclear phagocytic system as present in the liver and spleen; (d) toxicity (especially for positively charged polymer nano-carriers)

5. A distinct case of the vehicle-mediated CNS drug delivery employs specific cells carriers that can incorporate micro- and nano-containers (such as liposomes, micelles, and block-ionomer complexes) loaded with drugs and act as perfect Trojan horses by migrating across the BBB and carrying drugs to the site of action. It is documented that many neurological diseases, have in common an inflammatory component. The process of inflammation is characterized by extensive leukocytes (neutrophils and monocytes) recruitment. These cells have a unique property of migrating toward the site of inflammation via the processes known as diapedesis and chemotaxis.
6. Cerebral capillary endothelium expresses a number of efflux transport proteins, which actively remove a broad range of drug molecules before they cross into the brain parenchyma. Among them are: Pgp, MRP1, and BCRP, MOAT that belong to the ABC cassette (ATP-binding cassette) family.
7. The first generation is a low molecular weight Pgp inhibitors (cyclosporine A, verapamil, PSC833, etc.), which

compete for the active site with the therapeutic agent. The second generation inhibitors (LY335979, XR9576 and GF120918) are non-competitive inhibitors, which allosterically bind to Pgp, inactivating it and increasing drug transport to the brain. Despite their high efficiency in cell culture models, the small therapeutic range of these inhibitors, high in vivo toxicity, and fast clearance are the main obstacles for their therapeutic application. The third generations is a new class of inhibitors (nonionic polymer surfactants). These compounds are two- or three-block copolymers arranged in a linear AB or ABA structure. The A block is a hydrophilic PEG chain. The B block can be a hydrophobic lipid (BRIJs, MYRJs, Tritons, Tweens, and Chremophor), or a poly(propylene glycol) (PPG) chain (Pluronics®).

8. Recent findings suggest that effects of Pluronic® on drug efflux transport proteins involve interactions of the block copolymers with the cell membranes. Pluronic® at relatively low concentrations (e.g., 0.01 %) inhibits the Pgp ATPase activity, possibly due to conformational changes in the transport protein induced by the immersed copolymer chains in the Pgp-expressing membranes. In contrast, at high concentrations (e.g., 1 %), binding of Pluronic® to the membrane actually resulted in restoration of Pgp ATPase activity. In addition, Pluronic® block copolymers can affect mitochondria function and energy conservation in the cells. Recent studies have demonstrated that exposure to Pluronic® P85 induced significant decrease in ATP levels in BMVEC monolayers. Overall, the energy depletion (decreasing ATP pool available for drug transport proteins) and membrane interactions (inhibiting of ATPase activity of drug transport proteins) are critical factors collectively contributing to a potent inhibition of the drug efflux systems by Pluronic®.
9. Artificial hydrophobization of polypeptides with a small number of fatty acid residues (e.g., stearate or palmitate) has been shown to enhance cellular uptake in BMVEC. This effect can possibly be explained by the increased concentration of the polypeptides at the cell membrane, which promotes their binding with receptors.

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Gang Zhao, Xin Wei, and Dong Wang

Abstract

Macromolecular therapeutics have been developed to improve the efficacy and safety profile of varied therapeutic agents for different disease more than 50 years ago. *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymers as a drug delivery platform have been extensively studied and applied in the clinical trials. Recently, the application of HPMA copolymers in the treatment of non-cancerous diseases such like infectious diseases and musculoskeletal diseases has also been explored widely. In this chapter, different formulations of macromolecular therapeutics will be discussed. The design principles illustrated in the development of HPMA copolymer drug conjugates are also valid for other hydrophilic polymers, and they will be the principal focus of our discussion. Active targeting of HPMA copolymers based on targeting moieties and passively targeting HPMA copolymers based on the Enhanced Permeability and Retention (EPR) effect for cancer disease and Extravasation through Leaky Vasculature and subsequent Inflammatory cell-mediated Sequestration (ELVIS) mechanism for inflammation disease will also be discussed in this chapter.

Keywords

Cancer therapy • Central nervous system (CNS) • Hydroxypropyl methacrylamide HPMA copolymers • Targeting

51.1 Introduction

Water-soluble polymers have been widely used as carriers of drugs, genes, oligonucleotides and for modification of proteins, liposomes, surface of biomaterials. Drug conjugated to synthetic macromolecules was initiated by Jatzkewitz 60 years ago when researchers first used the water-soluble polymer polyvinyl pyrrolidone as a drug depot for the biologically active primary amine mescaline (Jatzkewitz 1955). Since then, the field was expanded and developed dramatically beyond depot effects. Numerous water-soluble polymer-drug

conjugates, PEG-protein conjugates, lysosomotropic polymer-drug conjugates and block copolymer micelles were synthesized in the 1960s and 1970s (De Duve et al. 1974; Abuchowski et al. 1977). “Polymer therapeutics” was coined by Ruth Duncan to describe polymeric drugs, polymer protein conjugates, block copolymer polymeric micelles to which a drug is covalently bound and polyplexes developed as non-viral vectors for gene interfering ribonucleic acid (siRNA) delivery (Duncan 2003). The rationales for polymer-drug conjugates include prolong the blood circulation time, reduce the side-effects of the drug, improve drug targeting and overcome drug resistance. Conjugation of low molecular weight drugs to polymers would prolong their resident time in blood circulation resulting in enriched bioavailability. The delivery system based on synthetic water-soluble polymer would render hydrophobic drugs better solubility and limit the cellular uptake to the endocytic route. Based on EPR

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(Enhanced Permeability and Retention) effect (Matsumura and Maeda 1986), polymer conjugation would facilitate the passive targeting of drugs to the solid tumor (Maeda 2010). The newly discovered ELVIS (Extravasation through Leaky Vasculature and Inflammatory cell-mediated Sequestration) mechanism (Yuan et al. 2012; Wang and Goldring 2011; Ren et al. 2011, 2014) explains macromolecular therapeutics' preferentially accumulate in inflammatory tissues (Liu et al. 2010) in general and inflammatory arthritis in particular (Wang et al. 2004; Yuan et al. 2012; Liu et al. 2008).

In this chapter, we will focus mainly on the development of *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymer drug conjugates as the design principles illustrated are also valid for other hydrophilic polymers. HPMA copolymers based macromolecular therapeutics are active against many cancer models and have been evaluated in several clinical trials (Kopeček et al. 2000). In 1994, HPMA copolymer-doxorubicin (FCE28068) was the first HPMA copolymer drug conjugate to enter clinical evaluation (Vasey et al. 1999). To achieve the controlled release and better solubility of paclitaxel, Pharmacia developed the HPMA copolymer-paclitaxel

(PNU166945) (Meerum Terwogt et al. 2001). Beyond oncology, HPMA copolymer is also applied to treat rheumatoid arthritis when conjugated with glucocorticoids (Yuan et al. 2012). In addition, there is great potential for these technologies to be extrapolated into neurological applications.

51.2 Structure of Macromolecular Therapeutics

The structure of macromolecular therapeutic agents can vary due to different types of the payload (Duncan 2003). While micelles, dendrimers, liposomes, nanoparticles and polymer-drug conjugates all share the concept of macromolecular therapeutics, they can be very different in their structures (Vikas et al. 2015). Different delivery systems are also applied to deliver different candidates because of their own unique properties. For example, macromolecular payloads prefer the liposomes, nanoparticles and micelle delivery systems, while polymeric delivery systems (e.g., HPMA copolymers) favor small molecular payloads (Fig. 51.1).

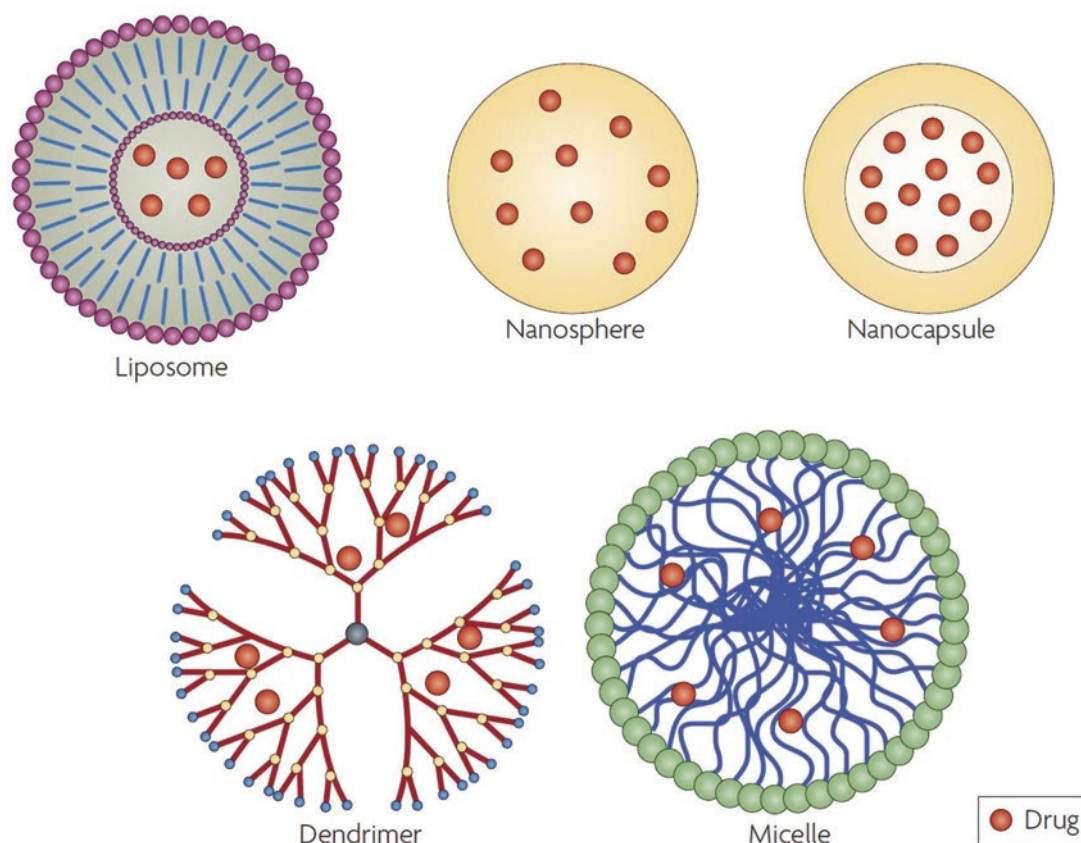


Fig. 51.1 A schematic representation of some macromolecular drug delivery systems. (a) Liposomes (~100–400 nm) are small spherical artificial vesicles typically made with lipid bilayers, (b) Nanosphere (~20–200 nm) are typically made with biodegradable polymers for sustained drug release, (c) Nanocapsules (~10–1000 nm) can encapsulate relatively large amounts of drugs and nucleic acids such as DNA, microRNA,

siRNA (small interfering RNA) and shRNA (small hairpin RNA), (d) Dendrimers (~3–20 nm) are monodisperse macromolecules that can be used to encapsulate or covalently conjugate drugs, targeting moieties and imaging agents, (e) Micelles (~10–100 nm) are self-assembled amphiphilic particles that can encapsulate both lipophilic or lipophobic drugs stabilized by surfactants (Adapted from Orive et al. (2009), with permission)

51.2.1 Liposomes

Liposomes have been defined as a vesicle for drug delivery since the 1960s (Bangham et al. 1965). Liposomes are composed of phospholipid membrane surrounded by the aqueous environment. The structure of the liposomes membrane is a double layer with fluidity. The shape of liposomes is basically spherical. Liposomes can be categorized into multilamellar vesicle (MLV) (Gentile et al. 2014), small unilamellar the liposome vesicle (SUV) (Lokappa and Ulmer 2011) and large unilamellar vesicle (LUV) (Moyano et al. 2008) according to the number of lipid layers and the size of the liposome. Most of the clinically used liposome formulations have to be decorated with inert water-soluble polymers (e.g., PEG, HPMA copolymer, etc.) to ensure their stealth against the mononuclear phagocyte system (MPS). While this modification may enlarge the size of the liposome, its half-life in circulation will be improved. The surface decorating polymers can also be used to introduce various targeting moieties or imaging probes. Liposome size can vary from 100 to 400 nm in diameter and plays a critical role in complement activation and MPS clearance of the delivery system (Drummond et al. 1999; Devine et al. 1994). Vesicles larger than 100 nm require additional strategies to prevent surface opsonization (Koo et al. 2005).

51.2.2 Nanoparticles

Nanoparticles can be categorized as drug nanoparticles, solid nanoparticles, polymer-based nanoparticles, lipid-based nanoparticles and nanocapsules. Drug nanoparticles are the dispersion of water-insoluble drug particles of drug formulations in the nanosize range in an aqueous environment. Drug nanoparticles can be achieved by breaking down the bigger particles by high-pressure homogenization method or by special crystallization techniques. Polymer-based nanoparticles are commonly composed of poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(lactic acid-co-glycolic acid) (PLGA), poly(ϵ -caprolactone) (PCL), and poly(methylmethacrylate) (PMMA). Chemical synthesis, salting-out, emulsification-diffusion, nanoprecipitation and freeze-drying methods are used to obtain polymer-based nanoparticles. Lipid-based nanoparticles mostly composed of fats or waxes and can be obtained by homogenization. For certain applications, nanoparticles may be tailored into different shape: namely sphere nanoparticles (Kocbek et al. 2007), cube nanoparticles (Verma and Ahuja 2015), rod-like nanoparticles (Wang et al. 2014), hollow spherical nanoparticles (Chen et al. 2014) and random shape nanoparticles.

51.2.3 Micelles

A formulation of micelle can be achieved by the self-assembly (aggregation) of amphipathic macromolecules

when they are added to aqueous solution. Critical micelle concentration (CMC), is defined as the concentration above which the micelle will form. The amphipathic macromolecules used for micelle formulations can be classified into three types: 1. The molecule is composed of the one polar end (hydrophilic group) and one non-polar end (hydrophobic group) (Koizumi et al. 2006). 2. Two polar ends with the non-polar center (Chen et al. 2013). 3. Two non-polar ends with the polar center (Nederberg et al. 2011). Shapes of micelles can be sphere (Chen et al. 2013), ellipsoid cylinder and monolayer micelle (Wiedwald et al. 2010). To improve micelle stability, Liu et al. prepared poly(2-cinnamoyl ethyl methacrylate-*b*-acrylic acid) which forms polymeric micelles in water (Henselwood and Liu 1997; Liu 1997) and cross-linked the core segment by photochemically induced polymerization of the cinnamoyl side groups which decreases the free volume of the core. The core density may increase and the size of the micelles might decrease because of the crosslinking.

51.2.4 Dendrimers

Dendrimers (Fig. 51.2) are highly uniformed, branched or star-shaped macromolecules. Dendrimers can be synthesized by divergent (Li et al. 2014) or convergent approaches (Kushwaha and Tiwari 2013). They are often of uniform molecular weight and very low polydispersities. Dendrimers also have modifiable surface functional group as well as internal cavities (Jansen et al. 1994). For example, Newkome et al. (Newkome et al. 1991) have synthesized a dendrimer containing hydrophobic interior and hydrophilic surface functionality. During or after synthesis of dendrimers, the drug molecules can be physically entrapped or chemically conjugated to the dendrimer (Thanki et al. 2013).

51.2.5 Polymer-Drug Conjugates

Polymeric-drug conjugates, are generally consisted of a polymeric backbone, active drugs, targeting moiety and signaling moiety, as illustrated in Fig. 51.3. The primary structure of the conjugates and the chemical bonds by which the components are linked to the backbone can be designed base on the conjugates applications (Miller et al. 2008), drug properties (Liu et al. 2010; Wang et al. 2007a) and biological rationales (Kopeček 1984; Kopeček et al. 1991; Putnam and Kopeček 1995). The shape of HPMA-copolymer conjugates (Nakamura et al. 2015), the length of the backbone (Nakamura et al. 2015), the number of the side chain and the length of the side chain (Kopeček et al. 2000) can be managed to some degree by different synthetic strategies. Reversible addition-fragmentation chain transfer (RAFT) polymerization is commonly used to manage the molecular

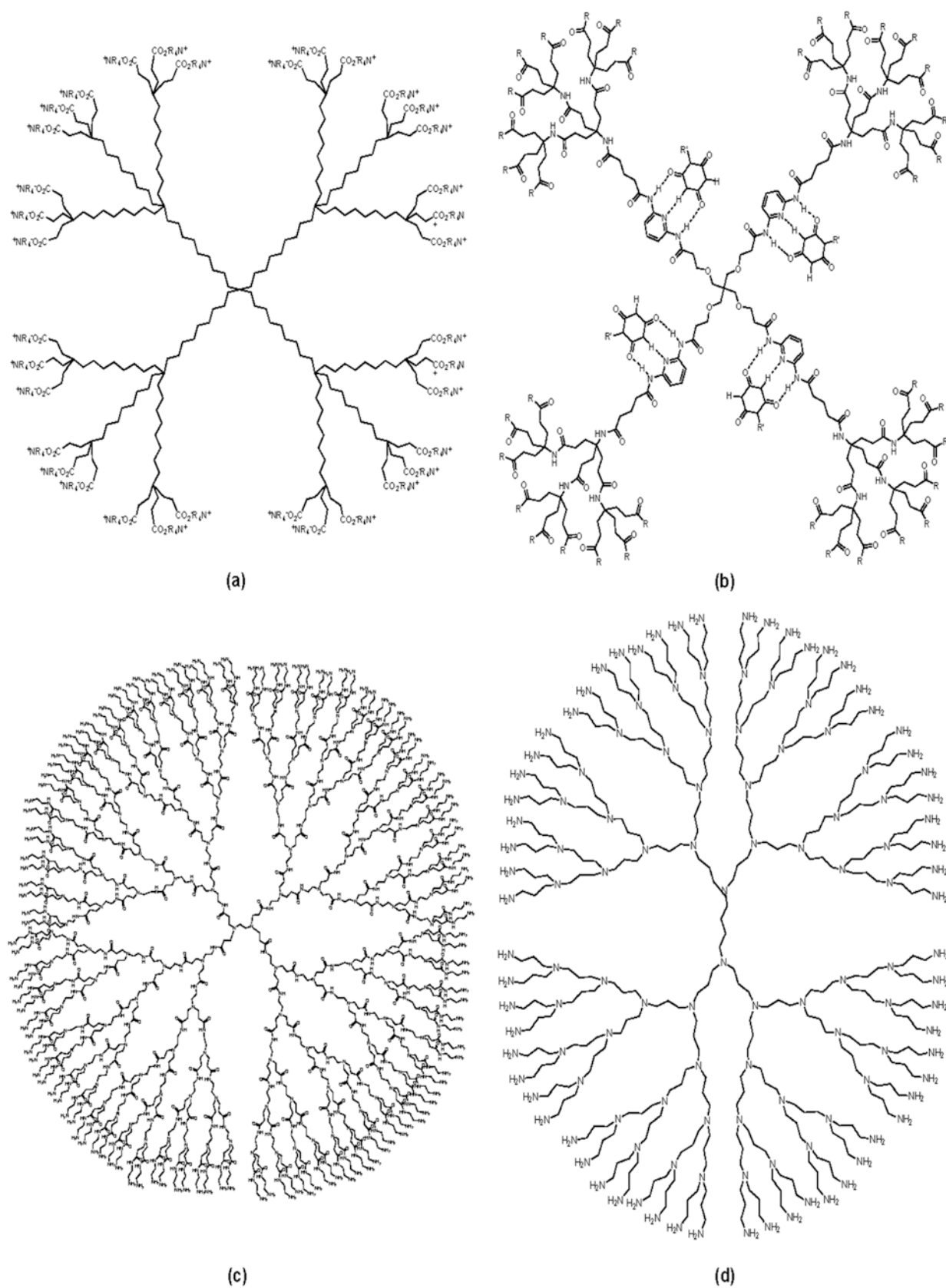


Fig. 51.2 Different families of dendrimers. (a) Unimolecular micelle. (b) Newkome's dendrimer with internal binding units. (c) PAMAM dendrimer. (d) POPAM dendrimer. (Adapted from Patri et al. (2002), with permission)

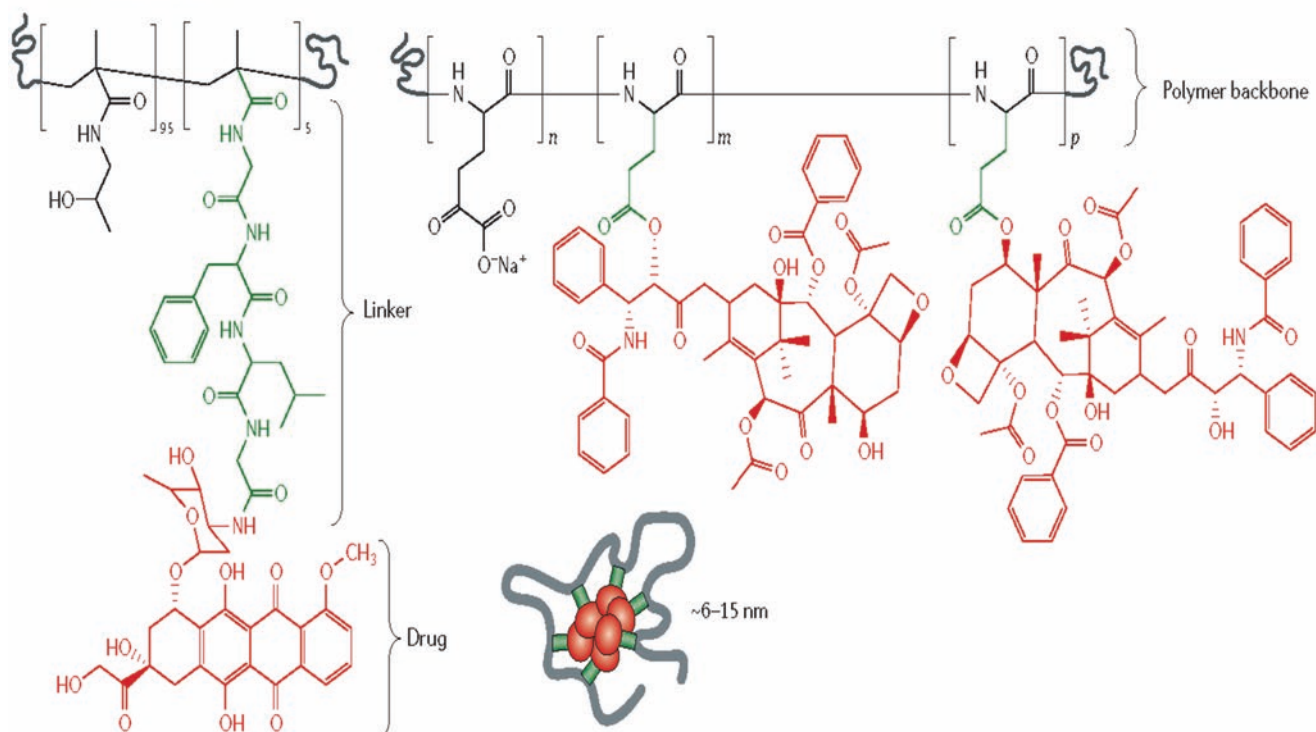
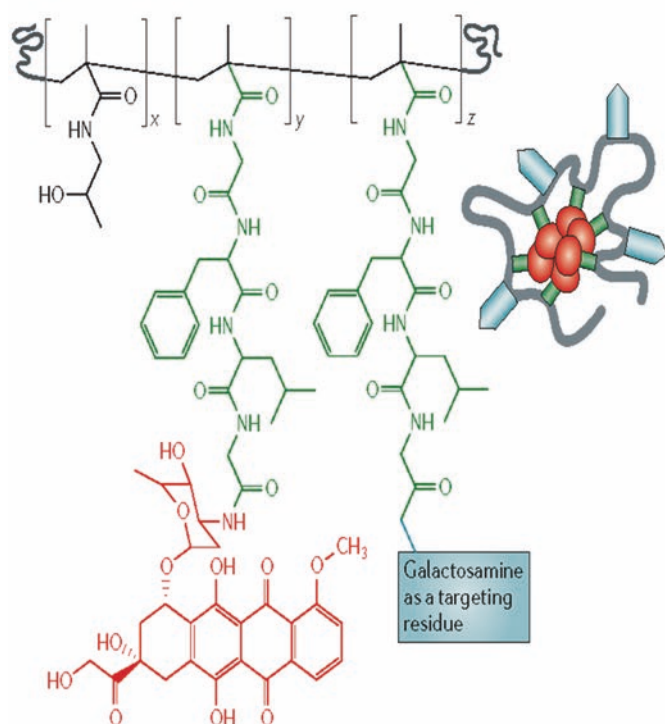
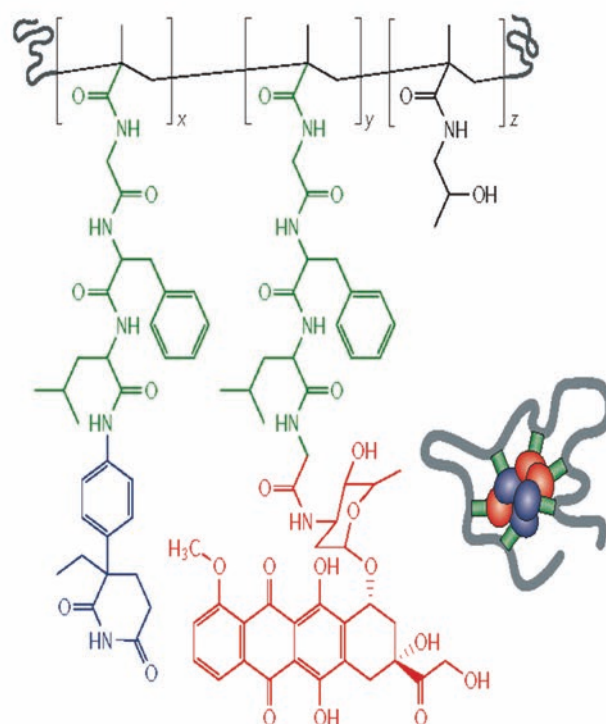
a Polymer-drug conjugate**b Targeted conjugate****c Polymeric combination therapy**

Fig. 51.3 Each panel shows both the detailed chemical structure and a cartoon of the general structure. The polymer backbone is shown in black, linker region in green, drug in red and additional components (for example, a targeting residue) in blue. (a) Two examples of more ‘simple’ polymer–drug conjugates containing doxorubicin (left) and

paclitaxel (right) that have progressed to clinical trial. (b) A multivalent receptor-targeted conjugate containing galactosamine (light blue) to promote liver targeting. (c) Polymeric combination therapy containing the aromatase inhibitor aminoglutethimide (red) and doxorubicin (blue) (Adapted from Duncan (2006), with permission)

weight and polydispersity of the polymer conjugates which will affect the distribution, accumulation and the elimination of the conjugates on living organism (Moad et al. 2008; Vikas et al. 2015).

51.3 Targetability of HPMA Copolymer Conjugates

The targeting of the polymeric conjugates to pathology is essential for a drug delivery system. The drug targeting mechanisms are generally categorized into active targeting and passive targeting (Zhou and Kopeček 2012).

51.3.1 Active Targeting

Active targeting is the method using the target moiety, such as antibody, specific low molecular weight ligands, etc., to direct the payload to preferentially accumulate at the site of pathology. Active targeting always takes advantages of the different pathological features of the lesion when compared to the normal tissue. The preferential interaction of the antigen expressed on the cell surface of a particular disease lesion and the targeting moiety of the delivery system will facilitate the active targeting process. As one may speculate, this strategy can lower the side effect of the drug by reducing unnecessary drug exposure to the normal tissues.

51.3.1.1 Antibody Mediated Targeting

Conjugate bound with specific antibodies is called antibody-targeted polymer conjugates. While such conjugation would significantly improve the polymer-drug conjugates' diseases targeting ability, the original antigen binding affinity of the antibodies conjugated to polymeric drug carriers can be compromised due to the chemical modification process (Tappertzhofen et al. 2014). Recently, a new method has been developed, attempting to address this problem (Pechar et al. 2011). A heterodimeric coiled coil structure can be formed with a highly specific interaction between peptide K ((VAALKEK)₄) and peptide E ((VAALEKE)₄). Benefiting from such molecular recognition, a cytostatic drug-bearing HPMA copolymer peptide E conjugate was synthesized, and self-assembled with peptide K tagged BCL1 leukemia cells specific scFv of B1 mAb, to obtain an antibody-polymer drug conjugate with largely intact antibody targeting ability.

51.3.1.2 Folate Mediated Targeting

Folate shows high affinity to its receptors, folate binding proteins. This unique property has established folate as a popular active targeting moiety to assist the intracellular transportation and drug/drug delivery systems (Campbell et al. 1991) (Kranz et al. 1995). The folate receptors are highly expressed on the cancer cells (Yoo and Park 2004), which would facili-

tate the active targeting of folate bearing polymer conjugates to the cancer lesion. The conjugates can be transported intracellularly via receptor-mediated endocytosis. Unlike liposomes, micelles and nanoparticles, the hydrophobicity of folate affect polymer-drug conjugate more because it can be hidden within the polymer random coil but not on the surface like in the other nanomedicine formulations. To overcome this limitation, researchers introduced the positive charges into the HPMA copolymer conjugates (Niewoehner et al. 2014). Folate receptor (FR) positive Hela cells were used to test the drug-cell binding affinity. In the presence of folate-modified cationic HPMA copolymers, FR antibody bounded cells decreased from 71.2% to only 34.0%. This indicates that the positive charge could probably amplify the binding efficiency of folate to its receptor due to the close proximity of conjugates to the cell surface by the electronic adhesion. To avoid non-specific binding by the positive charge in the circulation, the researchers applied charge shielding/deshielding approach. Charge shielding groups 2,3-dimethylmaleic anhydride (DMA) can be specifically hydrolyzed at tumor extracellular environment (pH 6.8). Then the conjugates show the charge-reversible ability, leading to the faster endocytosis into the cells of cancerous lesion than the normal tissue (pH 7.4). This technique may improve the folate-mediate targeting of HPMA copolymer conjugates to cancer.

51.3.1.3 Transferrin Mediated Targeting

Similar to folate, transferrin is another well-studied targeting moiety because of the overexpression of its receptor on the cancer cells surface (Tomita et al. 1991) and brain microvascular endothelial cells (Roberts et al. 1993). The binding of transferrin to its receptor would lead to its endocytosis. Iron in plasma will bind with transferrin and dissociate from it in the acidic compartment of the cell. Copolymers consist of the hydrophobic monomer lauryl methacrylate (LMA), a reactive anhydride functional methacrylate (TMA), and a large polyethylene glycol methacrylate monomer was copolymerized and conjugated to transferrin. This docetaxel containing, transferrin receptor targeting conjugate shows the enhanced cancer cell killing properties in in vitro cytotoxicity studies (Roy et al. 2014).

51.3.2 Passive Targeting

In 1979, the antitumor protein drug neocarzinostatin (NCS) was conjugated with a synthetic copolymer of styrene maleic acid copolymer (SMA) which was called SMANCS (molecular weight 16,000 g/mol) by Maeda (Maeda et al. 1979). This antitumor protein drug conjugate exhibited many unique properties, including prolonged half-life in circulation (20-fold), improved tumor-targeting capacity (2000 fold intra-tumor concentration than plasma), no immunogenicity and higher lipophilicity (Fang et al. 2011;

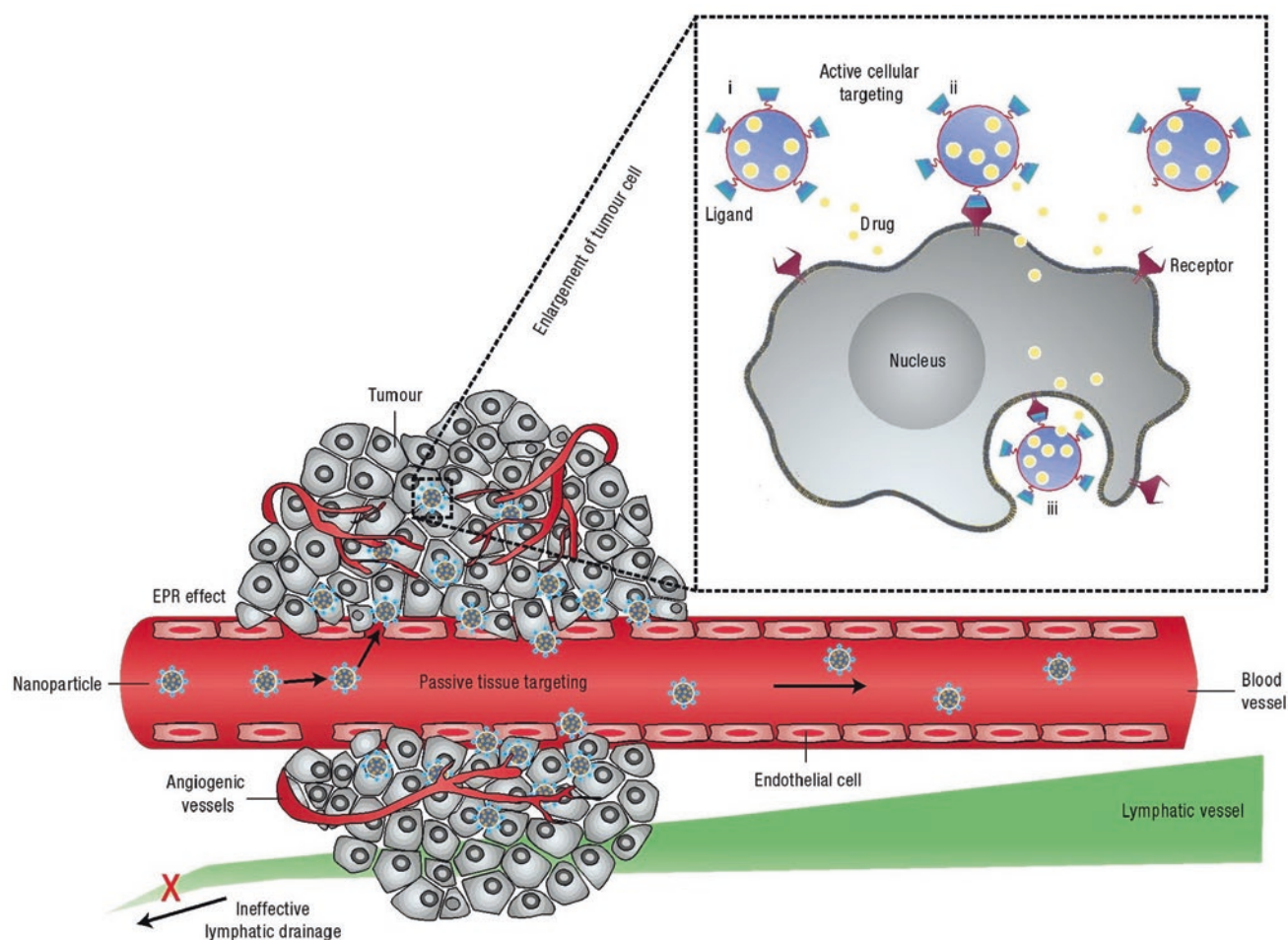


Fig. 51.4 Schematic representation of different mechanisms by which nanocarriers can deliver drugs to tumors. Polymeric nanoparticles are shown as representative nanocarriers (circles). Passive tissue targeting is achieved by extravasation of nanoparticles through increased permeability of the tumor vasculature and ineffective lymphatic drainage (EPR effect). Active cellular targeting (inset) can be achieved by func-

tionalizing the surface of nanoparticles with ligands that promote cell-specific recognition and binding. The nanoparticles can (i) release their contents in close proximity to the target cells; (ii) attach to the membrane of the cell and act as an extracellular sustained-release drug depot; or (iii) internalize into the cell (Adapted from Peer et al. (2007), with permission)

Maeda et al. 1984; Maeda and Konno 1997). These properties led to the conceptualization of the Enhanced Permeability and Retention effect (EPR effect) (Matsumura and Maeda 1986) showed in Fig. 51.4. With the advantage of EPR effect, SMANCS became the first macromolecular anticancer drug approved in 1993 in Japan (Fang et al. 2011; Maeda and Konno 1997). The EPR effect has been observed in the applications of many nanomedicine formulations, such as polymer conjugates, polymeric micelles and liposomes (Seymour et al. 1987; Dand et al. 2013). Most HPMAC copolymers utilize the EPR effect to achieve better tumor targeting and delivery of drugs. EPR effect is a tumor vasculature-dependent phenomenon. Most tumors are well vascularized with high density and the rapid growth of blood vessels leads to irregular vascular alignment and defects of the junction between endothelial cells. The increased local fenestration of macromolecules paired with the ill-developed lymphatic drainage at the tumor lesion lead to the local

accumulation of macromolecules over time (Folkman 1995; Fang et al. 2011; Hashizume et al. 2000; Jain 1999; Jain et al. 2002; Roberts and Palade 1997).

Wang et al. first described macromolecules' Extravasation through Leaky Vasculature and their subsequent Inflammatory cell-mediated Sequestration (ELVIS) (Wang and Goldring 2011). It explains the passive targeting of the HPMAC copolymer-dexamethasone conjugate (P-Dex) to inflammations (Yuan et al. 2012; Quan et al. 2010). Unlike the traditional understanding of EPR effect that macromolecules only can passively target to solid tumors (not inflammation) due to the leaky vasculature and impaired lymphatic drainage, they found although the extravasated macromolecules are cleared from the inflammatory tissue quickly, rapid internalization by inflammatory infiltrates and locally activated cells provide the mechanism for the sustained retention of the macromolecular drug conjugate at the site of inflammation.

51.4 Clinical Tests on HPMA Copolymer-Anticancer Conjugates

Cancer is the second leading cause of death in the United States. One of every four deaths in the United States is from cancer. In the past 60 years, synthetic water-soluble polymers, including HPMA copolymers have been used extensively as anticancer drug carriers due to their high biocompatibility and chemical functionality. All polymer conjugates used as anticancer agents in clinical trials are in effect macromolecular prodrugs. HPMA copolymers as an anticancer drug carrier are non-toxic, non-immunogenic and have multiple side chains to carry drug and targeting moiety payload (Duncan and Kopeček 1984; Duncan 2009; Kopeček et al. 2000). The rationale for HPMA copolymer anticancer drug conjugation, from the biological point, is to ensure that the drug would reach the pharmacological target and has limited access to sites of non-specific toxicity. Moreover, the drug must be released at a concentration and rate, which should be able to maximize its therapeutic action. It is important to control polydispersity as the molecule weight variation has a high impact on general and cellular pharmacokinetics with consequent implications for efficacy and toxicity. The antitumor HPMA copolymers target to the tumor through EPR effect which shows a significantly increased tumor drug concentration.

Many achievements have been made in the clinical evaluation of HPMA copolymer anticancer drug conjugate. For example, a Phase I clinical trial using HPMA copolymer-doxorubicin to treat breast cancer showed signs of activity coupled with fivefold decreased anthracycline toxicity in chemotherapy-refractory patients (Vasey et al. 1999). Phase II studies utilized 17 breast cancer patients, 29 non-small cell lung cancer patients and 16 colorectal cancer patients (Seymour et al. 2009). The study has expanded and confirmed the early indications that HPMA copolymer doxorubicin has therapeutic activity against some advanced solid neoplasms. Of the 14 evaluable patients with breast cancer 21 % had partial responses, and these were all anthracycline-naïve patients and it is possible that free doxorubicin could also have been effective in these patients. Three chemotherapy-naïve patients of 26 non-small cell lung cancer patients had partial responses. In contrast, none of the 16 evaluable patients with colorectal cancer responded (Seymour et al. 2009). HPMA copolymer doxorubicin has the extended plasma circulation of the polymer conjugate, an important prerequisite for passive accumulation within tumor tissues comparing with free doxorubicin and its delivery is determined largely by its kinetics and biodistribution (Duncan 2009). Failure of traditional chemotherapy results in recurrence and development of virulent multi drug resistance (Persidis 1999). To deal with this, the majority of cancers are being treated by a combination of drugs. HPMA copolymer-doxorubicin conjugate and HPMA copolymer-photosensitizer conjugate were the first combination therapy using polymer

bound to drugs (Krinick et al. 1994). Experiments results show that combination therapy achieved the better cure than either therapy alone (Kopeček et al. 2000; Zuluaga and Lange 2008; Matthew Peterson et al. 1996).

Paclitaxel is a well-known antitumor agent with proven efficacy in ovarian, breast and non-small cell lung cancer (Huizing et al. 1995). Intravenous administration of paclitaxel is hindered by poor water solubility of the drug and associated with unpredictable side effects. PNU166945 is a novel polymer-conjugate derivative of paclitaxel, which uses HPMA polymer as the polymeric carrier. This compound is water soluble, no toxicity and provides a wide platform for specific tumor targeting (Meerum Terwogt et al. 2001). HPMA copolymer paclitaxel was administered as a 1 h intravenous infusion every 3 weeks to a total of 12 patients in phase I clinical trial (Meerum Terwogt et al. 2001). However, this trial was discontinued before reaching dose-limiting toxicity because of there is potential neurotoxicity at higher doses found in the preclinical animal study (Meerum Terwogt et al. 2001). Plasma pharmacokinetic was measured over 48 h using HPLC and showed a linear relation for both HPMA copolymer-paclitaxel and released paclitaxel. The results suggest an improved pharmacokinetic behavior with potential controlled release of paclitaxel.

51.5 Non-cancerous Diseases

Beyond oncology, people are afflicted by many chronic conditions such as autoimmune diseases, musculoskeletal disorders and infectious diseases. Rheumatoid arthritis (RA) is a chronic, systemic and inflammatory autoimmune disease, which manifests in multiple joints of the body. One in five adults in the United States report having doctor diagnosed arthritis (CDC 2013). Currently, there is no cure for rheumatoid arthritis. Commonly used medications for treatment of the disease include non-steroidal anti-inflammatory drug (NSAIDs), glucocorticoids (GCs) and disease-modifying antirheumatic drugs (DMARDs) (Yuan et al. 2012). For oral administration of NSAIDs such as ibuprofen, only a small amount of drug can be absorbed and reach at the arthritic joints (Gallo et al. 1986; Mäkelä et al. 1981). The NSAIDs conjugated to the macromolecular carrier would target to the inflammation site after parenteral administration (Larsen et al. 1989). The therapeutic effects of NSAIDs are mediated primarily through nonselective inhibition of cyclooxygenase (COX), inhibiting both the cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). However, they will not prevent joint destruction and only show a small impact on inflammation and also the application of NSAIDs are limited due to the side effects, especially GI and renal toxicity (El Desoky 2001). Glucocorticoids (GCs), which are immunosuppressive and anti-inflammatory, have been widely used for controlling

the symptoms of inflammatory diseases (Kirwan et al. 1995). However, the side-effects associated with long-term GC treatment (e.g., immunodeficiency, weight gain, growth impairment, fluid retention and secondary osteoporosis, etc.) have hampered their clinical application (Canalis et al. 2007; Barber et al. 1993). To address this challenge, polymeric glucocorticoids have been proposed (Timofeevski et al. 1996). As a major breakthrough, HPMA copolymer-dexamethasone conjugate was designed and synthesized. Due to the high anti-inflammation potency and high toxicity, dexamethasone (Dex) was selected as the prototype drug candidate to be conjugated to HPMA copolymer via a hydrazone bond, which is acid-cleavable (Kratz et al. 1999). The general idea is that after systemic administration, HPMA copolymer-Dex (P-Dex) conjugate will passively target to the inflammatory joints and be quickly internalized by the synoviocytes via endocytosis and gradually release the free drug to exert its anti-inflammation effect locally without triggering systemic GC side effect of. While the *in vivo* data proved that this general design works very well, some chemistry problem remains. Due to the initial polymer analogous reaction approach that was taken, the drug content in P-Dex varies from batch to batch. To solve this problem, a new acid-cleavable, Dex containing monomer (MA-Gly-Gly-NHN=Dex) was synthesized to achieve precise control of Dex loading in the conjugate (Wang et al. 2007a). In the inflammatory joint, P-Dex treatment has a long-lasting inflammation resolution effect, which is critical and indirectly supports the long-term presence of Dex in the arthritic joints (Wang et al. 2007a; Liu et al. 2008). The mechanism underlining the P-Dex' targeting to arthritis is different from the classical EPR effect as the intimal lymphatic is intact in most of the arthritic joints and the passive targeting is initiated by their selective extravasation through the leaky vasculature of the inflamed synovial tissues (Yuan et al. 2012). As discussed in the passive targeting section, this novel passive targeting mechanism was called "ELVIS", which represent the macromolecular pro-drugs' Extravasation through Leaky Vasculature and the subsequent inflammatory cell-mediated Sequestration. The fast sequestration of P-Dex by the activated synoviocytes and potentially inflammatory infiltrates explains the passive targeting of P-Dex to inflammations (Quan et al. 2010; Wang and Goldring 2011; Yuan et al. 2012).

HPMA copolymer-drug conjugates have also been used to treat musculoskeletal diseases. Bone-targeting HPMA copolymer conjugates were designed for the treatment of osteoporosis and other musculoskeletal conditions (Wang et al. 2003, 2004, 2006, 2007b; Pan et al. 2006, 2008a, b; Miller et al. 2008). HPMA copolymer-D-Asp₈ conjugates show extensive targeting potential to the skeleton. D-Asp₈ (D-aspartic acid octapeptide) is an anionic peptide being used as bone-targeting moieties for HPMA polymer conjugates (Wang et al. 2003). It was showed that FITC-labeled

HPMA copolymer D-Asp₈ conjugate at 0.5 g/kg dose has a very strong binding at the high bone turnover sites such as primary spongiosa and metaphyseal areas in mice (Wang et al. 2003). The conjugate was also bound to the bones of osteopenic animal model in rat with the same dose in mice (Miller et al. 2008). Besides D-Asp₈ as bone targeting moieties, bisphosphonates are also commonly used as bone targeting moieties for HPMA copolymer conjugates (Russell 2011; Fleisch 1981). Instead of recognizing only resorption sites in skeletal tissues of D-Asp₈, alendronate (ALN) directs the delivery system to both formation and resorption sites (Wang et al. 2007b). Based on a previous study (Wang et al. 2003, 2006), alendronate-containing conjugates with relatively low molecular weight show higher binding to the bone than D-Asp₈ containing conjugates. Prostaglandin E₁ was selected as the first anabolic compound to be conjugated to the HPMA copolymer bone targeting polymers (Pan et al. 2006, 2008a). The high molecular weight, multiblock, backbone degradable bone targeted HPMA copolymer PGE₁ conjugate showed higher accumulation in the bone of OVX rat model and a higher efficacy in promoting bone formation in an osteoporosis rat model (Pan et al. 2008b, 2013).

51.6 Linker Chemistry

To achieve on-site drug release and reduce the drug toxicity to other organs, linker design between drug and HPMA copolymer are very important. There are several criteria for the linker design, such as the polymer-drug linker must be stable in the circulation until it reaches the targeted site. The polymer-drug linker should also be able to release the drug at optimum rate at the target (Duncan 2006). The use of unsuitable polymer-drug linkers which are either too stable to release drug on target site or not stable enough that degraded before the arriving the target site would lead to undesirable treatment outcome. pH-sensitive or peptidyl polymer-drug linkers are most frequently used in drug delivery (Duncan 2003, 2006). pH-sensitive linker was widely used to deliver anti-tumor agents as the tumor tissues are often associated with acidosis. Their eventual subcellular trafficking into the acidic endosomal/lysosomal compartments would provide another mechanism for the drug activation. One of the most widely used low pH sensitive linker is the hydrazone bond (Kratz et al. 1999). It is formed by a reaction between hydrazine and ketone in the presence of acidic catalyst. The hydrazone bond has been used to link doxorubicin onto HPMA copolymer to treat solid tumor. The drug was released within 48 h *in vitro* at pH 5 and it correlated well with the antitumor activity *in vivo* (Etrych et al. 2001). Another pH-sensitive linker being widely used is *cis*-aconityl linkage which is being reported in the use of anticancer drug immunoconjugates (Choi et al. 1999). Many pH-sensitive chemical structures

have been synthesized for drug delivery system. The release rate could be tuned to meet the therapeutic requirements. Besides tumor site release, the pH-sensitive linker has also been used as a cleavable structure in bone targeting and inflammation targeting (Wang et al. 2005; Yuan et al. 2012).

Speaking of bone targeting and on site release, cathepsin K-specific peptide linkage is also widely used in the bone-targeting copolymer conjugates. Cathepsin K is predominantly expressed in osteoclasts which make the cathepsin K-specific peptide the best linker candidate to release therapeutic agents from bone-targeting copolymer conjugates (Wang et al. 2005; Miller et al. 2008). Oligopeptide Gly-Phe-Leu-Gly is designed for degradation by the lysosomal cysteine proteases, particularly cathepsin B (Duncan et al. 1983). This linker is stable in the body circulation and can release the drug over a 24–48 h period in vitro and in vivo (Rejmanová et al. 1985).

Recently, new linkers that can be cleaved by tumor associated protease legumain have been developed (Stern et al. 2009). Self-immolative linkers, which form stable bonds between a macromolecular scaffold and its reporter groups are able to facilitate enhanced conjugate disassembly upon a specific activation event (Blencowe et al. 2011). These self-immolative linkers showed high loading capabilities and excellent solubility and stability characteristics. Bioresponsive coiled-coil peptide linkers which are noncovalent and biologically inspired are formed by heterodimerization of two complementary peptide sequences (Apostolovic et al. 2010). This linker offered several potential advantages including facile access to combination therapeutics and rapid production of compound libraries to screen for structure-activity relationships. Not only act as a linker, coiled coil peptides may also direct intracellular transport and trafficking (Apostolovic et al. 2010).

51.7 HPMA Copolymer for the Treatment of CNS Diseases

As described above, numerous macromolecular therapeutics, especially HPMA copolymer drug conjugates have been developed for the treatment of cancer and non-cancerous diseases. We believe some of the benefits of these delivery systems can be further extrapolated to diseases of the central nervous system (CNS). There is a wide range of pathological conditions associated with human brain, including infections, stroke, hypoxia, poisoning by teratogens (including alcohol), chronic degenerative disease and traumatic injury. Currently, no therapies are yet available to fully restore lost function or slow ongoing neurodegeneration in the brain. The poor brain permeability limited by blood brain barrier prevents the macromolecular therapeutics transport into the brain (Shlosberg et al. 2010; Pardridge et al. 1992). The blood brain barrier restricts the entry of macromolecules and low molecular

weight compounds from the periphery. Approximately 98% small molecular weight drugs and almost 100% larger molecular weight peptides and proteins do not cross the blood brain barrier (Pardridge 2004). To achieve a brain targeting drug delivery and sufficient therapeutic drug concentration in the brain, the presence of blood brain barrier seems to be the main limiting factor. In the past few years, some notable developments in systemic and local macromolecular drug delivery system for CNS diseases have been reported (Neuwelt et al. 2008). The advantage of systemic drug delivery is their non-invasive nature and use of traditional routes of administration. The drawback of the systemic drug delivery is the large dosages, which may be needed to achieve therapeutic drug concentration in the brain. To overcome this limitation, modification of a delivery vehicle with site-specific ligands that increase its penetration through blood brain barrier is frequently used (Weinstein et al. 1978; Béduneau et al. 2007). The pharmacokinetic properties of these drug delivery systems must increase the bioavailability of the drug and the time it remains in the circulation as well as reducing the drug clearance by the MPS (Siwak et al. 2002). To fulfill these requirements, polymeric drug delivery system seems to be one of the most promising solutions to shuttle drugs into the brain effectively (Liu et al. 2010).

Amphiphilic copolymers poly(*N*-(2-hydroxypropyl) methacrylamide-co-laurylmethacrylate) or P(HPMA-co-LMA) designed by Zentel group are able to mediate drug transportation to the brain (Hemmelmann et al. 2012). They show the P(HPMA-co-LMA) copolymers enhance the permeability of Rhodamine 123 across the blood brain barrier without affecting the integrity and barrier function. With the in vitro and in vivo experiments results, they showed that the delivery system is suitable for a host of potential drugs. The random P(HPMA-co-LMA) copolymer having 10 mol% of lauryl side chains is the most efficient delivery system (Hemmelmann et al. 2011, 2012).

HPMA copolymer based hydrogels have been invented to treat physical injury of the central nervous system specifically traumatic spinal cord injury (Fawcett and Asher 1999; Zhong and Bellamkonda 2008). The hydrogel is a biomaterial implantation at the injury site to support the injured neuron tissue and act as a platform for the axon to attach and grow. The hydrogel could also carry drugs for the local delivery (Geller and Fawcett 2002; Nisbet et al. 2008; Zhong and Bellamkonda 2008). Arginine-glycine-aspartic acid (RGD) peptide sequences and aminosugar sequences which could promote tissue organization and regeneration were conjugated to the HPMA copolymer hydrogel to treat CNS trauma (Woerly et al. 1993; Begovac and Shur 1990; Ruoslahti and Pierschbacher 1987; Plant et al. 1997). In vitro studies suggested that RGD containing HPMA copolymer hydrogels have the potential of facilitating tissues regeneration in the injured CNS such as spinal cord injury (SCI). In vivo studies confirmed the in vitro findings and showed that the hydrogels

were able to bridge tissue defects in the acutely injured spinal cord, support cellular ingrowth, angiogenesis, and axonogenesis within the structure of the hydrogel network, which was similar to those of the developing spinal cord (Woerly et al. 1999). To further investigate whether the hydrogel could restore the functions to the chronically injured spinal cord, HPMA copolymer hydrogel was implanted into the cavity that formed in the rat spinal cord 3 months after a severe injury when the functional deficit had reached a plateau (Woerly et al. 2001). The results showed that long-term implantation not only reduces the volume of the cavity but also induced tissue repair and axonal regrowth with extended long distance throughout the graft, accompanied by gradual functional recovery. This hydrogel offers two advantages: it can be combined with either genetically-engineered cells (Loh et al. 2001) or human stem cells and these cells develop successfully within the polymer before implantation.

All the findings above show that HPMA copolymer has the potential to be a CNS drug delivery carrier candidate. HPMA copolymer hydrogel system shows the potential to repair CNS lesions caused by trauma. It is a simple and safe scaffold for neuronal regeneration. It could also incorporate different growth factors and cells to further potentiate their repair capability. Amphiphilic copolymer P(HPMA-co-LMA) has the potential to transport drugs through blood brain barrier while maintaining the barrier integrity. It is the most efficient drug delivery system to delivery various drug crosses the blood brain barrier.

Traumatic brain injury (TBI) is a major cause of physical or cognitive morbidity or even mortality all over the world. The pathophysiology of TBI is complicated and consists of primary injury (mechanical damage to the head) and secondary injury mechanisms including cerebral edema, inflammation, and oxidative stress. Currently, there are no treatments. The synthetic glucocorticoid dexamethasone (Dex) is widely used for its anti-inflammatory and immunosuppressive effects. As we mentioned in the previous sections, Dex has a significant positive effect on inflammation. As a glucocorticoid, it could insert into the cell membrane and inhibit lipid peroxidation propagation reactions as part of the secondary injury of TBI. However, there are also significant challenges in using steroids as a therapeutic option for TBI. Dex, as a glucocorticoid, has many side effects after long term or short term treatment. Also, it is not water-soluble which is also a barrier in the treatment of TBI. In our group, we synthesized HPMA copolymer dexamethasone conjugate (P-Dex) to treat severe traumatic brain injury in mice. The preliminary results showed the accumulation in cerebral cortex after traumatic brain injury. P-Dex also showed a significant positive effect on preventing the activation of microglia and inhibition of lipid peroxidation at the injured cortex, which could reduce the damage of cerebral cortex caused by reactive oxygen species. The preliminary study validates the significant potential of P-Dex in the treatment of traumatic brain injury.

51.8 Summary

In the past decades, polymer therapeutics have been developed for not only anti-tumor treatment but also used to treat numerous other diseases. The advantages of macromolecular therapeutics have been recognized for its diverse functionality, targetability and potential to improve the treatment of a variety of diseases (Li and Wallace 2008; Liu et al. 2010; Yuan et al. 2012; Hemmelmann et al. 2012; Rihova and Kubackova 2003). As being discussed in this chapter, targeted drug delivery to the skeleton, inflammation, tumor and CNS system using HPMA copolymer has great potential for further therapeutic development.

51.9 Review Questions

1. What is the ELVIS mechanism?
2. What is the structural difference between liposomes and micelles?
3. Describe three targeting moieties used to modify the copolymers to achieve active targeting property and what are they used for.
4. Describe two linkers used for the HPMA copolymer drug conjugates and what are they designed for.

51.10 Answers

1. HPMA copolymers' passively targeting and retention at the site of inflammation can be attributed to their Extravasation through Leaky Vasculature and subsequent Inflammatory cell-mediated Sequestration. This process is termed as ELVIS mechanism.
2. Liposomes are spherical vesicles composed of phospholipid bilayer membrane encapsulating an aqueous core. Both the aqueous core and the lipid bilayer can be used for drug loading purpose.
Micelles are formed by aggregation of surfactant molecules (e.g., lipids, amphiphilic polymers) with a hydrophobic core and a hydrophilic corona. The hydrophobic core can be used for drug loading purpose.
3. Refer to 3.1 Active targeting.
4. Refer to 6. Linker chemistry.

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Abstract

Central nervous system (CNS) diseases represent a class of complex disorders for which cures have been largely unmet due to the general lack of knowledge regarding underlying pathogenic mechanisms. Gene-based therapies directed at ameliorating neurodegenerative diseases exhibit great potential due to rapid scientific advances made regarding delivery modalities, neurosurgical methods, neuroimaging, and molecular biological manipulation. Given these breakthroughs, the diseased CNS presents a neuroimmunological challenge as gene delivery many times requires invasive surgical procedures and a majority of the gene delivery platforms incite transient, and sometimes, inflammatory events that possess the potential to exacerbate disease-related processes. In this chapter, we will discuss the most current literature on gene therapy for CNS disorders by detailing the neuroimmunological profiles of presently available gene transfer platforms, approaches that have been made to minimize vector-mediated inflammation, and current state of gene therapy clinical trials for neurodegenerative diseases.

Keywords

Gene therapy • Immunotherapy • Neurodegeneration • Vaccination • Viral vector

52.1 Vector Selection Rationale

To derive an informed decision in the selection of an appropriate gene therapeutic vehicle for the treatment of a specific neurologic disease, the following must be considered: vector

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capacity, tropism, genome maintenance, vector-mediated transgene expression duration and levels, and safety profile. The size of the therapeutic transcription unit is many times employed as an initial criterion to focus vector choice. This category also includes a given vector's ability to harbor multiple transcription units, thereby potentially affording reconstruction of a complex biochemical pathway (i.e., dopamine biosynthesis for Parkinson's disease). Potential applications for several presently available vector platforms are restricted by insert size limitations and are sometimes excluded if multi-gene delivery is a prerequisite for therapy.

Cell type specificity is also an important issue when developing a gene-based therapeutic intervention for neurodegenerative disorders. It would be most beneficial if the vector of choice could transduce and express in cell types that comprise only the specific disease-affected pathway. Vector tropism can be regulated through modulation of cellular receptor interactions by one or more of the following approaches: alteration of virus docking proteins, utilization of alternate viral serotypes, pseudotyping, and introduction

of tethered ligands for cellular receptors into the viral envelope. Once a vector is optimally targeted to the brain region of interest, therapeutic transgene expression can be restricted to selected cell populations via the utilization of cell type-specific promoters and/or transcriptional elements (Wagner et al. 1992; Zatloukal et al. 1992; Zabner et al. 1999; Toyoda et al. 1998; Beer et al. 1998). Strict spatial control of transgene expression is vital to ensure the correct cells will manufacture the gene product. This control, in turn, reduces the risk that ectopic transgene expression will occur and lead to untoward effects on adjacent neurological pathways.

Most neurodegenerative disorders evolve insidiously over many years thus requiring gene-base modalities to impart therapeutic benefit for several decades of an individual's lifetime. To this end, a vector genome should be stably maintained within the transduced cell for extended periods of time. Vector genome maintenance is, therefore, a critical factor in selection of an appropriate gene therapy vehicle for neurodegenerative diseases. Vector genomes can exist as episomes and/or integrated forms within nuclei of host cells. Mitotically active cells, such as those of the progenitor and glial lineages, eventually exhibit diminished episomal vector-mediated transgene expression. However, the post-mitotic property of CNS neuronal populations does not exclude the utilization of episomal vectors since genomes can be maintained without progressive therapeutic vector loss due to mitosis. Integrating vectors circumvent this issue but their use raises safety concerns including their potential to transactivate proximal proto-oncogenes and to disrupt essential host genes via insertional mutagenesis.

Another similar issue regarding vector selection relates to the desired levels and duration of gene product expression for treatment of neurodegenerative disorders. Depending upon the vector and transcriptional elements chosen, pharmacologic or physiologic levels of transgene expression can be achieved for time periods of short or long duration. As with other selection criteria, the decision of which level/duration of expression is preferred rests heavily on what aspect of the particular disorder is to be targeted and at which time during the disease course the intervention is to be implemented. Early interventions may require maintenance of long-term physiologic levels of transgene expression (i.e., neuroprotective strategies) since the neural networks may be primarily intact at this time. A vector/promoter combination that safely and stably maintains gene expression at nearly physiologic levels in the CNS would serve as a potential candidate for such early treatment approaches. Treatment modalities that are implemented after presentation of clinical symptoms may require long-term pharmacologic levels of transgene product to restore function to a brain region decimated by disease.

Safety is of utmost concern regarding the application of novel gene therapeutic strategies within the brain. Many

presently available vectors trigger immunogenic and/or inflammatory responses when introduced into the CNS. These responses are known to arise from the humoral and/or cell-mediated arms of the immune system, and the magnitude differs depending upon which vector type is employed. For example, repeat administration of early generation viral vectors has been shown to lead to lower transgene expression and serious inflammation, likely the result of a primed immune system (Byrnes et al. 1996). Therefore, a vector that is stably maintained and that can express its transgene for extended periods of time would be a more favorable choice as a gene therapeutic vehicle for neurodegenerative conditions. Another aspect that is often overlooked regarding gene therapy safety is the role of transgene products in the elaboration of immune responses and toxicity. Transgene products that are of foreign origin, ectopically expressed, or pharmacologically expressed harbor the potential to induce cytotoxicity and/or immune responses. Research addressing these issues is imperative to elucidate the role of transgene products in the elaboration of these potentially harmful responses, and how such responses can be successfully circumvented. Utilization of regulatable transcriptional or post-transcriptional elements in delivery vectors to provide "fine-tuning" of therapeutic transgene expression levels is a way to minimize harmful clinical outcomes.

52.2 Gene Transfer Platforms

Genetic material can be delivered to cells by two broad classes of vectors: nonviral and viral. Nonviral gene transfer modalities include the use of plasmid DNA directly or in an encapsulated state. Such approaches are deemed the safest due to the lack of inflammatory and/or immunogenic components, but require repeated administration to maintain therapeutic gene expression. Viral vectors exploit the evolutionary achievements of viruses to propagate, package and transfer genetic material from cell to cell and organism to organism. Via genetic modification of mammalian viruses, it is possible to specifically target gene expression in desired cellular populations. Adenovirus, Adeno-associated virus (AAV), lentivirus, and Herpes simplex virus (HSV)-based vectors as they have been utilized for central nervous system (CNS)-directed gene therapy will be discussed below. These vector types represent a subset of the more commonly used viral vectors for CNS gene delivery.

52.2.1 Nonviral Gene Transfer

Although viral gene delivery systems have proven more efficient for use in the nervous system, viral vector production requires special expertise and equipment, and remains time

and labor intensive. As an alternative to viral vectors, transfection techniques utilizing cationic lipids have shown promise for use with cells of the nervous system and may prove to exact fewer adverse effects. Conventional nonviral systems (reviewed in Anwer et al. (2000)), which rely on passive cell targeting through charge interactions, possess several practical advantages over viral delivery of transgenes. These include decreased production time and costs as well as greater safety, since there is no risk of viral vector recombination.

For more than a decade, there have been a variety of reports demonstrating the use of lipid reagents for transfection of cells comprising the nervous system with very modest transfection efficiencies, even after optimization (Holt et al. 1990; Jiao et al. 1992; Kaech et al. 1996; Loeffler et al. 1990; Matter-Sadzinski et al. 1992). However, more recently our laboratory showed dramatically improved transfection efficiencies in the mouse CNS using the cationic lipids Tfx-10 and Tfx-20 (Promega) to deliver DNA with a microprocessor-controlled injector, suggesting further work towards optimization of lipid transfection is warranted for gene transfer applications requiring direct intracranial administration of therapeutic DNA-lipid complexes (Brooks et al. 1998).

Less invasive methods of delivering nonviral vectors to the CNS would be more desirable. Despite the minimal inflammatory profile of intracranial delivery of DNA-lipid complexes, the potential exists for surgery-induced adverse events, while the relatively limited volume of distribution obtained from stereotactic infusion makes the approach rather infeasible when attempting to treat diseases that involve expansive regions of the brain. To that end, methodologies have been developed to enable the injection of modified DNA-lipid complexes into the bloodstream that are designed to hone to the brain, and sub-regions thereof, to more widely deliver their therapeutic payload. Nonviral gene delivery systems in particular lend themselves to targeting strategies aimed at achieving higher transfer efficiency and tissue specificity, since they can withstand a wider range of chemical and physical conditions used to incorporate targeting moieties. They also allow for more flexibility and ease-of-use when mixing targeting ligands with plasmid DNA and the lipid delivery reagents.

To approach systemic infusion of targeted DNA-encapsulated liposomes, the liposome formulation requires further modification. Whereas in the case of direct stereotactic injections, cationic liposome-DNA complexes can be used, such complexes highly aggregate in physiological saline leading to inefficient and variable dissemination of the transfection complex when delivered via the bloodstream (Matsui et al. 1997; Mahato et al. 1997). The use of neutral (uncharged) liposomal mixtures in combination with inert and biocompatible polymer polyethylene glycol (PEG) produces sterically stabilized liposomal structures (Papahadjopoulos et al. 1991). Inclusion of PEG is also believed to prevent the binding of

opsonins circulating within the bloodstream, thereby significantly decreasing phagocytic cell recognition and clearance (Moghimi and Patel 1992). Physical targeting of these “pegylated liposomes” through the use of ligands and antibodies has been employed to enhance the efficiency of gene transfer to many specific cell types (reviewed in Anwer et al. (2000)), including the CNS (Pardridge 2003).

The blood–brain barrier (BBB) has long-served as a major obstacle to liposome-based gene delivery to the CNS via systemic administration. Zhang and colleagues have employed a pegylated immunoliposome-mediated method for systemic administration of plasmids expressing tyrosine hydroxylase (TH) in a rodent 6-OHDA model of Parkinson’s disease (PD; Zhang et al. 2004). A plasmid construct expressing TH, the rate-limiting enzyme in the dopamine biosynthetic pathway, was encapsulated within pegylated immunoliposomes and injected systemically into Parkinsonian rats. High-level transgene expression within the striatum and transient correction of apomorphine-induced rotation behavior were observed, indicating that these nonviral gene transfer reagents were capable of traversing the BBB. In addition, a time-course experiment showed that TH enzyme activity within the striatum diminished substantially from Day 3–9 post-treatment. In a recent follow-up study, this group demonstrated that transient gene expression profiles derived from immunoliposome-targeted gene delivery appear to be the result of plasmid degradation *in vivo* (Chu et al. 2006). Immune responses generated against pegylated immunoliposomes are thought to be minimal *in vivo*, but if multiple injections are required to subvert disease progression, then the adaptive immune response may play a role in clearing and/or degrading this gene-based therapeutic.

Recent development of using exosomes as a small interfering RNA (siRNA) carrier, although still in its early days, may be considered a major advance for the field of macromolecular drug delivery. Alvarez-Erviti et al. delivered siRNA to the mouse brain by systematic injection of targeted exosomes (Alvarez-Erviti et al. 2011). To reduce immunogenicity, they prepared exosomes from self-derived immature murine dendritic cells. Targeting was achieved by engineering the dendritic cells to express a fusion of exosomal membrane protein Lamp2b and a neuron-specific rabies viral glycoprotein (RVG) peptide. Purified exosomes were loaded with exogenous siRNA by electroporation. The authors demonstrated *in vitro* and *in vivo* that intravenous injection of RVG-targeted exosomes delivered Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) siRNA specifically to neurons, microglia and olivodendrocytes in the mouse brain, resulting in a specific gene knockdown. To show the therapeutic potential of their approach, they showed that delivery of RNAi targeting Beta-secretase 1 (BACE1), which mediates the formation of β -amyloid plaque and is associated with Alzheimer’s disease pathogenesis, led to BACE1 inhibition

and a significant decrease in brain β -amyloid levels of wild-type mice.

Apparently, exosomes have several advantages over existing non-viral delivery vehicles. First, exosomes derived from a patient's own cells should display less immunogenic properties than any foreign delivery carriers. Second, exosomes are relatively stable in the blood as they avoid opsonins, coagulation factors and complement, most likely attributed to their surface expression of CD55 and CD59 (Clayton et al. 2003). Third, dendritic cell-derived exosomes express the tetraspanin CD9 on their surface (Thery et al. 1999), which facilitates direct membrane fusion with the target cell and contents-delivery directly into the cytosol. This mode of entry bypasses the endosomal-lysosomal pathway and circumvents the need for endosomal-escape strategies. Finally, the small size of exosomes (50–100 nm in diameter) allows the particles to evade rapid clearance by the mononuclear phagocytes systems which clears particles >100 nm in size and enhance passage through vessel fenestrations and passage through the extracellular matrix.

52.2.2 Adenovirus Vectors

Adenoviruses (Ad) are a family of non-enveloped, double-stranded DNA viruses that generally cause mild respiratory infections in mammals. Over 50 serotypes have been identified. Ad-based vectors are attractive candidates for gene delivery to the CNS, as high vector titers can be generated, and these episomally maintained vectors can efficiently infect and express transgenes in a variety of cell types including the non-dividing cells of the CNS (Akli et al. 1993; Bajocchi et al. 1993; Davidson et al. 1993; Le Gal La Salle et al. 1993; Smith et al. 1996a, b, c). First generation vectors consisted of constructs that lacked genes for the potent regulatory proteins E1A, E1B, and E3. Although high titers could be obtained (approximately 10^{13} viable particles per ml) the immunological and physiological complications that resulted from Ad vector transduction in the CNS were serious. This robust response was due to enhanced cytotoxic T lymphocyte (CTL) activity to viral proteins and/or the expressed transgene product (Michou et al. 1997; Tripathy et al. 1996). Vigorous inflammation precludes repeated administration of this vector. As a consequence of these findings, second generation vectors were developed that additionally lacked the E2A gene. The resultant inflammatory response elicited by these vectors was reduced but still fairly substantial. In an effort to completely remove viral genes from the system, helper virus-dependent or so-called “gutless” forms of Ad vectors were developed (Mitani et al. 1995; Parks et al. 1996; Morsy et al. 1998; Hardy et al. 1997). These forms possess a large transgene capacity (up to 28 kb) and do not express any viral proteins. High multiplicities of infection (MOIs) have

been used to infect greater than 85 % of neurons with little if any cytotoxicity or adverse physiological effects up to 7 days post-infection (Cregan et al. 2000). Gutless Ad vectors direct expression of therapeutic genes in vivo with moderately high efficiency among gene therapy vectors. Gutless Ad constructs, but not first-generation Ad vectors, mediate sustained transgene expression in the brain even in the presence of anti-Ad immunity (Thomas et al. 2000, 2001). Another study, conducted by Zou and colleagues, compared transgene expression from first-generation Ad vectors to gutless Ad vectors in brain. Two months following transduction, the helper-dependent vectors exhibited higher transgene expression and elicited lower numbers of brain-infiltrating macrophages and T cells than first generation Ad vectors (Zou et al. 2000), further speaking to the improved safety profile of this Ad vector iteration.

52.2.3 Adeno-Associated Virus Vectors

Adeno-associated virus (AAV) is a non-pathogenic member of the parvovirus family. Its single-stranded DNA viral genome requires co-infection with either adenovirus or HSV for its own replication/propagation. Wild-type AAV encodes two viral gene products, Rep and Cap, which function in replication/integration and structural stability, respectively. The inverted terminal repeats (ITR) sequences promote extrachromosomal replication and Rep-mediated genomic integration of the flanked transgene. Newer methods of packaging have led to titers approaching 10^9 transducing units/10 cm plate and have eliminated the need for helper Ad which assures that negligible levels of contaminating Ad helper virus are present in the preparation (Xiao et al. 1998). Once packaged, the unique Cap protein of AAV allows for high-degree virion purification. Helper virus-free AAV vectors elicit transient and minimal inflammatory reactions when delivered in vivo, an observation likely due to the lack of associated viral gene expression and the facility to highly purify vector stocks (Xiao et al. 1997; Lo et al. 1999; Bueler 1999; Kaplitt and Makimura 1997).

Recombinant AAVs utilizing the cis-acting 145 bp ITRs to flank vector transgene cassettes provide a packaging capacity of up to 4.7 kb for foreign DNA. In muscle, molecular assembly of the AAV vector genomes occurs through intermolecular recombination between two ITRs, leading to genome concatemerization (Yang et al. 1999). This aspect of AAV vector biology has led to attempts to develop transsplicing or recombination-based overlapping vector approaches for the transfer of large therapeutic genes. Transsplicing or hybrid dual AAV vectors have been successfully exploited to reconstitute oversized gene expression. Dual AAV vectors are generated by splitting a large transgene expression cassette in two separate halves each packaged in

a single normal size (<5 kb) AAV vector (Duan et al. 2001; Thery et al. 1999; Yan et al. 2000). The reconstitution of the full-length expression cassette is achieved upon coinfection of the same cell by both dual AAV vectors followed by one of the following: ITR-mediated tail-to-head concatemerization of the 5' and 3' genomes followed by splicing (dual AAV trans-splicing vectors); homologous recombination between overlapping regions contained in 5' and 3' genomes (dual AAV overlapping vectors); or a combination of the two (dual AAV hybrid vectors). Recently Dickson group have developed a triple trans-splicing vector system, in which three independent AAV vectors carrying sequential exonic parts of the human Duchenne muscular dystrophy (DMD) coding sequence (Koo et al. 2014). The three exonic parts of DMD coding sequence are able to express the full-length protein as a result of trans-splicing events conjoining three vectors via their inverted terminal repeat sequences. This method of triple-AAV-mediated trans-splicing could be

applicable to the delivery of any large therapeutic gene (>11 kb) into postmitotic tissues (muscles or neurons) for the treatment of various genetic diseases.

The existence of numerous characterized AAV serotypes allows for use of alternative capsid types in the event serotype-specific neutralizing antibodies are generated or extant, thus extending the utility of the vector platform in the setting of pre-existing AAV immunity. Moreover, each serotype exhibits distinct patterns of transduction within the major tissues of mammals (Table 52.1). In general, AAV1, 5 and 9 exhibit higher transduction efficiencies than vectors packaged with other serotype capsid in all regions assessed within the CNS (Alisky et al. 2000; Burger et al. 2004; Aschauer et al. 2013; Markakis et al. 2010). AAV4 transduces specific cell types, such as ependyma and astrocytes in the subventricular zone, with higher efficiency (Liu et al. 2005; Benraiss et al. 2012). More recently, AAV9 has been shown to cross the BBB and transduce neurons and glia in

Table 52.1 Comparison of various AAV serotypes in CNS transduction

Serotype	Transduction efficiency	Tropism	Cytotoxicity	References
AAV1	Substantia nigra: +++	Neurons: +++	Immunogenicity: +	Markakis et al. (2010), Aschauer et al. (2013)
	Striatum: +++	Microglia: ++		
	Hippocampus: ++	Astrocytes: ++		
	Auditory cortex: +	Oligodendrocytes: +		
AAV2	Substantia nigra: +	Neurons: ++	Immunogenicity: +	Markakis et al. (2010), Aschauer et al. (2013)
	Striatum: +	Microglia: +		
	Hippocampus: +	Astrocytes: +		
	Auditory cortex: +	Oligodendrocytes: +		
AAV4	Subventricular zone: +	Neurons: ++	Immunogenicity: +	Benraiss et al. (2012), Liu et al. (2005)
		Astrocytes: ++		
		Ependyma: ++		
AAV5	Substantia nigra: +++	Neurons: ++	Immunogenicity: +	Markakis et al. (2010), Aschauer et al. (2013)
	Striatum: +++	Microglia: +		
	Hippocampus: +++	Astrocytes: ++		
	Auditory cortex: ++	Oligodendrocytes: +		
AAV6	Substantia nigra: ++	Neurons: ++	Immunogenicity: +	Markakis et al. (2010), Aschauer et al. (2013)
	Striatum: +	Microglia: +		
	Hippocampus: +	Astrocytes: +		
	Auditory cortex: ++	Oligodendrocytes: +		
AAV7	Substantia nigra: +++	Neurons: ++	Immunogenicity: +	Taymans et al. (2007)
	Striatum: +++	Microglia:		
	Cortex: +++	Astrocyte: +		
		Oligodendrocytes:		
AAV8	Substantia nigra: ++	Neurons: ++	Immunogenicity: +	Aschauer et al. (2013)
	Striatum: ++	Microglia: +		
	Hippocampus: ++	Astrocytes: +++		
	Auditory cortex: +++	Oligodendrocytes: +++		
AAV9	Substantia nigra: NA	Neurons: ++	Immunogenicity: +	Aschauer et al. (2013)
	Striatum: +++	Microglia: +		
	Hippocampus: +++	Astrocytes: +		
	Auditory cortex: +++	Oligodendrocytes: +		

+: minimum; ++: medium; +++: maximum; NA: data not available in publication

the brain and spinal cord following intravenous injection (Duque et al. 2009; Foust et al. 2009; Gray et al. 2011). The varying serotypes not only dictate transduction efficiency, but they have been shown to modulate transgene expression kinetics. The underlying mechanism(s) is unknown, but may be due to differences in virion uncoating and intracellular trafficking within the transduced cell (Thomas et al. 2004).

AAV vectors, when delivered into the CNS, are capable of expressing transgene products for periods of time in excess of 4 years. This has been demonstrated in both rodent and non-human primate studies (Kaplit et al. 1994; Bjorklund et al. 2000; Chirmule et al. 2000; During et al. 2001; Guy et al. 1999; Lo et al. 1999; McCown et al. 1996; and Bankiewicz personal communication). The pattern and duration of gene expression in these studies suggests that the AAV vectors once introduced into host cells adopt a transcriptional configuration that supports stable expression. This characteristic could lie in the physical status of the AAV vector genome (integrated vs. episomal) following transduction of a given host cell. The ability of wild-type AAV to specifically integrate into human chromosome 19 is intriguing if this property could be translated to AAV-derived vectors (Kotin et al. 1990). The ability to integrate creates the potential for stable, long-term expression of a transgene for the treatment of neurological disorders. Wu and colleagues presented a method for detecting integrated AAV vector genomes and provided some evidence that integration into CNS cells occurs, however, the specificity of this integration site is yet undetermined (Wu et al. 1998). More recent data generated by the laboratory of Mark Kay suggest that concatenated, non-integrated AAV genomes exist in greater abundance than integrating forms (Chen et al. 2001). To this end, more thorough studies detailing the genome status of AAV vectors in vivo must be conducted.

The risk of integration-induced oncogenesis appears to still be a theoretical concern despite a recent report. In long-term rodent studies with AAV vectors there was a significant increase in the incidence of hepatocellular carcinoma in mice treated with an AAV vector (Daly et al. 2001; Donsante et al. 2001). Systematic investigation was conducted to establish the genesis of the AAV vector/tumor association. These studies employing sensitive quantitative polymerase chain reaction (PCR) failed to yield evidence that the tumors arose from an insertional mutagenesis event. In a separate study, mice were injected with varying doses of AAV vectors with no evidence of hepatic tumors. These data argue that the hepatic malignancies likely arose by administration of contaminants with the AAV vector stocks and not because of genomic perturbations induced by the vector. However, more recent data compel reexamination of the propensity of AAV to produce chromosomal alterations. In that study, which was entirely performed in cultured human HeLa cells, AAV vector proviral integrations were found associated with chromosomal deletions and rearrangements most frequently on chromosome 19,

nearby but not precisely at the site of wild-type AAV integration (Miller et al. 2002). Multiple explanations for this finding are unexplored and thus its significance is unclear. Assuming safe integration of AAV vectors is demonstrated, a major limitation that will likely persist is the limited transgene size capacity exhibited by these vectors. This shortcoming will prove troublesome when cell-specific promoters and/or transcriptional enhancer elements must accompany the desired therapeutic gene for the purposes of directed expression. One group has attempted to circumvent this issue by developing heterodimeric AAV vectors where transfer of a transcription unit of approximately 9 kb can be achieved (Sun et al. 2000).

52.2.4 Lentivirus Vectors

Replication-defective lentiviral vectors derived from Human Immunodeficiency Virus (HIV) are another promising gene transfer vehicle for CNS applications as they can transduce both dividing and non-dividing cells, do not encode viral proteins, have an approximate 9-kb insert capacity, and are capable of sustaining stable gene expression for greater than a year (Kordower et al. 1999, 2000; Reilly 2001; Wang et al. 2000). To ensure against the generation of replication-competent HIV virions, the packaging system has been divided into four plasmids and four accessory HIV genes have been deleted from those packaging-assisting plasmids. Titers with this approach yield stocks of $1\text{--}5 \times 10^9$ infectious units (Bensadoun et al. 2000; Naldini et al. 1996b).

HIV-based lentiviral vectors when pseudotyped with the vesicular stomatitis virus (VSV) G protein (VSV-G) are principally neurotropic (Desmaris et al. 2001). Entry, proviral derivation and expression appear to occur efficiently in non-dividing neurons without the requirement for disruption of the nuclear envelope (Naldini et al. 1996a, b). Whereas, lentiviral vectors integrate chromosomally in dividing cells, the status of the proviral genome in neurons is less well studied. However, recent evidence garnered from the creation of feline immunodeficiency virus (FIV)-derived vectors devoid of integrase activity suggests that in non-dividing cells, transgene expression levels are equivalent to vectors capable of integrating into the host cell chromosome (Saenz et al. 2004). Efforts to test whether lentiviral vector proviral genomes can be rescued by HIV infection have also been informative. If the vectors retain necessary packaging sequences they are inefficiently rescued (Evans and Garcia 2000; Follenzi et al. 2000). However, self-inactivating (SIN) vectors, which possess major deletions within the U3 and U5 regions of the long terminal repeat (LTR) regions of the lentiviral genome, are unable to be rescued (Iwakuma et al. 1999; Yu et al. 1986).

Lentiviral vectors have been examined with respect to their potential elicitation of immune responses in the brain and within visceral organs. The data available indicate that in the CNS lentiviral vectors are minimally immunogenic, if

optimized vector production and purification protocols are followed (Baekelandt et al. 2003). Systemic immunization preceding injection of lentiviral vectors into the CNS determined that pre-existing anti-lentiviral immunity, regardless of the transgene, did not affect transgene expression (Abordo-Adesida et al. 2005). Furthermore, Abordo-Adesida and colleagues showed that the transgene, but not the virion or vector components, is chiefly responsible for providing antigenic epitopes to the activated immune system, following systemic immunization with lentivirus.

Even though they are more limited in number, rodent and non-human primate studies performed to date have shown that HIV vectors encoding for glial cell line-derived neurotrophic factor (GDNF) provide robust functional and structural neural protection in models of PD and provide an encouraging approach for gene therapy. Kordower and colleagues injected lentiviral vectors expressing GDNF into the striatum and substantia nigra of nonlesioned aged rhesus monkeys and young adult rhesus monkeys treated 1 week prior with the PD symptom-inducing neurotoxicant, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; Kordower et al. 2000). Treatment with these vectors augmented dopaminergic functioning and reversed a set of behavioral deficits. More recently, Brizard et al. were able to utilize a similar GDNF-expressing lentiviral vector to demonstrate functional reinnervation from remaining dopaminergic nerve terminals in the 6-hydroxydopamine (6-OHDA) rat model of PD (Brizard et al. 2006).

Lentivirus vectors have also been used in the development of potential Alzheimer's disease (AD) therapeutics. Dodart and colleagues recently delivered varying alleles of the apolipoprotein E (apoE) gene, which encodes for a lipid-binding protein with Amyloid beta ($A\beta$) fibrillogenesis modulation activity, to AD mouse models to determine if expression of different alleles led to differential amyloid deposition (Dodart et al. 2005). Lentiviral delivery of the apoE4 allele, one that is believed to enhance the risk of human AD, led to increased $A\beta$ deposition as measured by immunocytochemistry and enzyme-linked immunosorbent assay (ELISA). Conversely, lentiviral vector-mediated expression of the apoE2 allele resulted in a significant reduction in amyloid burden, which is suggestive of a potent dominant negative effect of apoE2 over mouse apoE on brain $A\beta$ deposition. These findings are exciting but more experimentation is warranted to determine the actual biological function of apoE and how modulation of its activity could alter normal physiology.

52.2.5 Herpes Simplex Virus Vectors

Herpes simplex virus type 1 (HSV-1) is a naturally neurotropic virus capable of establishing latent infection within neurons, but also possesses the ability to infect a wide range of cellular targets. The cellular receptors responsible for

virion docking and uptake have been cloned, including the herpes virus entry mediator A (HveA) and C (HveC, nectin-1; Kwon et al. 1997; Montgomery et al. 1996; Hsu et al. 1997; Warner et al. 1998; Geraghty et al. 1998). Not surprisingly, these receptors (or homologues) are expressed on a variety of cell types. The HSV life cycle involves long periods of latency, and to that end, the virus has evolved a number of elaborate and highly efficient mechanisms to avoid detection and elimination by immune cells (Banks and Rouse 1992). These properties have led to the development of two forms of HSV-1-based delivery vectors capable of *in vivo* and *in vitro* gene transfer to the nervous system: recombinant and amplicon vectors (Dobson et al. 1990; Andersen and Breakefield 1995; Andersen et al. 1992; Breakefield and DeLuca 1991; Breakefield et al. 1992; Fink et al. 1992; Glorioso et al. 1994; Wolfe et al. 1992).

52.2.5.1 HSV Recombinant Vectors

Recombinant HSV vectors comprise a wild-type HSV genome rendered replication defective via disruption/deletion of an indispensable viral gene(s). Typically, the immediate-early gene loci, which encode for potent transactivation proteins that initiate the viral lytic cycle, are targeted for insertion of therapeutic transcription units via homologous recombination (Marconi et al. 1996, 1999). Following construction, recombinant vectors are packaged into infectious virions using an engineered eukaryotic cell line that supplies the absent viral gene product(s) *in trans* (Andersen et al. 1992; Dobson et al. 1990). The genome of recombinant vectors, at present, can accommodate approximately 30 kb of genetic material. Recombinant vectors are also attractive gene transfer vehicles for CNS disorders because they can be propagated to relatively high titers (10^8 – 10^9 plaque-forming units/ml). In addition, the threat of insertional mutagenesis is greatly diminished as the vector genome persists episomally within post-mitotic cell nuclei.

Immune responses arising from infusion of recombinant HSV vectors can arise from any of the following sources: viral particle components, co-purified packaging cell debris, low-level *de novo* viral gene product expression, and transgene expression itself. What appears to be a major source of immune response elicitation is related to *de novo* expression of the intact viral transcription units. Recombinant HSV vectors, although replication-defective, harbor virtually intact segments of the HSV genome. These open reading frames (ORFs) are expressed at low levels even in the absence of immediate-early gene products, augmenting the potential for antigen processing and subsequent major histocompatibility complex (MHC) Class I presentation (Kriskey et al. 1998; Johnson et al. 1992). Detailed assessment of immune responses elicited against HSV recombinant vectors within the brain have been lacking. However, insights may be gleaned from studies employing the amplicon vector platform, which have received, immunologically speaking, more investigational attention.

52.2.5.2 HSV Amplicon Vectors

The HSV-1 amplicon is a uniquely designed eukaryotic expression plasmid that harbors two non-protein encoding virus-derived elements: an HSV origin of DNA replication (OriS) and the cleavage/packaging sequence ("a" sequence) (Federoff et al. 1992; Frenkel 1981; Frenkel et al. 1982; Geller and Breakefield 1988; Geschwind et al. 1994; Spaete and Frenkel 1982, 1985; Stow and McMonagle 1982). Both *cis* sequences are specifically recognized by HSV proteins to promote the replication and incorporation of the vector genome into viable viral particles, respectively. This highly versatile plasmid can be readily manipulated to contain desired promoters, enhancers, and transgenes of substantial size (~130 kb) (Wade-Martins et al. 2001). Heterologous transcription units either singly or in combination can be cloned into the amplicon plasmid using conventional molecular cloning techniques, and the resultant construct is packaged into enveloped viral particles for subsequent transduction of cells or tissues.

Amplicon plasmids are dependent upon helper virus function to provide the replication machinery and structural proteins necessary for packaging amplicon vector DNA into viral particles. An engineered replication-defective HSV derivative that lacks an essential viral regulatory gene has conventionally provided helper packaging function. These helper viruses are similar to the recombinant HSV vectors discussed above in that they retain a majority of the HSV genome. The final product of helper virus-based packaging contains a mixture of varying ratios of helper and amplicon virions. The titers obtained from helper virus-based amplicon packaging range from 10^8 to 10^9 expressing virus particles/ml. More recently, helper virus-free amplicon packaging methods have been developed by providing a packaging-deficient helper virus genome via a set of five overlapping cosmids or a bacterial artificial chromosome (Fraefel et al. 1996; Saeki et al. 1998, 2001; Stavropoulos and Strathdee 1998). This packaging strategy requires the co-transfection of eukaryotic cells that are receptive to HSV propagation (i.e., BHK, Vero) with packaging-incompetent HSV genomic DNA, amplicon DNA, and any accessory HSV genes shown to enhance amplicon titers (Bowers et al. 2001). Crude vector lysates are then purified by a series of ultracentrifugation steps and titered by expression or transduction-based methodologies (Bowers et al. 2000). The titers obtained from helper virus-free amplicon packaging typically range from 10^7 to 10^8 expressing virus particles/ml. The lack of contaminating helper virus in these stocks, and thus loss of immunosuppressive proteins like ICP47, has also made the HSV amplicon a powerful delivery platform for infectious disease and cancer vaccines (Hocknell et al. 2002; Tolba et al. 2001, 2002; Willis et al. 2001).

Similar to recombinant HSV vectors, immune responses arising from infusion of HSV amplicon stocks can arise from

several sources and such responses are dependent upon the packaging system employed. Immune response-eliciting sources include viral particle components, co-purified packaging cell debris, low-level *de novo* viral gene product expression (helper virus-based packaging only), and the expressed transgene. Early generation helper virus-based packaging methods lead to vector stocks that contain substantive levels of contaminating helper virus. Due to the identical physical properties of amplicon and helper virus particles, preferential purification of amplicon particles is not possible. The replication-defective helper virus, comparable to the recombinant HSV vectors described above, expresses viral proteins at low levels within transduced cells. These viral proteins exhibit cytotoxic activity and can potentially undergo antigenic processing and subsequent immune presentation.

Wood and colleagues were the first investigators to examine the immune responses elicited against early iterations of packaged HSV amplicon stocks. For their studies they utilized an amplicon expressing the reporter gene product β -galactosidase and packaged using a recombinant HSV helper virus (*tsK*), which possessed a temperature-sensitive mutation in the ICP4 gene locus (Wood et al. 1994). Although *tsK* is replication-defective at the non-permissive temperature of 39 °C, viral gene product-associated cytotoxicity remains observable. Administration of these amplicon stocks induced a vigorous inflammatory response. Elevated MHC Class I expression and microglial activation was evident by 2 days post-infection, which was followed by MHC Class II cell recruitment, T cell activation, and macrophage influx at 4 days following delivery of helper virus-contaminated amplicon stocks.

In a more recent study, our laboratory described the innate responses elicited upon stereotactic delivery of HSV amplicon vectors packaged via two different methods (Olschowka et al. 2003). C57Bl/6 mice were injected with sterile saline, β -galactosidase-expressing amplicon (HSVlac) packaged by a conventional helper virus-based technique, or a helper virus-free HSVlac preparation. Animals were sacrificed at 1 or 5 days post-transduction and analyzed by immunocytochemistry and quantitative Reverse transcription polymerase chain reaction (RT-PCR) for various chemokine, cytokine, and adhesion molecule gene transcripts. All injections induced inflammation with blood-brain barrier opening on Day 1 that was similarly enhanced following all treatments. By Day 5, mRNA levels for the pro-inflammatory cytokines (IL-1 β , TNF- α , IFN- γ), chemokines (MCP-1, IP-10) and an adhesion molecule (ICAM-1) had fully resolved in saline-injected mice and to near baseline levels in mice receiving helper virus-free HSVlac. In contrast, mice injected with helper virus-packaged amplicon stocks elicited elevated inflammatory molecule expression and immune cell infiltration even at Day 5. In aggregate, these studies demonstrated helper virus-free ampli-

con preparations exhibit a safer innate immune response profile when delivered directly to the brain, presumably due to the absence of helper virus gene expression, and provide support for future amplicon-based CNS gene transfer strategies. What remains to be assessed experimentally in greater detail is the role, albeit minor, that virion structural components, packaging cell-derived debris and transgene product appear to play in the activation of the innate immune response by helper virus-free amplicon stocks. In addition, the role of pre-existing HSV immunity in modulating host responses to brain-delivered HSV vectors remains understudied.

52.3 Neuroimmunotherapy

Awareness of brain “immunocompetence” has increased significantly in recent decades. Immunomodulatory molecules are expressed in the brain by microglia, astrocytes and neurons in the presence of an inducing stimulus, including the infusion of gene transfer vectors. Additionally, macrophages, T cells, and B cells traverse the blood–brain barrier and survey the brain as part of their normal physiologic function (Hickey 2001). Furthermore, chronic activation of brain-resident inflammatory processes has been shown to underlie the pathophysiology of several neurodegenerative diseases such as AD and PD. One of the central features of AD is the excessive accumulation of amyloid beta ($A\beta_{1-42}$), a 42-amino acid peptide, in extracellular senile plaques. Moreover, the AD brain is decorated with complement proteins that bind to $A\beta$ peptides and aid in opsonization for eventual clearance by microglia. In response to accumulating $A\beta$, microglia in the AD brain express an array of potent cytokines and chemokines that are likely neurotoxic and may contribute to cell loss (Hanisch 2002; McGeer and McGeer 2003; Strohmeyer and Rogers 2001).

Inflammatory processes may also contribute to the degeneration observed in PD brain. PD is characterized by the destruction of dopamine-containing neurons in the substantia nigra (SN), resulting in a loss of dopaminergic afferents to the basal ganglia and eventual motoric dysfunction (Olanow 2003; Olanow et al. 2003). Pathologically, PD brain exhibits a region-specific accumulation of Lewy bodies, which are intraneuronal inclusions comprised of aggregated α -synuclein and other cellular proteins (Hald and Lotharius 2005). A variety of pro-inflammatory mediators have been detected in human PD and animal models of the disease (reviewed by McGeer and McGeer (2004)). Moreover, epidemiological evidence has shown that chronic treatment with nonsteroidal anti-inflammatory drugs (NSAIDs) reduces the risk of AD and PD compared to those who take NSAIDs on a non-regular dosing regimen (Standridge 2004; Schiess 2003). While it appears that numerous cytokine and chemokine molecules are closely associated with and likely contribute to neurodegeneration in AD and PD, it is uncer-

tain whether these inflammatory mediators impart beneficial effects during pathogenesis.

A promising, yet inherently complex, means of preventing $A\beta$ accumulation in AD or α -synuclein in PD relates to the use of the host immune system to mount specific immune responses against the self-peptides, $A\beta$ and α -synuclein (Klyubin et al. 2005; Masliah et al. 2005). The findings from a variety of immune-based strategies speak to the promise of such approaches, but also reveal the potential for morbid complications, especially in the case of AD vaccination. Therefore, it is imperative that as immunotherapeutics are designed to prevent and/or treat neurodegenerative diseases the underlying inflammatory processes are taken into account.

52.3.1 Immunotherapeutic Approaches to Treating Neurodegenerative Diseases

Two principal immunotherapeutic strategies for diseases afflicting the CNS have been pursued: passive and active vaccine-based approaches. Passive immunization involves the transfer of antiserum, purified antibodies, or an antibody-encoding gene via gene delivery to a recipient to prevent the accumulation of or to promote the removal of a central pathogenic factor. For example, passive immunization achieved by administration of $A\beta$ -specific antibodies has shown promise in preclinical studies. The laboratories of Drs. Holtzman and Ashe have independently reported the benefits of systemic delivery of $A\beta$ -specific antibodies to AD mice (Dodart et al. 2002; Kotilinek et al. 2002). Treated mice exhibited a marked reduction in amyloid accumulation and a significant improvement in memory-oriented behavioral tests. Using a similar passive immunization approach, Bard et al. showed that peripherally administered monoclonal antibodies against the amyloid β -peptide enter the CNS of platelet-derived growth factor promoter (PDAPP) mice (Bard et al. 2000). Additionally, these passively administered antibodies promoted the clearance of pre-existing amyloid, thus reducing the plaque burden. Prophylaxis against amyloid pathology required multiple injections of high-titer antibody in all studies. Approaches that provide for the stable production of $A\beta$ -specific antibody in situ, such as via gene transfer, may represent a more viable therapeutic option.

52.3.2 Single Chain Antibodies as Passive Immunotherapeutics

One gene-based passive vaccination approach that may serve in this capacity involves the use of single-chain antibodies (scFv's). ScFv's are composed of the minimal antibody-binding site formed by non-covalent association of the V_H

and V_L variable domains joined by a flexible polypeptide linker. Human scFv-phage libraries are available and allow for high affinity human scFv antibodies to be selected from combinatorial libraries. Thus far, phage display has proven to be a powerful tool for rapidly generating and isolating recombinant antibodies. Selection by phage display entails binding of phage populations expressing antibody molecules on the tip of the phage particle to a specified antigen (i.e., α -A β peptide). The antigen is immobilized on a microtiter plate well, incubated with phage and then washed to remove non-specifically bound phage. Phages are eluted from antigen, re-expanded and the entire process is repeated for several more cycles, a process termed “panning”. At each cycle, the fraction of specifically bound phage increases while the diversity of antibody sequences decreases until the population consists of only phages that can bind antigen with roughly equivalent panning efficiencies (Malone and Sullivan 1996). The phage clones identified with strong affinity to the target antigen are then amplified in *E. coli* (Haidaris et al. 2001).

Sequences encoding eucaryotic secretion signals (i.e., kappa light chain leader) can be appended to the scFv genes and subsequently be cloned into gene transfer vectors. Cells transduced by a given gene transfer vector will act as a nexus of scFv expression, leading to a gradient of secreted scFv to act extracellularly on pathogenic factors such as A β . Further antibody engineering makes it possible to manipulate the genes encoding these antibodies to allow for expression within mammalian cells. Genetically fusing the scFv to intracellular targeting signals allows for specific subcellular expression (Zhu et al. 1999). These intracellular antibodies, termed intrabodies, are capable of modulating target protein function in at least three important ways. Intrabodies can: (1) block or stabilize macromolecular interactions; (2) modulate enzyme function by sequestering substrate, occluding an active site or keeping the enzyme in an active or inactive conformation; and (3) divert proteins to alternative intracellular compartments (for review (Richardson and Marasco 1995)). Intrabodies have been utilized for both phenotypic and functional knockouts of target molecules. For example, scFv's directed against the extracellular domain of ErbB-2 Receptor Tyrosine Kinase 2 (ErbB-2) fused to an endoplasmic reticulum (ER) signaling domain have been expressed intracellularly and successfully target to the ER lumen. These targeted scFv's were capable of binding newly synthesized ErbB-2 and preventing its transit through the ER to the cell surface resulting in functional inactivation of this protein (Beerli et al. 1994). Below we will discuss in details scFv preclinical development in a number of neurodegenerative diseases.

52.3.2.1 Prion Disease

A number of studies have reported to use scFv to target prion proteins (Leclerc et al. 2000; Heppner et al. 2001; Cardinale

et al. 2005; Donofrio et al. 2005; Luginbuhl et al. 2006; Filesi et al. 2007; Flego et al. 2007; Miyamoto et al. 2007; Padiolleau-Lefevre et al. 2007; Polymenidou et al. 2008; Wuertzer et al. 2008; Campana et al. 2009; Muller-Schiffmann et al. 2009; Shimizu et al. 2010; Skrlj et al. 2010, 2011; Fujita et al. 2011; Petsch et al. 2011; Zhang et al. 2011; Kubota et al. 2012; Moda et al. 2012). Many of the scFvs are capable of inhibit cellular prion protein (PrP^c) aggregation and reduce prion protein scrapie associated (PrP^{Sc})-related cellular toxicity. Our group has led an effort to develop passive immunomodulation therapy with recombinant AAV2 delivered anti-PrP^c scFv in a mouse model of prion disease (Wuertzer et al. 2008). Our results showed that the secreted anti-PrP^c scFvs could bind to PrP^c and prevent the formation of the proteinase K-resistant PrP^{Sc}. Treatment with rAA2-scFv delayed the onset of the prion disease in a mouse TSE model. Another approach of deliver for scFv is through scFv expressing cells. Donofrio et al. produced RD-4 rhabdomyosarcoma cells expressing anti-PrP 6H4 scFv (Donofrio et al. 2005). Filesil et al. generated PC12 cells that express secreted version of anti-prion scFv (Filesi et al. 2007). In both studies, the secreted anti-PrP scFv inhibited PrP^{Sc} formation. More recently, Fujita and colleagues established a Ra2 microglial cell line expressing anti-PrP 3S9scFv. Microglia are known to infiltrate the prion lesions. Delivery of the anti-PrP scFv by Ra2 microglial cells showed a slight but significant improvement in survival time when the microglial cells were injected into mice 7 weeks after 22 L scrapie prion infection (Fujita et al. 2011).

52.3.2.2 Alzheimer's Disease

The first anti-A β scFv for the treatment of Alzheimer's disease was produced based on the variable regions of an anti-A β IgM 508 antibody (Frenkel et al. 2000). This scFv, named 508 F(Fv), could lead to the disaggregation of A β fibrils and prevent toxic effects in cultured PC-12 cells. By screening a naïve human scFv phage library with A β 1–28 and A β 1–40 Liu et al. selected two positive scFv clones, H1v2 and C1, which bound to the N-terminal or C-terminal of A β , respectively (Liu et al. 2004). Liu et al. also showed that H1v2 but not C1 could inhibit A β aggregation in vitro (Liu et al. 2004). Likewise, engineered scFv antibody based on mAb WO-2, which recognize A β 2–8 could inhibit A β fibrils formation, disaggregate A β fibrils, and reduce A β oligomer toxicity (Robert et al. 2009).

Anti-A β scFvs can be easily delivered with AAV vectors. Tg2576 mouse AD model injected with rAAV2 expressing anti-A β scFv had detectable scFv in the hippocampal neurons 1-year post injection. The mice with rAAV2-CAscFv59 injection showed decreased levels of amyloid deposits without obvious neurotoxicity (Fukuchi et al. 2006). Another study of AAV1 delivered anti-A β 1–16, A β 40, and A β 42 achieved widespread scFv expression in the mouse

brain. Intracranial delivery of AAV1 expressing anti-A β scFvs did not show any adverse effect and achieved about 20–50 % reduction in amyloid deposits in CRND8 mice (Levites et al. 2006). Wang et al. demonstrated that intramuscular delivery of rAAV2-scFv against A β was also safe and effectively reduced total A β levels in the brain (Wang et al. 2009). We intrahippocampally delivered rAAV1-A β -scFv in a 3xTg-AD mouse model and found reduced A β and hyperphosphorylated tau levels and improve cognitive functions (Ryan et al. 2010). All above studies demonstrated the safety and efficacy of AAV delivered long-term expression of anti-A β scFvs as a viable AD treatment option.

52.3.2.3 Huntington Disease

In the past decade, various intracellular anti-Huntingtin (Htt) immunotherapies have been developed as a potential treatment options for Huntington disease (HD) (Butler et al. 2012), including anti-Htt scFvs. A scFv (C4) recognizing the 17 N-terminal Htt was identified by screening the human phage display library (Lecerf et al. 2001). Co-expression of this C4 scFv and the expanded repeat Htt-polyQ-GFP reduced the number of Htt aggregates in COS-7 cells (Lecerf et al. 2001). In organotypic slice cultures, co-transfection of the C4 scFv could reduce the malonate-induced cell death in mutant Htt-expressing cells (Murphy and Messer 2004). Further study showed that C4 scFv specifically targets the soluble portion of the mutant Htt N-terminal fragment and therefore may shift the equilibrium of the mutant Htt aggregation (Miller et al. 2005). The C4 scFv had been tested in vivo in a *Drosophila* HD model that expresses Htt exon 1-Q93. The survival rate of this model with scFv expression increased by 70 %. The C4 scFv expression also prevented neurodegeneration and prolonged the lifespan of the HD flies (Bortvedt et al. 2010; Wolfgang et al. 2005). Striatum delivery of AAV2/1 expressing C4 scFv in B6.HDR6/1 mice reduced mutant Htt aggregates in neurons. Striatal expression of C4 scFv seemed to be well tolerated and did not exhibit any adverse effects in mice (Snyder-Keller et al. 2010). However, the efficacy of the AAV delivered C4 scFv decreases over time and age of the animals. In an effort to improve the efficiency of C4 scFv, Butler and Messer generated a scFv fusion protein with a PEST domain. The C4 scFv-PEST fusion protein retained the anti-mutant-Htt function and has increased proteasomal degradation of the mutant Htt (Butler and Messer 2011). The collective data have established the efficacy of C4 scFv in targeting the mutant Htt in vitro and in vivo.

52.3.2.4 Parkinson's Disease

α -synuclein (SYN) has become a major target for PD immunotherapy as aggregated SYN-containing Lewy bodies in nigral dopamine neurons (Mezey et al. 1998; Spillantini et al. 1997). Our laboratory has generated conformation-

specific humanized scFvs against SYN. These scFvs can be efficiently expressed in mammalian cells through transductions with HSV vector carrying the scFvs expression cassette (Maguire-Zeiss et al. 2004). Emadi et al. identified 10 anti-SYN scFvs by screening naïve human phage display libraries. A strong scFv recognizing the C-terminal of SYN could inhibit SYN aggregation in vitro (Emadi et al. 2004). Co-expressing of anti-monomeric SYN scFv and SYN in mammalian cells prevented SYN aggregation and reduced SYN-mediated cellular toxicity (Lynch et al. 2008; Zhou et al. 2004). Similarly, scFv against oligomeric SYN could also prevent SYN aggregation and rescue cytotoxicity (Emadi et al. 2007).

52.3.3 Active Vaccination

In contrast to passive means of preventing pathogenic protein accumulation in the CNS, active immunization involves using antigen to stimulate the host to produce vigorous immune responses, including the elicitation of antibodies and cytotoxic cells. This more conventional immunization method is many times preferable since long-term immune memory to a specific antigen can be readily maintained.

52.3.3.1 Alzheimer's Disease

Recent publications have highlighted the potential of active A β peptide-based immunization in the treatment of AD (Janus et al. 2000; Morgan et al. 2000; Schenk et al. 1999). Schenk et al. used a transgenic model that overexpresses a mutant Amyloid Precursor Protein (APP) V717F mini-gene driven by the platelet-derived growth factor promoter (PDAPP; Schenk et al. 1999). PDAPP mice immunized and boosted with A β _{1–42} peptide with complete and incomplete Freund's adjuvant, respectively, before the onset of AD-like pathology, were protected from development of plaque formation, neuritic dystrophy, and astrogliosis. Treatment of older PDAPP animals (11 months) with existing pathology resulted in reduced plaque burden and slower progression of AD-like neuropathology. A β -directed vaccination was assessed by other laboratories in a model of AD that overexpresses mutant human β APP₆₉₅ (K670N/M671L; Swedish mutation) and presenilin-1 (PS1) mutations under the control of the hamster prion promoter (PSAPP; Morgan et al. 2000; Takeuchi et al. 2000). Neuropathologically, these animals demonstrate compact amyloid plaques but no dramatic neuronal loss in either the hippocampus or association cortices. Behaviorally, PSAPP mice exhibit learning and age-related memory deficits as amyloid accumulates. Following vaccination with A β , PSAPP mice were protected from learning and age-related deficits as well as a partial reduction in amyloid burden. There were no apparent deleterious effects of the vaccination.

These groundbreaking studies provided impetus for the implementation of Phase I and II clinical trials where human aggregated A β and a potent adjuvant (QS-21) was used to immunize individuals with advanced AD. While some of the patients enrolled in the Phase II trial generated anti-A β antibody titers (Lee et al. 2005), the trial was halted due to the occurrence of aseptic meningoencephalitis in a subset of responders (based on the antibody titer) and non-responders. Postmortem analyses of two patients that died showed that the encephalitic response was characterized by infiltrating CD4 cells but not CD8s, giant nucleated cells, or macrophages (Ferrer et al. 2004; Gilman et al. 2005). Most intriguing was the observation that these brains harbored low A β plaque load strongly suggesting that active A β immunization could alter A β accumulation patterns (Nicoll et al. 2003). This trial not only demonstrated that immunization against A β may be effective but underscored the requirement that AD vaccines require fine-tuning to greatly diminish the likelihood of eliciting brain inflammation.

52.3.3.2 Parkinson's Disease

The discovery that SYN can transmit from cell to cell in a prion-like fashion suggests that immunization might be a viable option for the treatment of PD with synucleinopathies. The first SYN vaccine that enters clinical testing is AFFITOPE® PD01 developed by AFFiRiS (Masliah et al. 2005). The AFFITOPE® PD01 vaccine consists of a peptide carrier conjugate and is formulated with aluminum hydroxide as immunological adjuvant. The SYN peptides are 7 amino acids in length, thus too short to induce a SYN-specific T cell response avoiding T cell autoimmunity. T cell helper epitopes of the carrier protein provide T cell help to activate the B cell response. AFFITOPE® PD01 has been designed to prevent cross-reactivity of the antibodies induced with the other synuclein family members such as β -syn. Preclinical studies involving the subcutaneous administration of AFFITOPE® PD01 have demonstrated the induction of a SYN-specific humoral immune response. Experiments done in transgenic mouse models of synucleinopathies showed that immunization with AFFITOPE® PD01 reduces the level of cerebral SYN and attenuated SYN-triggered neuropathological alterations including neuronal cell loss and dendritic density. Furthermore, PD01 treated animals show superior cognitive functions as assessed by the Morris water maze test. In the phase I clinical trial, two different doses of PD01 (15 and 75 μ g) were safe and well tolerated, meeting the primary endpoint of the trial. Fifty percent of the vaccinated patients generated SYN-specific antibodies as measured in serum samples. Additionally, vaccine-induced antibodies were detectable in cerebrospinal fluid. Furthermore, analysis of clinical endpoints revealed a trend towards functional stabilization of the vaccinated groups compared to non-

vaccinated control patients. The promising phase I clinical data warrant later phase clinical testing.

52.4 Current Neurological Gene Therapy Clinical Trials

Gene therapy is a promising option for a number of neurodegenerative diseases. The potential benefits of using viral platform for correcting diseases are enormous, and as a result considerable efforts have been made to develop and test in patients (Table 52.2).

52.4.1 Parkinson's Disease

PD presents an excellent clinical target for viral vector gene therapy as PD exhibits defined locations of pathology and a specific neurotransmitter deficit. To date, several gene therapy approaches have been developed to treat the major motor symptoms of PD, all with the use of AAV or lentivirus vector platform (Table 52.2). Each of the clinical approaches has focused on aspects of the basal ganglia.

The first PD gene therapy approach has attempted to ameliorate motor symptoms by altering the neurochemical conductivity of neurons mediating the motor behavior. AAV/GAD (Aromatic L-Amino Acid Decarboxylase) was delivered to the subthalamic nucleus. GAD catalyzes the synthesis of gamma aminobutyric acid, the major inhibitory neurotransmitter in the CNS, potentially providing lost inhibitory control in the basal ganglion motor system, thus restoring appropriate transynaptic balance (Luo et al. 2002). Given the positive safety and tolerability findings in a Phase I study (Kaplitt et al. 2007), a Phase II trial was initiated with 45 subjects that included both treatment and sham groups (LeWitt et al. 2011). Although the results proved significant on the primary measure (UPDRS motor-off scale), the effects were quite modest. Moreover, many other measures were not significant, even though five subjects were removed from the analysis due to injection targeting issues. The modest nature of these Phase II results led to a discontinuation of the program.

A second approach has attempted to attain therapeutic level of dopamine by overexpressing enzymes that contribute to dopamine synthesis. One clinical investigation used AAV2 to deliver L-DOPA-converting enzyme AADC (Aromatic L-Amino Acid Decarboxylase) in the putamen. In the Phase I clinical trials, no toxicity occurred from the vector infusion into the putamen, but the therapeutic outcomes were modest at best (Christine et al. 2009). A long-term follow-up to the phase I study (Christine et al. 2009) found a significant elevation in the positron emission tomography (PET) value 4 years after the vector delivery (Mittermeyer

Table 52.2 Gene therapy clinical trial for neurodegenerative diseases

Study	Phase	Viral vector	Therapeutic gene	Delivery target	Results
<i>Parkinson's disease</i>					
Kaplitt et al. (2007)	I	AAV2	GAD	Subthalamic nucleus	Well tolerated; advanced to PhII
LeWitt et al. (2011)	II	AAV2	GAD	Subthalamic nucleus	Mixed results; program suspended
Christine et al. (2009)	I	AAV2	AADC	Putamen	Well-tolerated; improved motor rating scales
Muramatsu et al. (2010)	I	AAV2	AADC	Putamen	Well-tolerated; no further testing
Marks et al. (2008)	I	AAV2	Neurturin (CERE-120)	Substantia nigra and putamen	Well-tolerated; advanced to PhII
Marks et al. (2010)	II	AAV2	Neurturin (CERE-120)	Putamen	Well-tolerated; mixed results
Kells et al. (2010)	I	AAV2	GDNF	Putamen	NA
Rafii et al. (2014)	I/II	Lentivirus	AADC, TH and GTPCH	Putamen	Well tolerated with moderate motor behavior improvement; Open label
<i>Alzheimer disease</i>					
Tuszynski et al. (2005)	I	Retrovirus	NGF	Ex vivo implantation; forebrain	Well tolerated; improved in the rate of cognitive decline
Rafii et al. (2014)	I	AAV2	NGF (CERE-110)	Nucleus of Meynert	Safe and well-tolerated for 2 years

et al. 2012). Another identical Phase I trial was initiated that used the same AAV-AADC vector (Muramatsu et al. 2010). Again there were no adverse events attributed to the vector infusion. But the outcome measures reflected only modest improvements. To overcome the efficacy issue, another Phase I trial has been initiated with the goal of obtaining greater putamen transgene expression through increased dosage and CED (convexional enhanced delivery) technique (Clinical Trials. Gov identifier: NCT01973543). This ongoing Phase I study should provide more definitive evidence as to whether a significant level of efficacy can be achieved with an improved gene delivery method. A different open label clinical study used a lentiviral vector expressing AADC as well as TH (tyrosine hydroxylase) and GTP-CH1 (guanosine 5'-triphosphate cyclohydrolase1) (ProSavin), with the latter two enzymes also significantly contributing to dopamine's synthesis (Palfi et al. 2014). In this phase I/II study, no serious adverse events related to the study drug or surgical procedure were found. A significant improvement in mean UPDRS part III motor scores off medication was recorded in all patients at 6 month and 12 months compared to baseline.

The third gene therapy strategy has employed a neurotrophic factor delivery approach to confer trophic support to the dopaminergic and surrounding neuronal populations. The first trophic factor approach has used AAV2 to deliver neurturin (CERE-120), related to glial cell line-derived neurotrophic factor (GDNF). Both the phase I (Marks et al.

2008) and phase II (Marks et al. 2010) trials found no adverse effects but did not find significant improvement in the primary endpoint measures up to 12 months post AAV infusion. However, 15–18 months later, significant improvements were found. A subsequent Phase II study was initiated where the AAV2-neurturin was infused into both putamen and the substantia nigra. However, this double-blind trial failed to demonstrate significant changes in the primary endpoint, precluding a further Phase III trial. Another ongoing neurotrophic factor gene therapy has used AAV2 to express GDNF. In this clinical trial, an improved delivery approach, CED, has been adopted to broaden the transgene coverage area (Kells et al. 2010).

52.4.2 Alzheimer's Disease

The gene therapy approaches for AD have been focused on nerve growth factor (NGF). The first clinical trial of NGF therapy was ex vivo NGF gene delivery in eight individuals with mild AD. Autologous fibroblasts genetically modified to express human NGF were implanting into the forebrain (Tuszynski et al. 2005). Evaluation of the Mini-Mental Status Examination and AD Assessment Scale-Cognitive subcomponent suggested improvement in the rate of cognitive decline. PET scans showed significant increase in cortical 18-fluorodexyglucose after treatment. Brain autopsy

from one subject indicated robust growth responses to NGF. These positive findings suggested that long-term growth factor treatment had the potential to improve symptoms and modify neurological disease progression.

Another clinical study of NGF gene therapy employed AAV2 to deliver NGF (CERE-11) directly to the forebrain region (Rafii et al. 2014). The Phase I clinical trial of CERE-110 with dose escalation demonstrated that surgical delivery of CERE-110 to the brain results in the long-term expression of bioactive NGF. Clinicians also observed apparent stabilization of brain cell metabolic activity in treated subjects, which may reflect a slowing of cell deterioration. The treatment was well tolerated at all doses levels. The positive results of Phase I trial supported the initiation of an ongoing multicenter, double-blind, placebo-controlled Phase II trial.

52.4.3 Safety of Gene Therapy

The first gene transfer trials were approved in 1989, but the field has yet to live up to its promise and hype. The initial progress in gene therapy in the 1990s was followed by a string of tragedies. The first was in 1999, when 18-year-old Jessie Gelsinger died of complications from an inflammatory response shortly after receiving a dose of experimental adenovirus vector in a gene therapy trial for a rare liver disorder. His death halted all gene therapy trials in the US for a time, sparking a much-needed discussion on how best to regulate experimental trials and report health problems in volunteer patients. Between 1999 and 2006, the use of retrovirus to deliver gene therapy to patients for various immune disorders caused nine cases of leukemia (including one death) and three patients acquired a bone marrow disease (Pike-Overzet et al. 2006). In 2007, a 36-year-old woman with a 15-year history of rheumatoid arthritis, died of fungus infection and subsequent multi organ dysfunction after receiving an investigational gene therapy product in a clinical trial (Evans et al. 2008). A gene intended to reduce inflammation and disease was delivered into the affected joint using recombinant AAV2 vector expressing TNF- α antagonist. These unfortunate incidents raised important safety concerns, and the federal and local regulatory agencies such as FDA (Food and Drug Administration), RAC (Recombinant DNA Advisory Committee), IRB (Institutional Review Board) and IBC (Institutional Biosafety Committee) have since developed comprehensive requirements for gene therapy clinical trials to ensure the right and safety of human research subjects.

All the clinical trials of CNS gene therapies that have been tested so far display a favorable safety profile; the most consistent adverse events reported for all the studies were related to the stereotactic surgical procedure, which was similar to the stereotaxic surgical procedure used for deep brain stimulation approved by European regulatory agencies and FDA. Additionally, a substantial placebo effect observed in

all three Phase II PD studies indirectly verified the safety of stereotaxic procedure (Palfi et al. 2014; Markakis et al. 2010; LeWitt et al. 2011).

Another concern in the field involves the potential impact of preexisting neutralizing antibodies to viral vectors. However, with regard to the brain, neutralizing antibodies so far has appeared not to contribute to any local inflammatory reaction, transgene expression, or long-term bioactivity (Bartus 2012).

Immune responses against the transgene are perhaps a greater concern. Most gene transfer trials would deliver wild-type human genes, which would not be expected to generate an immune response under normal circumstances. However, in standard gene replacement approaches in patients with genetic diseases, the patients may not produce any of the missing protein in any form. In this case, the treated patients might view the wild-type protein expressed from vector-transduced cells as a foreign protein. This could cause a humoral response limiting the efficacy of a secreted protein, and/or a cytotoxic T-lymphocyte response against transgene-expressing cells. Recent studies of using AAV9 to deliver GFP to the brain in rats and nonhuman primates indicated that transduction of antigen-presenting cells, such as astrocytes, within the CNS could lead to immune-mediated clearance of transduced cells (Ciesielska et al. 2013; Samaranch et al. 2014). Not only could this cause a loss of transgene expression, but the destruction of transduced cells could lead to deleterious adverse effects. In nonhuman primates injected with AAV9/GFP vectors (modeling the expression of a foreign antigen), treated animals developed severe ataxia requiring euthanasia within a few weeks of injection (Samaranch et al. 2014). Thus, it is advisable to place restrictions on trial subjects to exclude those that would be at high risk of developing an immune response against the wild-type expressed protein.

52.4.4 Efficacy of Gene Therapy

Despite the promising safety profile of the gene therapy trials for CNS diseases in Phase I, all the phase II clinical trials either yielded mixed results or displayed modest improvement that did not meet the primary end points (LeWitt et al. 2011; Palfi et al. 2014; Marks et al. 2010). In light of the consistent disconnect between preclinical and clinical success in gene therapy, we need to take a step back from the clinic and reexamine the predictive power of animal models, the approaches of gene delivery, and the selection criteria of trial patient subjects.

The fact that most of gene therapy programs produced robust and positive data in the most widely accepted animal models of neurodegenerative diseases but failed to replicate the magnitude or consistency of these effects in patients necessarily raises a question of whether the predictive validity of these models is sufficient for establishing preclinical proof of concept. In the case of PD, the overreliance of acute toxin-based PD models (e.g., 6-OHDA and MPTP model) limits

our exploration of treatment options and models that encompass more the pathogenic variables. As more than 90 % of PD cases are idiopathic and the pathogenesis is multifactorial, derived from environmental factors acting on genetically predisposed individuals with aging, a double/multiple-hit model that can incorporate both environmental insults and genetic susceptibility will provide better prediction for the therapeutic potential of gene therapy.

Another major concern highlighted in the PD gene therapy trials included weak transgene expression and protein product transport issues in the pathological brain tissue. In addition to improved delivery technologies such as CED, there are foreseeable advantages of alternating the vector serotype or using engineered vectors to improve transduction directly. Current AAV gene therapy clinical trials all use AAV serotype 2. As we discuss previously, AAV2 has the relatively weakest transduction efficiency in neurons and glial cell types compared to other serotypes. Although AAV2 displays a desirable safety profile and has obtained regulatory approval for clinical testing, it is worthy the research effort to develop improved vectors for better transduction.

The third consideration for the improvement of the efficacy of gene therapy is the selection criteria of patient subjects for trials. A recent exploratory analysis of the AAV2-neurturin phase II data suggests that earlier-stage PD subjects (defined here as those 5 or fewer years postdiagnosis) showed much greater clinical benefit from AAV2-neurturin than did those treated at 10 or more years postdiagnosis (Bartus et al. 2013). This raises the age issue in the gene therapy strategy for neurodegenerative diseases, particularly with regard to neurotrophic factor intervention. It is of great importance to determine whether earlier-stage patients might indeed respond far better and more reliably than those currently enrolled in experimental treatments. Progress in clinical biomarker research, especially for presymptomatic neurodegenerative diseases, will undoubtedly shape the earlier intervention efforts and may help establish surrogate endpoints to allow a more biometrically equilibrated scoring system.

52.5 Future Outlook

The future of gene-based immunotherapy for neurological disease remains mired in many unknowns. The most informed choice of vector platform, gene regulation system, and immunotherapeutic strategy will depend on numerous non-mutually exclusive factors: the specific neurodegenerative disease, the disease stage, the underlying inflammatory mechanisms associated with the disease, the interplay between those events and those inherent to the proposed vector systems following CNS delivery, and the therapeutic transgene itself. Another major challenge that faces this field involves the heterogeneity of human immune responses. It is therefore imperative that research continues

to better understand vector associated antigens and transgene product related immune responses in the context of the host nervous system. Thus far, the safety profile of AAV and more limited experience with lentiviral vector transduction portend demonstration of clinical benefit. However, delivery and anatomical issues unique to the targeted disease entity remain a challenge that is likely surmountable. We envision that the pre-screening of clinical trial participants would allow for the derivation of custom therapeutics (O'Toole et al. 2005; Rosenberg 2005). These considerations will be important in the conceptual design and the clinical implementation of gene-based immunotherapeutics for diseases afflicting an organ system formerly regarded, and unfortunately so, as an immunoprivileged region of the human body.

52.6 Review Questions

1. What are the two formats of non-viral gene transfer modalities
2. What is the rationale for viral vector selection?
3. What are the potential safety issues for gene therapy in CNS disorders?
4. What are the currently common viral vector platforms for gene transfer?
5. List three vectors that have been developed to increase packaging capacity of AAV virus?
6. Which AAV serotypes can effectively transduce microglia?
7. What are the two forms of HSV-1 based delivery vectors cable of gene transfer to the nervous systems?

52.7 Answers

1. lipid and exosomes
2. vector capacity; tropism; duration and levels of transgene expression; safety profiles
3. surgery complications; preexisting neutralizing antibodies; immune response against the transgene
4. Adenovirus, lentivirus, HSV, and AAV
5. dual trans-splicing vector; dual overlapping vector; dual hybrid vector
6. AAV1 and AAV4
7. recombinant HSV vector and Amplicon vector

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Abstract

A wide range of neuroimaging techniques have been developed which aid in the clinical diagnosis of neurological diseases and are valuable tools for research into basic physiological mechanisms and effects of new therapies to combat neurological disease. This is a rapidly evolving field as new imaging techniques with enhanced sensitivity and specificity are constantly being developed and made available to the clinician and research community. This chapter will provide an introduction to the basic mechanisms, capabilities and applications of neuroimaging methods, allowing the neuroscience student to gain an appreciation of the range of available imaging techniques and the potential for use as serial non-invasive monitors of the progression and treatment of neurodegenerative disease in animals and humans.

Keywords

Magnetic resonance imaging • Magnetic resonance spectroscopy • Neurodegeneration • Positron emission tomography • Single photon emission computed tomography

53.1 Introduction

Neuroimaging techniques include nuclear medicine studies such as positron emission tomography (PET) and single photon emission computed tomography (SPECT). Morphological imaging methods include magnetic resonance imaging (MRI) and computed tomography (CT). Studies of brain physiology and biochemistry include PET, SPECT, CT perfusion, MRI perfusion, magnetization transfer MRI (to assess myelin loss), ^1H and ^{31}P magnetic resonance spectroscopy (MRS) and magnetic resonance spectroscopic imaging (MRSI). These methods provide a broad array of techniques for serial non-invasive measurement of alterations in brain physiology and biochemistry, which are early and sensitive indicators of many neurological diseases. Although struc-

tural imaging techniques are not always sensitive indicators of neurological disease, functional and molecular imaging methods, including SPECT, PET and physiological MR techniques, have great value as they can reveal abnormalities before structural atrophy or focal CNS lesions are visible.

Neuroimaging techniques can be broadly classified by the information content of the images. Morphological information can be obtained from MRI and CT, sensitivity and specificity of MRI for detecting brain lesions is improved over CT (Brugieres et al. 1995) due to improved soft tissue contrast of MRI (Ell et al. 1987; Kim et al. 1996). In addition, MRI or CT can be combined with nuclear medicine techniques to provide anatomical detail allowing clear identification of the location of radioactive probes. Physiological information, including tissue perfusion mapping, mapping of receptor densities, and mapping of tissue biochemistry can be accomplished using MRI, MRSI, SPECT, and PET. Functional information, mapping of neuronal activity, can be obtained using functional MRI and PET, as well as other imaging modalities not covered in this chapter including electroencephalography (EEG), which records the electrical activity

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of the brain and magnetoencephalography (MEG) which provides spatially resolved maps of the electrical activity of the brain. This chapter will cover the basics of signal generation, signal acquisition and some examples of the information obtained in MRI, MRSI, SPECT and PET as an introduction to this broad and fascinating field.

53.2 Principles of Imaging

53.2.1 Basic Principles of MRI

MRI methodology is a complex and intriguing field of study. In this chapter, the basic mechanisms of MRI signal production and acquisition are introduced to allow the student of the neurosciences to develop a base understanding. Excellent textbooks are available for more advanced study of the details (Haacke et al. 1999; Liang and Lauterbur 2000; Slichter 1996; Gillard et al. 2005).

53.2.1.1 Signal Source

The signal in magnetic resonance imaging (MRI) and spectroscopy (MRS) rely on the magnetic properties of nuclei (Baier-Bitterlich et al. 1998). Nuclei with a non-zero *spin quantum number* ($I \neq 0$) will possess a *magnetic moment vector* μ . The magnetic moments are a result of the paramagnetic fields produced by the interaction of charged particles within the nucleus. The spin quantum number of the nucleus will be zero ($I=0$) if both the mass number and the atomic number of the nucleus are even. These nuclei will not have a nuclear magnetic moment and hence are not observable using magnetic resonance techniques. Any nucleus with an odd atomic number or an odd mass number will have a non-zero spin quantum number ($I \neq 0$). These nuclei will have a nuclear magnetic moment and thus can produce a signal.

For nuclei with $I=1/2$, including ^1H observed in MRI, the magnetic moment vector μ of the nuclei will either be parallel or anti-parallel to the externally applied magnetic field (Fig. 53.1). The magnetic moment of a nucleus can be related to its angular momentum by the magnetogyric ratio of that nucleus defined by (Harris 1983; Krane 1987):

$$\gamma = \frac{\mu}{I} \quad (53.1)$$

where μ is the nuclear magnetic moment vector of a nuclear isotope, and I is the angular momentum (spin) quantum number of a nucleus. The magnetogyric ratio is constant for each nuclear isotope. The sum of all magnetic moments is the macroscopic magnetic moment, named *the Net Magnetization Vector* \mathbf{M} , which is defined as:

$$\mathbf{M} = \sum (\mu_+ + \mu_-) \quad (53.2)$$

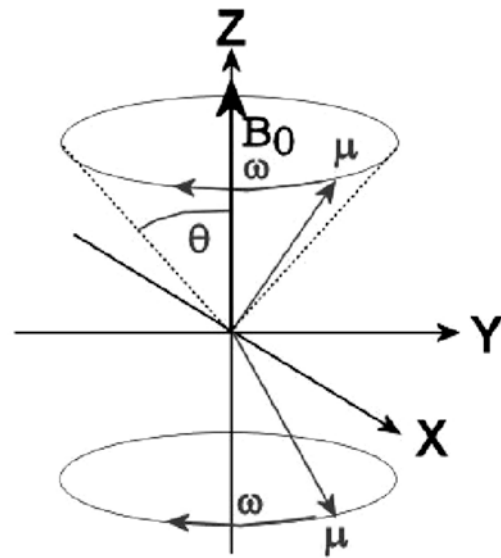


Fig. 53.1 The precession of the nuclear magnetic moment vectors μ about the static magnetic field vector B_0 . The angle of the precessional cone is given by θ . The precessional frequency is given by the Larmor frequency ω

where μ_+ and μ_- represent magnetic moments in the parallel and anti-parallel state for a two quantum states ($m_I = \pm 1/2$) of a spin $1/2$ magnetic nucleus ($I = 1/2$) such as the proton.

53.2.1.2 Precession and the Larmor Equation

The two states of different energy are not exactly parallel and anti-parallel to the external magnetic field. The magnetic moments are at an angle θ relative to the magnetic field due to the quantized energy states of the subatomic particles. The force of the magnetic field B_0 on the magnetic moments μ forces these nuclear magnetic moments into a precessional motion (Fig. 53.1). The frequency of precession, also called *Larmor* frequency, is proportional to the magnetic field according to the Larmor equation:

$$\omega = \gamma * B_0 \quad (53.3)$$

where ω is the rotational frequency of the net magnetization produced by excitation of the nuclear magnetic moments (rad/s), and γ is the gyromagnetic ratio, constant for a given nucleus. B_0 is the magnetic field strength at the nucleus measured in Tesla (T) or Gauss (G). $1 \text{ T} = 10,000 \text{ G}$. As an example, the resonance frequency of protons is 64 MHz at 1.5 T.

The net magnetization vector \mathbf{M} is the source of MR signal. The time evolution of the net magnetization vector \mathbf{M} is the signal source in magnetic resonance. Motion of \mathbf{M} will create an oscillating magnetic field. This oscillating magnetic field will create an induced voltage in a receiving coil. This induced voltage in the receiving coil over time is the signal.

53.2.1.3 Resonance

When a nucleus is exposed to a secondary magnetic field (B_1) that has an oscillation identical in frequency and direction to its own Larmor precession, the nuclei absorb energy from the external source. The secondary magnetization is applied at the Larmor frequency, which is typically in the radiofrequency (RF) range, thus the brief application of the B_1 field is referred to as an RF pulse. Absorption of energy from the RF pulse results in a phase coherence in individual nuclei during the process of changing from the low energy to the high energy state. The net effect of B_1 is a tipping of M_0 (net equilibrium magnetization) away from the Z (longitudinal) axis (parallel to B_0) towards the X,Y (transverse) plane. The force of B_0 on the transverse component of M now rotates M_{xy} at the Larmor frequency. The rotating magnetization causes a voltage in a coil “tuned” to the Larmor frequency to pick up the signal by magnetic induction (Fig. 53.2). The resulting angle between the Z axis and M (typically 90°) is called *flip* angle and depends upon the amplitude and duration of the RF pulse.

53.2.1.4 Signal Detection and Time Evolution

Faraday’s law of induction states that if a receiver coil is magnetically coupled to an oscillating magnetic field, a voltage is induced in the receiver coil. Therefore, as M precesses at the Larmor frequency in the transverse plane, a voltage is induced in the coil. This voltage constitutes the *MR signal*. The magnitude of the signal depends on the magnitude of M in the transverse plane. The amount of magnetization in the

longitudinal plane gradually increases (T_1 relaxation). At the same time, but independently, the net magnetization in the transverse plane gradually decreases due to loss of phase coherence (T_2 relaxation). As the magnitude of transverse magnetization decreases, so does the magnitude of the voltage induced in the receiver coil (Fig. 53.2). The evolution and decay of the net magnetization, M is picked up by the receiver coil resulting in the magnetic resonance signal, the *free induction decay (FID)*.

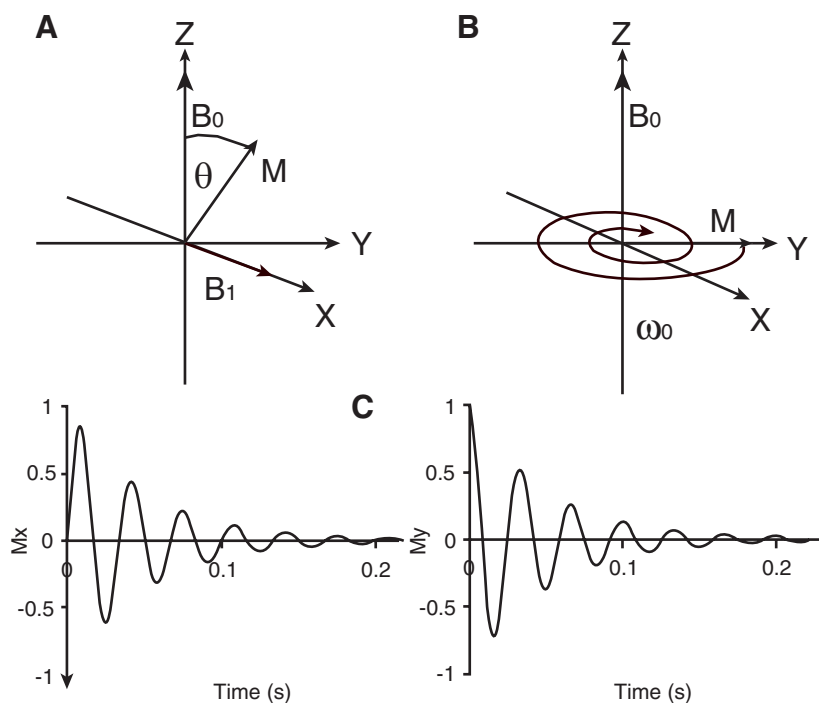
When the RF pulse is switched off, M realigns with the Z axis at a rate $(1/T_1)$ slower than the rate of signal loss $(1/T_2)$. In order to do so, M must transfer the energy gained to the surrounding molecules. This process is referred to as T_1 relaxation (spin–lattice relaxation). As T_1 relaxation occurs, M returns to *equilibrium* (M_0).

53.2.1.5 Magnetic Field Gradient used to Generate MRI

The magnetic field dependence of the signal’s frequency is used as a basis for spatial encoding of the magnetic resonance signal. This is done using magnetic field gradients. Magnetic field gradients cause a linear shift in magnetic field with position. This allows one to use a single chemical species, such as water, as a signal source for imaging (MRI). Sophisticated spatial encoding schemes have been developed to allow multidimensional spatial encoding.

The Larmor equation demonstrates the basis of these encoding schemes. As the magnetic field changes, so does the frequency. In one dimension, application of a magnetic field gradient of 10 gauss/cm (1 Tesla (T)=10,000 gauss (g))

Fig. 53.2 Application of a secondary magnetic field (B_1) rotating at the Larmor frequency has the effect of tipping the net magnetization (M) away from the equilibrium position (M_0) along the longitudinal axis and towards the transverse plane. After the brief application of B_1 , rotation of M in the transverse (X-Y) plane at the Larmor frequency (B) induces a Free Induction Decay (FID) signal in a pickup coil (C)



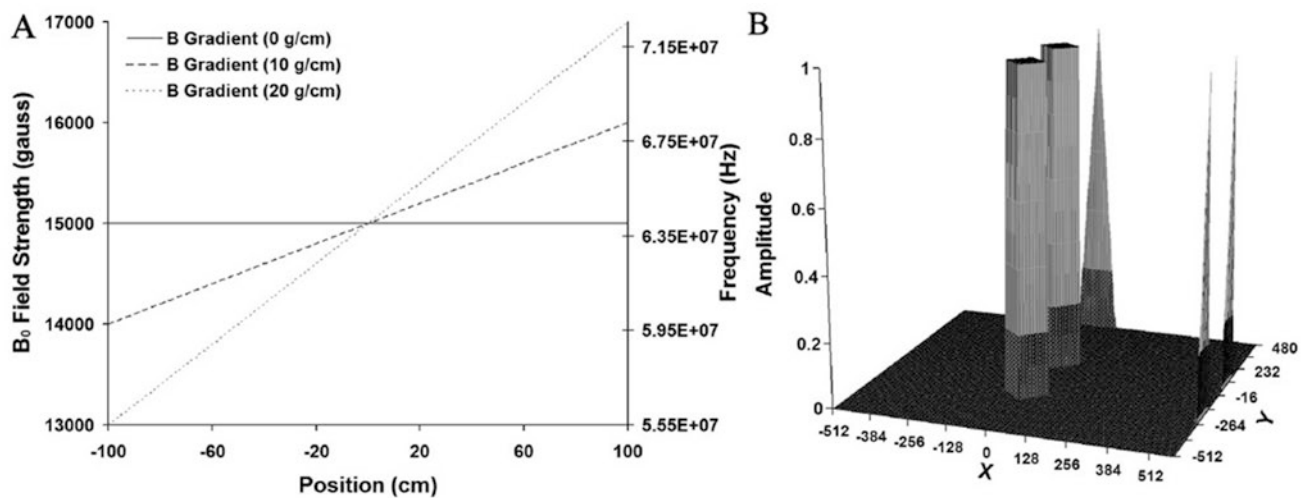


Fig. 53.3 Magnetic field gradients used for frequency encoding spatial position. (a) Magnetic field strength as a function of position in the magnet for three gradient strengths. (b) Arbitrary object within the MRI

system (*center*) and the frequency readout (signal) from a gradient applied during signal acquisition along the x-axis (rear) and the y-axis (*right*)

produces a field shift in the water frequency of 64 MHz (1.5 T) of 42,667 Hz/cm. The basis of the use of the magnetic field gradient for spatial encoding is displayed in Fig. 53.3.

53.2.1.6 Fourier Transform: Frequency Analysis of the Time Domain MR Signal

The time domain MR signal is difficult to visually interpret, hence the signal is transformed into a spectrum which is intensity on the vertical axis and frequency on the horizontal axis. This is performed mathematically by the Fourier transform. The Fourier transform is performed according to the equation:

$$f(\omega) = \int_{-\infty}^{\infty} f(t) e^{-i\omega t} dt \quad (53.4)$$

where ω = frequency (radians/s), t = time (s), $i = \sqrt{-1}$, $f(\omega)$ is the frequency domain signal (spectrum) and $f(t)$ is the time domain signal (MR signal, free induction decay or spin echo).

53.2.1.7 Spin Echo

Signal loss in the free induction decay (FID) can be partially refocused using a second application of B_1 with a duration and amplitude that produces a 180° flip angle of \mathbf{M} (180° pulse). This has the effect of placing individual magnetic moments, which are no longer in phase with the central frequency, in a position to realign with spins at the central frequency.

Pulsed magnetic resonance experiments are represented by parallel time lines of the applied pulses and the signal (Fig. 53.4a). We will introduce this formalism here to explain the spin-echo and, in the next section, the spin-echo MRI pulse sequence. The first line is the time line of the applied

B_1 pulses, also referred to as radiofrequency, or RF pulses because they are applied at the Larmor frequency, which are typically in the radiofrequency range.

53.2.1.8 Spin-echo Magnetic Resonance Imaging (MRI)

Three dimensions of spatial encoding are required for MRI. These three dimensions are independently applied by (1) signal production from a thin slice by using a frequency selective excitation in the presence of a magnetic field gradient (slice selection), (2) acquiring the spin-echo signal in the presence of a magnetic field gradient (frequency or read-out gradient) and (3) acquiring the spin-echo signal after producing a spatially dependent phase shift which is incremented in the second dimension (phase encoding gradient). The pulse sequence diagram showing the application of the frequency selective RF pulses, the slice, phase and frequency encoding gradients and the spin-echo signal acquired are shown in Fig. 53.5. The spin-echo acquisition is repeated for each value of phase encoding gradients to fill in the second dimension of the single slice MRI, requiring repetition of the sequence 256 times for 256×256 image resolution. The raw MRI is acquired in the time domain, which requires Fourier transform of the signal into the frequency domain for display (Fig. 53.6).

53.2.2 Modification of Signal Intensity

The power and versatility of magnetic resonance techniques lie in the ability to modify signal intensity based on the biophysical properties of the spins of interest (primarily tissue water). Signals can be “weighted” (signal amplitude altered)

Fig. 53.4 Time evolution of the spin-echo signal. (a) Pulse sequence diagram showing (top) the timing of the applied 90 and 180° RF pulses (B_1) and (bottom) the time evolution of the spin-echo signal. (b) Vector diagram of the time evolution of the spin-echo signal. Clockwise from top-left: Equilibrium magnetization is rotated by the 90° RF pulse, signal evolves and individual spins lose phase coherence, reducing signal intensity, a 180° RF pulse is applied which places out of phase magnetic moments in position to rephase at a time (τ) equal to the time of dephasing before the 180° pulse, allowing the refocusing of the signal (spin-echo)

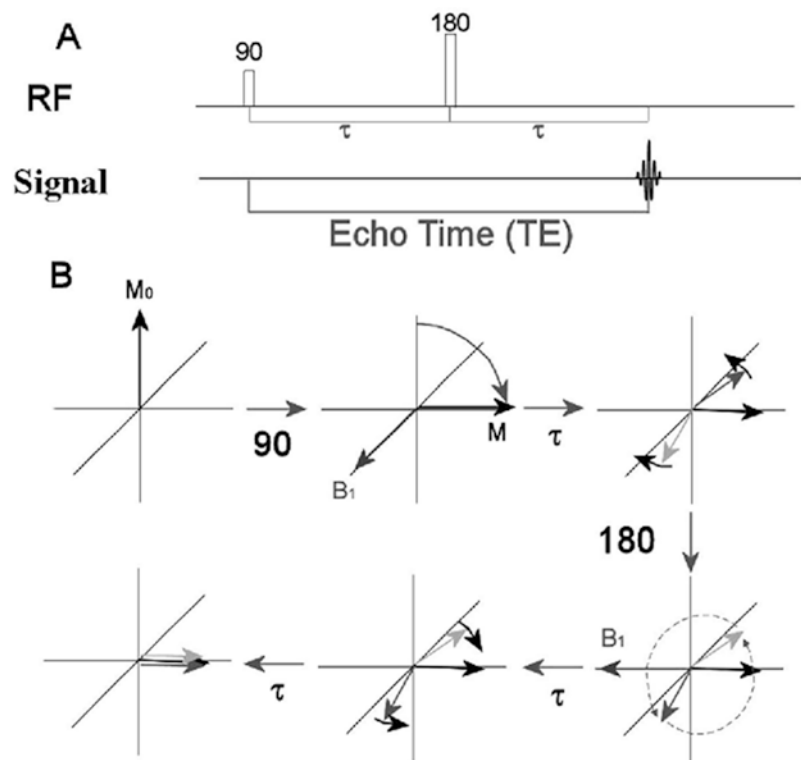
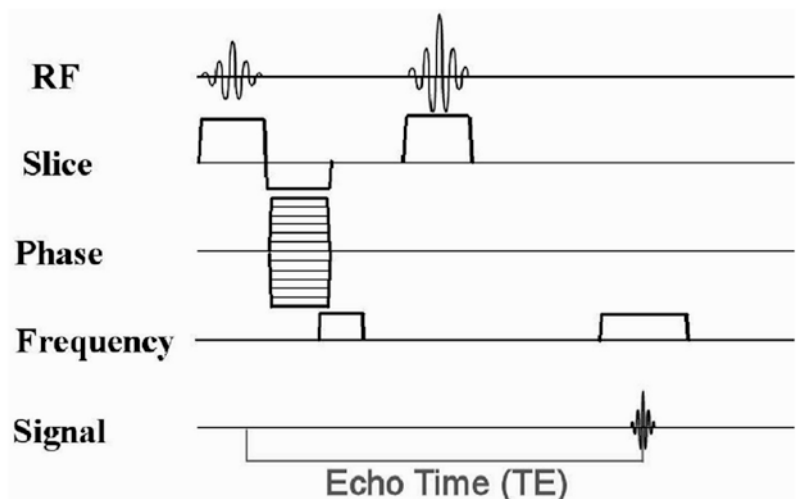


Fig. 53.5 Pulse sequence for the generation of spin-echo MRI



based on the magnetic relaxation properties (T_1 and T_2), water diffusion (random molecular motion), or the interaction of the hydration layers of membranes and proteins with free water (magnetization transfer). Tissue perfusion can be mapped by the introduction of contrast agents or inversion of signal from water in arterial blood supply of the tissue. In addition, signal can be suppressed from tissue based on frequency (fat or water suppression), position (suppressing motion artifacts from unwanted tissue), or preferentially viewed based on moving vs. static water (angiography or suppression of motion from flowing blood).

53.2.2.1 Magnetic Relaxation, T_1 and T_2

Spin-Lattice Relaxation (T_1)

T_1 relaxation is the process of releasing the energy absorbed by the magnetic nuclei during the application of the RF pulse. This process occurs by releasing the energy to the surrounding molecules (lattice) returning the spin system to the equilibrium condition (M_0). The rate at which this process occurs in tissue depends on the molecular environment and varies with tissue type, providing a means of generating contrast between tissues with otherwise similar signal intensities

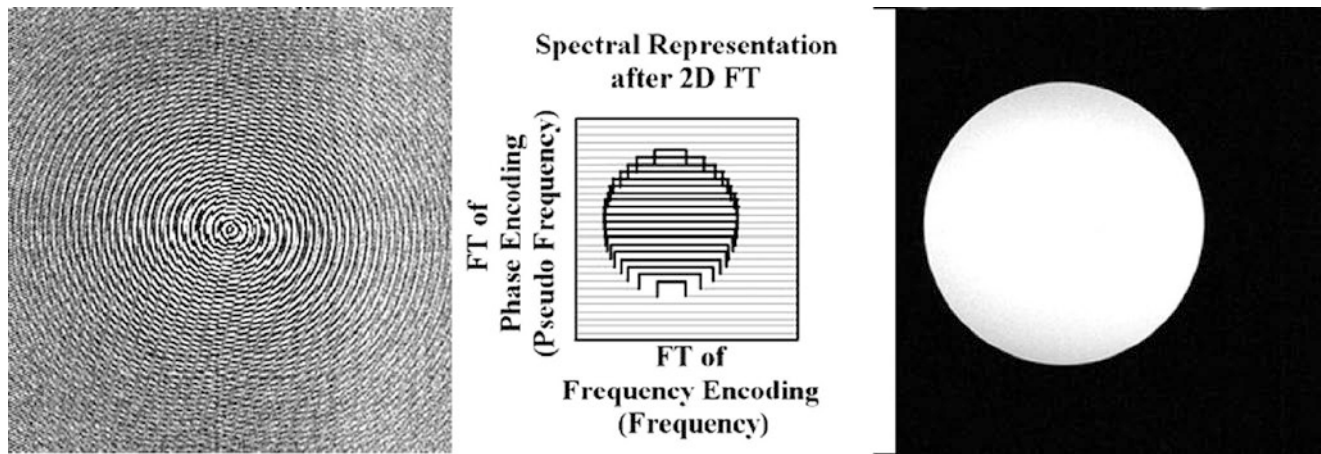


Fig. 53.6 MRI signal, 2 dimensional Fourier transform and grey scale representation of the Fourier transformed data. *Left*: grey scale representation of the single slice MRI of a spherical phantom filled with

water. *Center*: spectral representation of the Fourier transformed data. *Right*: image represented in gray scale, the standard viewing method

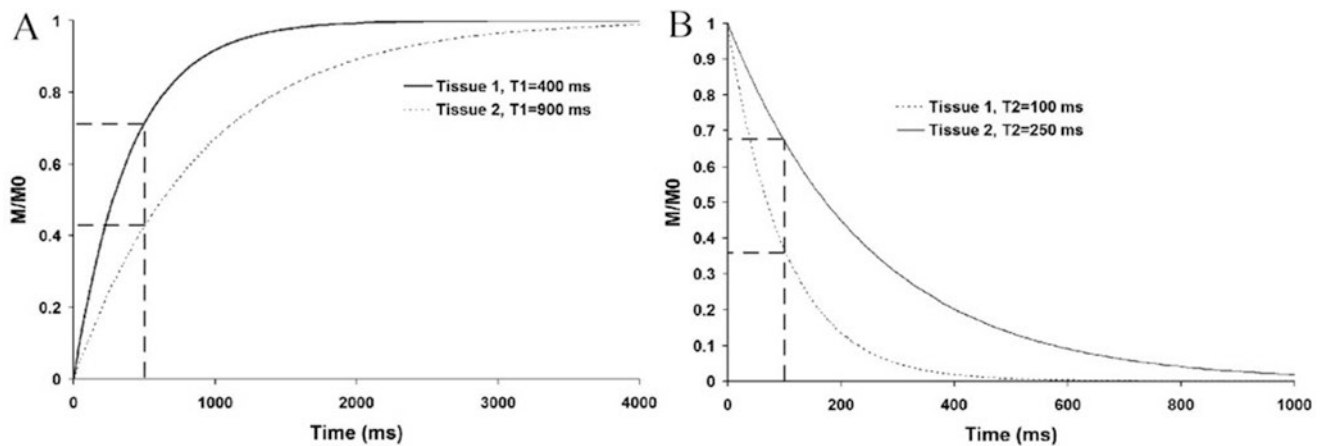


Fig. 53.7 T_1 and T_2 relaxation curves. (a) Recovery of the net magnetization along the Z (B_0) axis after a 90° RF pulse. Dotted lines demonstrate the amount of Z magnetization after a 500 ms delay (TR), proportional to the signal intensity after the second 90° RF pulse. This demonstrates the contrast available in tissue of similar water content (M_0) with different T_1 by reducing TR. (b) decay of signal in the trans-

verse plane (X-Y) with echo time (TE). *Dotted lines* demonstrate the amount of longitudinal (X-Y) magnetization (signal) with a 100 ms spin echo (TE), proportional to the signal intensity during the acquisition of a 100 ms echo. This demonstrates the contrast available in tissue of similar water content (M_0) with different T_2 by increasing TE

(water content). T_1 relaxation occurs exponentially after an RF pulse with a time constant of T_1 according to the equation:

$$M_z(t) = M_0(1 - e^{-t/T_1}) \quad (53.5)$$

where: $M_z(t)$ is the time dependent amplitude of the Z component of the magnetization (along B_0), which approaches the equilibrium magnetization (M_0) over time (t) with the time constant T_1 . Since MRI acquisition typically requires multiple excitations, adjusting the repetition time (TR) between successive excitations reduces the net magnetization according to TR/T_1 and alters the T_1 based contrast. As a result, signal intensity within each volume element in the

MR image (voxel) has intensity modified by the average T_1 of tissue within the voxel according to the repetition time of the acquired image. This occurs as illustrated in Fig. 53.7a. Figure 53.7a shows the Z-magnetization recovery curves for two different tissues, one with a T_1 of 400 ms and one with a T_1 of 900 ms. In order to recover full magnetization, a delay (TR) of $5 \times T_1$ is required. Using a short TR (500 ms, dashed lines) the tissue with the 400 ms T_1 has recovered to 71 % of M_0 while the tissue with the 900 ms T_1 has only recovered to 43 % of M_0 . The signal intensity will be proportional to the recovery of the net magnetization after the second 90° RF pulse, providing a signal that is 65 % more intense for the shorter T_1 tissue even if the original signal

intensity (M_0 , proportional to water content) is identical, providing improved tissue contrast and a mechanism for detecting altered cell content (pathology) within tissue.

Spin-Spin Relaxation (T_2)

The spin echo (Sect. 53.2.1.6) demonstrates that the 180° RF refocusing pulse reverses off-resonance effects (spins at a slightly different frequency than the Larmor frequency) after the 90° RF pulse allowing a signal to be generated at a time after the original signal loss. However, the natural process of energy exchange between adjacent molecules causes a loss of signal coherence by exchange of spin states with loss of phase coherence. This loss of signal cannot be refocused. The exchange of spin states with no net loss of energy in the spin system is referred to as spin-spin relaxation (T_2). Signal loss as a function of echo time (or TE) is given by:

$$M = M_0 e^{-TE/T_2} \quad (53.6)$$

where TE=echo time and T_2 is the average T_2 of the tissue. As a result, signal intensity within each volume element in the MR image (voxel) has intensity reduced by the average T_2

of tissue within the voxel according to the echo time of the acquired image. T_2 effects on the signal intensity in two different tissues with the same water content (M_0) at an echo time of 100 ms is shown by the dashed lines in Fig. 53.4b. Tissue with shorter T_2 (100 ms) refocuses 37 % while the longer T_2 tissue (250 ms) refocuses 67 % of the original signal, demonstrating significant contrast between tissues of different T_2 using a long echo time acquisition. This demonstrates the contrast available in tissue of similar water content (M_0) with different T_2 by increasing TE. It should also be noted that the signal intensity will be modified at the same time by TR and T_1 through the choice of TR, thus a long TR is typically chosen for purely T_2 weighted images. An example of T_2 weighted imaging and calculation of a T_2 map from multiple TE images from mouse brain can be seen in Fig. 53.8.

Multiple echoes may be refocused from one excitation by application of multiple 180-degree RF pulses, allowing the acquisition of a series of echoes within one TR. For example, if one echo is acquired at very early echo time and a second echo is acquired at 100 ms, two images can be acquired. If long TR is used so that the second echo is purely T_2 weighted, then the first echo with almost no T_2 weighting due to short

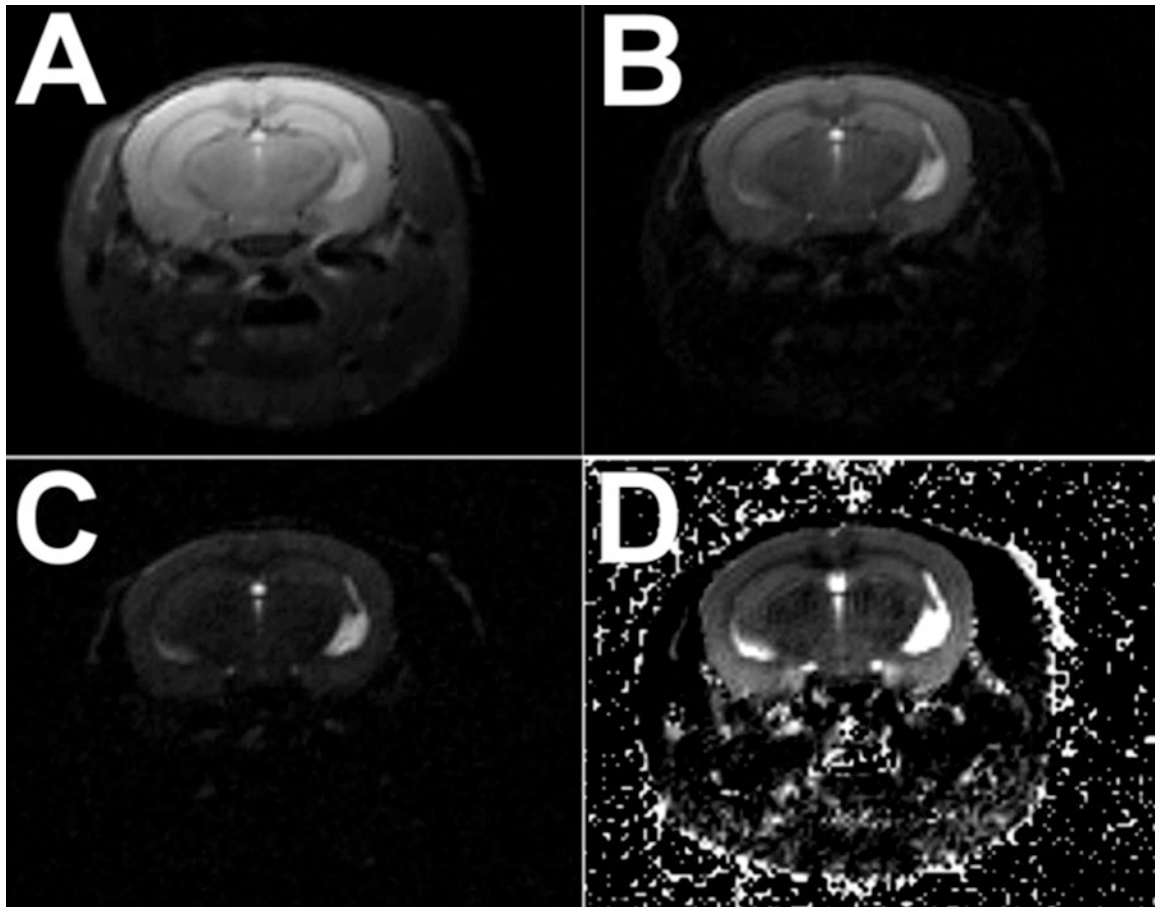


Fig. 53.8 T_2 mapping data set, 3/8 T_2 weighted images with (a): TE=15 ms, (b) TE=60 ms, (c) TE=120 ms and (d) T_2 map created by fitting eight echo imaging sequence to (53.6)

TE and no T_1 weighting due to the long TR will have contrast based purely on the tissue water content (M_0). This image would be referred to as *proton density weighted*, as the signal intensity in each voxel is proportional to the free water content.

53.2.2.2 Diffusion

Symmetrical application of magnetic field gradients around the refocusing pulse in a spin echo will refocus the signal from static molecules. However, microscopic molecular motion (Brownian motion) will cause dephasing of individual magnetic moments to a degree which is dependent on the freedom of the molecular motion of the water in cells. Freedom of the molecular motion is affected by the size and shape of the cell and is modified in different cell types. Signal intensity is modified by:

$$ADC_w = \log(S_0 / S) * b \quad (53.7)$$

where b is the strength of the diffusion weighting, and ADC_w is the apparent diffusion weighted coefficient of water. A high b -value produces low signal intensity in tissue with a high ADC_w . ADC_w is given in mm^2/s , a value which depends on the temperature and viscosity of the liquid or solid under measure. Since we are typically looking at water in MRI, the diffusion of water is what is measured. Free water, at typical body temperature (38°C) is approximately $2.2 \times 10^{-3} \text{ mm}^2/\text{s}$. However, water in tissue is not free to diffuse as a result of barriers, the cell membrane, and is reduced according to the size and shape of the cell. Shorter distances between cell walls result in a lower diffusion rate. Areas of free water in tissue, such as cerebral spinal fluid or large arteries and veins, show similar diffusion rates to free water. Tissue diffusion values in cells range from 0.6 to $1.0 \times 10^{-3} \text{ mm}^2/\text{s}$. In addition, if the cell is not spherical, which is typically the case in brain, ADC_w is dependent on the direction of diffusion weighting.

Diffusion can be measured separately in various directions by application of diffusion gradients in different directions during individual measurements. In order to obtain an approximation of the average diffusion of water in an asymmetrical cell, measurement of diffusion in three orthogonal directions (for example, along X, Y, and Z directions) are obtained and averaged. However, this is an approximation and a true value can only be obtained by determination of the **diffusion tensor**. This requires a minimum of six individual measurements. This can be understood once the student is familiar with the definition of a tensor.

A tensor is a mathematical representation of a three dimensional shape using a three by three matrix (Fig. 53.9, (53.8)). The matrix represents the total magnitude of the object in each of nine directions, three of which are equal due to symmetry (e.g. $D_{xy} = D_{yx}$) of the other three cross components, reducing the problem to six dimensions. Hence,

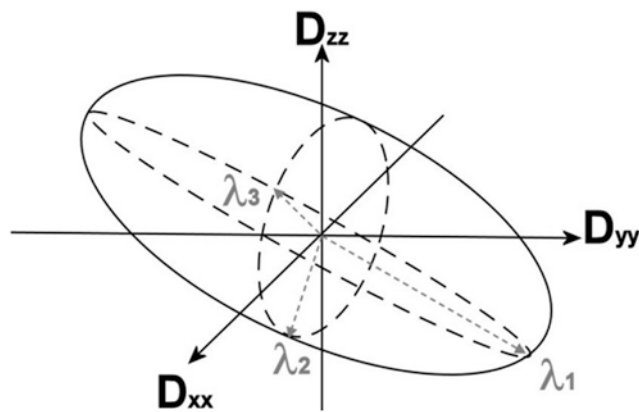


Fig. 53.9 Example of a non-spherical cell with directional dependence of diffusion. Primary laboratory frame of reference are given by x, y and z axes, while the primary axes of diffusion are given as λ_{11} (major), λ_{22} (intermediate) and λ_{33} (minor). Determination of the values of elements of the diffusion tensor allows determination of the magnitude and direction of the primary axes of diffusion (eigenvalues)

six directions of diffusion measurement are the minimum needed to define the diffusion tensor. More measures can be used to improve the accuracy of the measurement through cross correlation of multiple measures.

$$\vec{D} = \begin{bmatrix} D_{xx} & D_{xy} & D_{xz} \\ D_{yx} & D_{yy} & D_{yz} \\ D_{zx} & D_{zy} & D_{zz} \end{bmatrix} \quad (53.8)$$

The diffusion tensor can then be *diagonalized* to generate a diffusion tensor of the form:

$$\vec{D} = \begin{bmatrix} \lambda_1 & 0 & 0 \\ 0 & \lambda_2 & 0 \\ 0 & 0 & \lambda_3 \end{bmatrix} \quad (53.9)$$

to determine the primary *eigenvalues* (λ_1 , λ_2 , λ_3) as designated in Fig. 53.9. These data can be used to generate pixel by pixel maps of mean diffusivity ($D_{av} = 1/3 * (\lambda_1 + \lambda_2 + \lambda_3)$) and fractional anisotropy (FA), which is given by the equation:

$$FA = \frac{1}{\sqrt{2}} \sqrt{\frac{(\lambda_1 - \lambda_2)^2 + (\lambda_2 - \lambda_3)^2 + (\lambda_1 - \lambda_3)^2}{\lambda_1^2 + \lambda_2^2 + \lambda_3^2}} \quad (53.10)$$

FA ranges from zero for a spherical cell to one for an infinitely long cell with zero cross sectional area.

Determination of the principle eigenvalues (λ_1 , λ_2 , λ_3) also allows determination of the principle direction of the cell orientation, which is the orientation of λ_1 in the laboratory (X, Y, Z) frame. Principle direction of the cell can be used to determine fiber orientation in white matter tracts and provide additional delineation of anatomical structure. A map of λ_1 orientation is produced using a red-green-blue encoding in

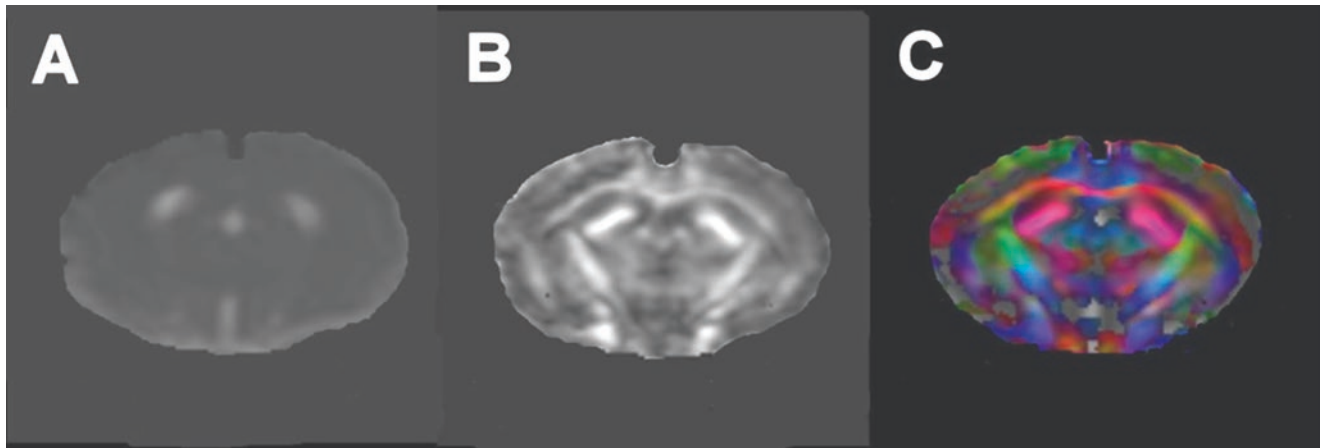


Fig. 53.10 Diffusion tensor imaging results from a mouse brain imaging study at 7 T. (a) mean diffusivity (D_{av}) (b) Fractional Anisotropy (FA), (c) Color coded FA orientation map

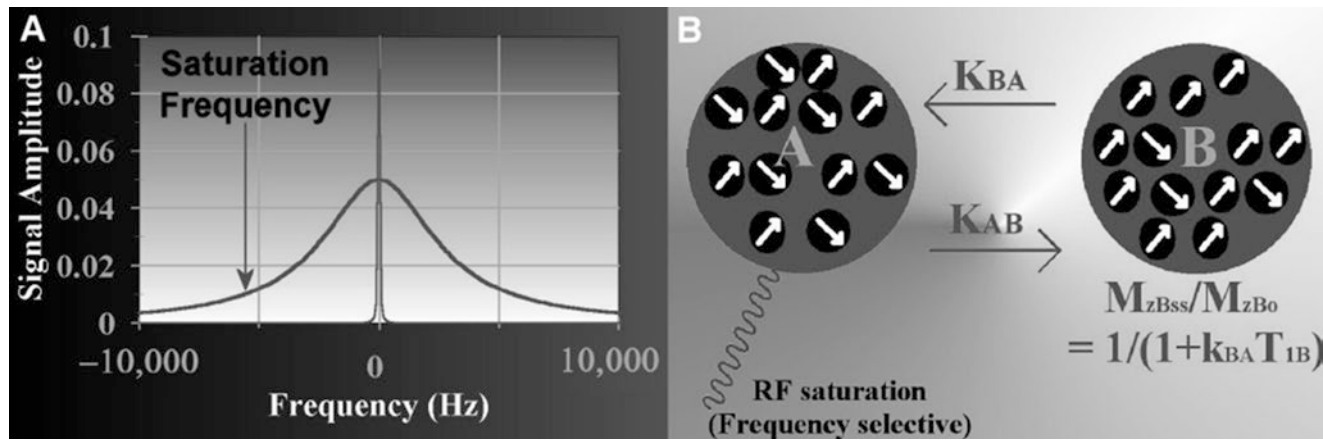


Fig. 53.11 Magnetization transfer MRI. (a) Off-resonance application of a saturation pulse will saturate the signal from macromolecules with short T_2 (broad line) while leaving the narrow water resonance (center line) unaffected. (b) Saturation of macromolecules (spin pool A) causes

reduction of the free water signal (spin pool B) measured as reduction in intensity of MRI (M_{zBss}/M_{zBo}) inversely proportional to the exchange rate of free water with the water in the macromolecular hydration layer (k_{BA}) times the T_1 of free water (T_{1B})

each pixel to represent the direction of the primary eigenvalue, with red representing L-R orientation, blue representing inferior-superior (foot to head) direction and green representing the anterior-posterior direction. Intermediate angles are represented by linear combinations of the three primary colors. An example of DTI from a mouse brain showing the D_{av} , FA, and RGB encoded λ_1 direction maps are displayed in Fig. 53.10.

53.2.2.3 Magnetization Transfer

T_2 of protons on molecules are dependent on molecular motion. Large molecules such as membranes and proteins with slow molecular motion have a very short T_2 (less than 1 ms). As a result, the linewidth of these molecules is very broad compared to free water. The relationship between linewidth (full width at half maximum) and the T_2 of molecules is given by:

$$\Delta\nu = \frac{1}{\pi * T_2} \quad (53.11)$$

Where $\Delta\nu$ =linewidth (full width at half maximum). As a result, selective saturation of the protons on large molecules and membranes without affecting the magnetization of free water can be accomplished by a narrow bandwidth RF pulse 4–20 kHz off resonance from the free water signal. Application of the saturation pulse for several seconds is required for a steady state to develop between the saturated macromolecules and exchange of the free water with the hydration layer of the macromolecules, developing a partial saturation (signal loss) in the free water. Signal loss by this mechanism is proportional to the exchange rate of water in the hydration layer of the membranes and macromolecules (Fig. 53.11). Determination of the degree of magnetic saturation is done using two images, one without the saturation

pulse and one with the saturation pulse. The two images are then divided to determine the spatial distribution of the magnetization transfer ratio (MTR) according to the equation:

$$MTR = \frac{M_0 - M_{ss}}{M_0} \quad (53.12)$$

where M_{ss} = signal intensity in the presence of steady state saturation and M_0 = signal intensity in the absence of the off-resonance macromolecule saturation. A more complete description of the magnetization transfer effect can be found in a review article by Henkelman et al. (2001).

53.2.2.4 Perfusion

Cerebral perfusion is a marker for metabolic activity of the brain with normal hemodynamic function. Cerebral perfusion has been used extensively for detection of disease and mapping of brain activation in health and disease. MRI methods for mapping cerebral perfusion include (1) qualitative bolus tracking after injection of magnetic contrast agents (also referred to as dynamic contrast enhanced, or DCE MRI) and (2) quantitative arterial spin-tagging. Each method is described along with the relative strengths and weaknesses, below.

Bolus Tracking (or dynamic contrast enhanced, DCE) Perfusion MRI

Injection of a bolus (typically in 5 s or less) of magnetic contrast agent causes signal intensity changes in tissue as the bolus passes through the tissue. Magnetic contrast agents used are typically chelated gadolinium (e.g. Gd-DTPA) complexes, which reduce T_1 and T_2^* of blood and tissue, increasing signal

in T_1 and reducing signal in T_2^* weighted images. Advanced rapid magnetic resonance imaging techniques, such as echo planar imaging (EPI) are capable of acquiring multiple slices covering the human brain in 1–3 s, allowing the dynamic tracking of the spatial distribution of the injected contrast agent. Signal intensity versus time is then analyzed for each voxel (volume element) in each acquired image to determine (1) time to peak contrast (mean transit time, MTT), (2) area under the bolus passage signal, proportional to cerebral blood volume (CBV). By mass analysis, cerebral blood flow (CBF) can then be determined by the relation:

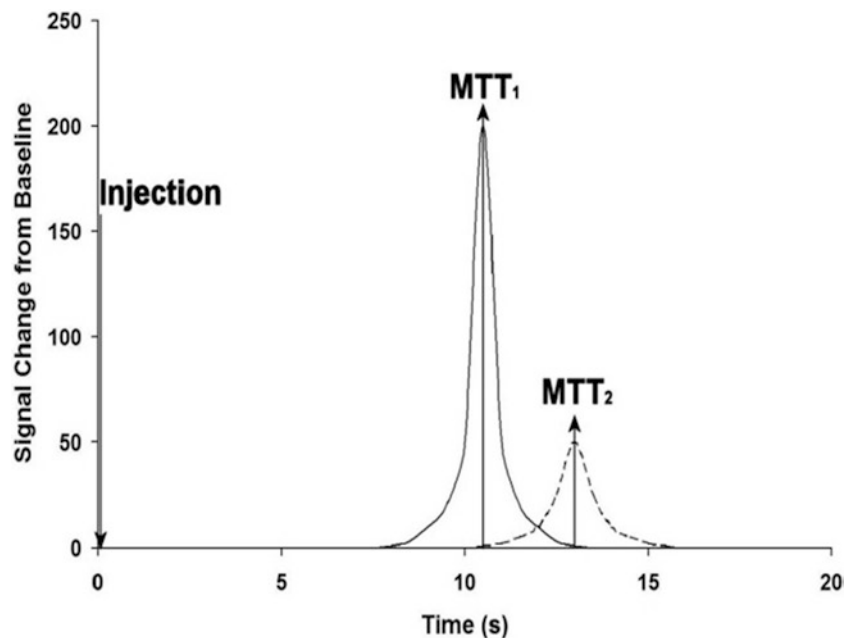
$$MTR = \frac{CBF}{CBV} \quad (53.13)$$

Examples of the signal change over time after bolus injection is shown in Fig. 53.12 for a representative normal flow area (MTT_1) and an area with compromised cerebral perfusion (MTT_2). While this method is robust, it suffers from a number of issues related to absolute quantitation of the CBF including, but not limited to partial volume effects, nonlinear relationship between signal intensity change and contrast agent concentration, and determination of the input function of the bolus required for accurate quantitation. In clinical practice, MTT maps are the preferred method for visualizing areas of reduced CBF.

Arterial Spin-Tagged Perfusion MRI

Quantitation of the CBF can be an important factor in studies of therapeutic intervention. The method of arterial spin-labeled (ASL) perfusion MRI allows quantitative determination of the perfusion rate in units of ml/100 g tissue/min. However, the technique requires exceptional instrumental

Fig. 53.12 Simulated examples of the time course of signal changes after Gd-bolus administration. Voxelwise analysis of the time series of images is used to create maps of mean transit time (MTT), cerebral blood volume (CBV), corresponding to the area under the curve, and cerebral blood flow (CBF) according to (53.13). The delayed curve (MTT_2) corresponds to an area of compromised CBF



stability, as the result depends on accurate determination of the difference between two images on the order of a few percent. With the appropriate acquisition parameters to achieve sufficient signal to noise ratio (>100) and current instrument stability, this technique is feasible, especially in high field clinical systems. Currently, arterial spin-tagged perfusion measures are not widely used in clinical settings.

Arterial spin-tagged perfusion MRI is performed by inverting the water protons in the blood of the arteries supplying blood to the tissue of interest. Typically, in brain, the carotid arteries are used as the supply. The original description of the method (Detre et al. 1992; Williams et al. 1992) uses a continuous narrow band RF inversion of the protons in the arterial blood, outside of the imaged slice, before and after excitation to cause a steady-state loss of signal in cerebral tissue. The signal loss is related to tissue perfusion (Detre et al. 1992) by:

$$f = \frac{\lambda}{T_{\text{lapp}}} \left(1 - \frac{M_{\text{ss}}}{M_0} \right) \quad (53.14)$$

$$\frac{1}{T_{\text{lapp}}} = \frac{1}{T_1} + \frac{f}{\lambda} \quad (53.15)$$

Where:

And: f = flow rate, λ = water extraction fraction of tissue from blood (0.8–1.0), M is the MRI signal intensity with (M_{ss} = steady state) and without (M_0) inversion of the protons in the water of the inflowing blood. One potential complication with this method is the effect of magnetization transfer, which occurs with the continuous inversion pulse. This can be balanced by reversing the gradient so that the spatial location of the inversion is above the head when acquiring the M_0 image. This effect is reduced, but not eliminated using more recent pulsed arterial spin labeling techniques (Kim 1995; Kwong et al. 1995).

53.2.2.5 Relaxometry, Quantitative DWI and Quantitative MT

Perfusion is not the only quantitative method available using magnetic resonance techniques. T_1 , T_2 , apparent diffusion coefficient of water (ADC_w), and MT effects can be quantitatively mapped using the appropriate series of measurements. For example, acquisition of MRI data collected in a series of echo times can be processed to create an image, which is a quantitative map of T_2 (Fig. 53.8). Each effect requires its own set of measures, but in principle all methods for modifying signal intensity in MR can be quantified. With the exception of DWI, quantitative mapping is not typically done in a clinical setting due to the time required for additional measures and additional processing required for these data. Advances in MRI are increasing the speed of acquisition, which may allow quantitative

techniques to precisely define alterations in neuronal cell alterations, physiology and metabolism as research into multidimensional quantitative MRI defines the sensitivity and specificity of combining these methods to diagnose neurological disease.

53.2.2.6 Functional MRI

Functional activation of brain regions causes a flux in the oxygen level and the perfusion rate in the activated tissue. These dynamic changes generate dynamic contrast changes in the MRI signal. This was first demonstrated in 1992 (Kwong et al. 1992) and has developed into a popular method among neuroscientists for mapping task related activity as well as brain functional connectivity.

The mechanism leading to signal change in MRI is the reduction of deoxyhemoglobin in the blood in the activated area. This effect occurs because the vasculature reacts to the increased demand for oxygen and glucose in the activated area by overcompensating, a physiological process known as “luxury perfusion”. During luxury perfusion, the deoxyhemoglobin levels in the blood drop. Deoxyhemoglobin is paramagnetic and will cause reductions in T_1 and T_2/T_2^* . The signal intensity change with brain activation has been referred to as the Blood Oxygenation Level Dependent (BOLD) effect.

Images are acquired continuously during an fMRI study using single shot methods such as echo planar MRI. This allows a single slice to be acquired in tens of milliseconds and allows the entire brain volume to be imaged every second or two. The result is thousands of images that require alignment, timing corrections, and statistical analysis for determining areas of activation. Such computational power was not widely available until the 1990s. Functional MRI is normally performed using a task-based protocol with images taken continuously and the task performed with a precise timing synchronized to the image acquisitions. This “block design” allows the statistical analysis of images with pixelwise correlation between signal intensity changes and the timing of the task. An example can be seen in Fig. 53.13.

Figure 53.13 shows the result from an alternating decision/motor task where the subject is presented a number from 1 to 5 and presses the corresponding finger on both hands. A recording device on each hand with separate buttons for each finger record the responses. This task was performed repeatedly during the time period indicated in red. Between the decision/motor task, subjects rested while an alternating red-green checkerboard pattern flashed in the eyepieces used for presentation inside of the magnet. Activation of the visual cortex correlated with the green time bars as seen in the blue-green overlay on the MRI. Bilateral activation of the motor strip as well as some frontal cortex activity is apparent during the decision/motor task.

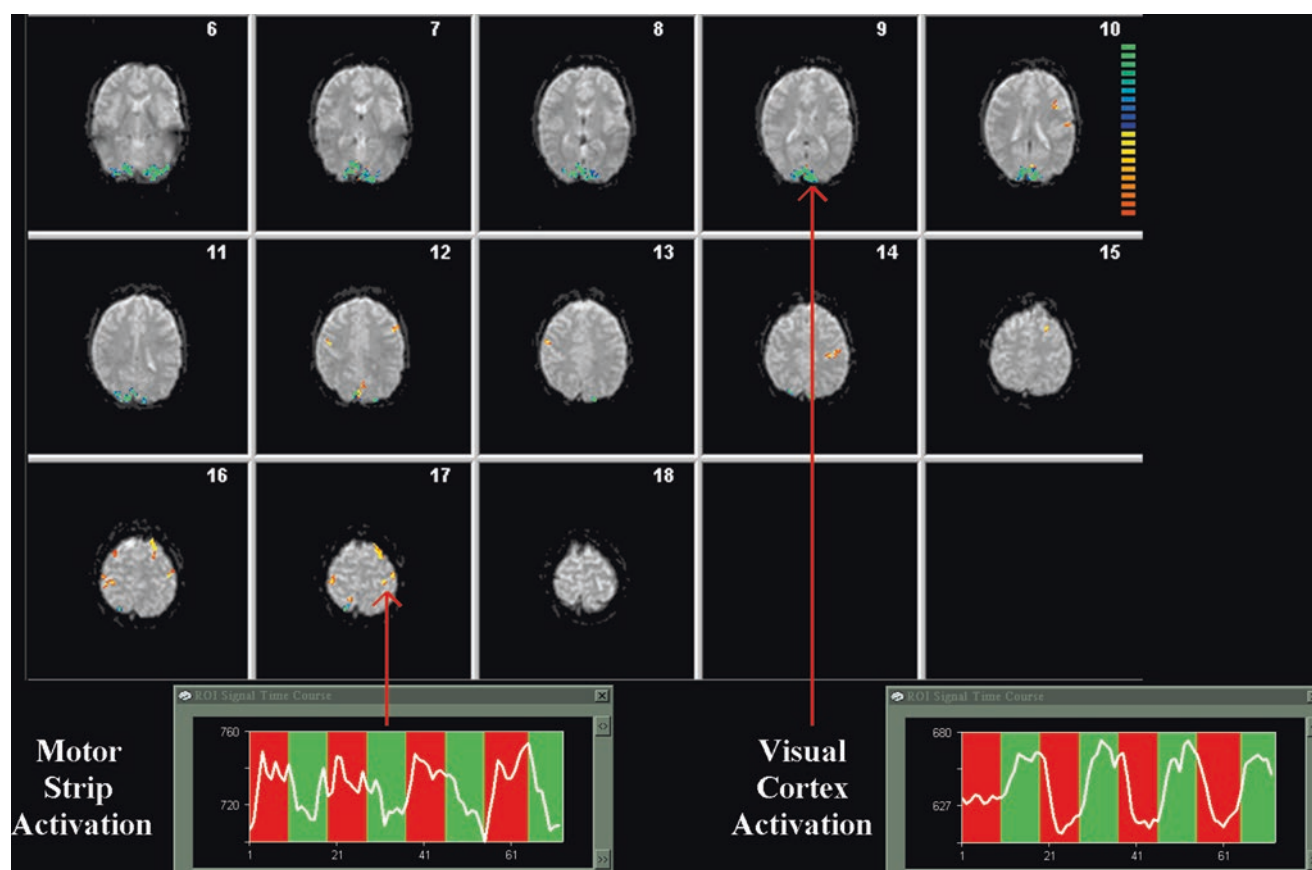


Fig. 53.13 Result from a functional MRI scan with dual protocols

53.2.3 Magnetic Resonance Spectroscopy (MRS) and Spectroscopic Imaging (MRSI)

Prior to the development of MRI in the 1970s magnetic resonance was a well-developed technique used for chemical analysis by spectroscopic analysis of chemicals in sample preparations. This capability has been extended to image guided localized magnetic resonance spectroscopy of tissue in-vivo. Localized spectroscopy uses frequency selective pulses in the presence of a gradient for volume selective excitation and spectroscopic imaging uses phase encoding gradients for spatial encoding of the spectroscopic signals. Many sophisticated pulse sequences have been developed for in-vivo localized spectroscopy and are in use in research and clinical MRI systems worldwide. The difficulties of in-vivo spectroscopy are primarily low spatial resolution due to the low signal intensity. As a result long acquisition times are required to overcome the low signal intensity by signal averaging. Despite this difficulty, in-vivo spectroscopy is unique in the ability to perform non-invasive analysis of chemical composition of tissue without biopsy and allows the serial non-invasive determination of tissue chemistry with treatment. While any nucleus with

a magnetic moment can be studied using MRS and/or MRSI, here we will focus on the most frequently used nucleus for study of brain, the proton (^1H).

Frequency shifts of nuclei on different molecules (spectroscopy) are caused by the electron cloud of a molecule. Electron cloud density, and hence field shifts due to the electron cloud, are specific to each molecule. According to the Larmor equation, this results in a frequency shift of the ^1H signal for each proton on each molecule, developing a specific “frequency fingerprint” for each molecule. The areas of the peaks are proportional to the concentration of each molecule in the region sampled. These areas are analyzed by fitting the peak areas with one of a variety of spectroscopic analysis software packages. An example of the spectrum from a region of mouse brain acquired at 7 T is shown in Fig. 53.14.

^1H MRS applies frequency selective pulses in order to generate signal from a single volume of interest defined using MRI. The signal is acquired in the absence of a magnetic field gradient in order to detect the minor frequency shifts due to the electron clouds around atoms (chemical shift). ^1H MRSI uses either a slice selective excitation or volume selective excitation with spatial encoding over the region using phase encoding gradients. This produces an

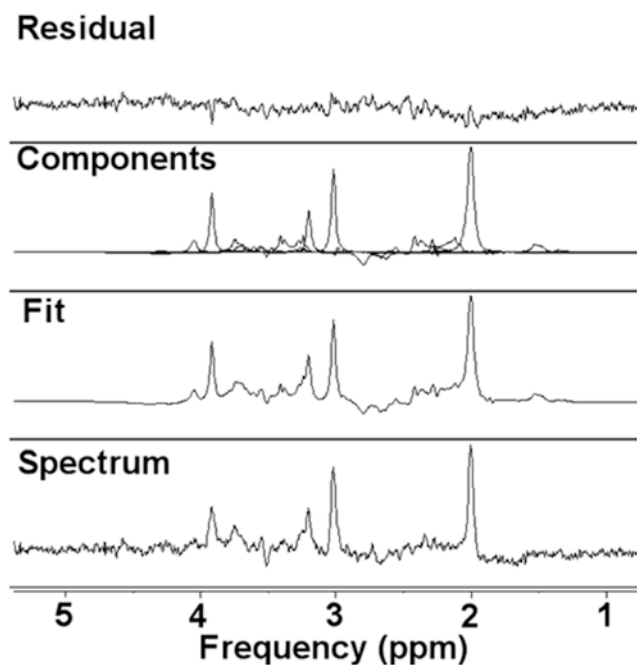


Fig. 53.14 Example of a ^1H MR spectrum from a ^1H MRSI data set with a nominal spatial resolution of $2.34\ \mu\text{l}$ ($1.5 \times 1.25 \times 1.25\ \text{mm}$) in mouse brain. Original: Spectrum obtained from the region. Fit: Overall fit using models of known spectra of metabolites including, but not limited to N-acetyl aspartate (NAA), glutamate (Glu), glutamine, creatine (Cre), choline (Cho) and myoinositol (mI). Components: Individual peaks contributing to the overall spectra of metabolites and Residual: Subtraction of the fit from the original spectrum. The frequency scale is given in ppm ($(\text{frequency}/\text{Larmor frequency}) \times 10^6$) to create a frequency scale insensitive to the magnetic field strength

array of volume selective spectra over the region selected. This technique provides improved spatial resolution and the ability to reformat data post-acquisition to allow precise localization of spectra within the slice or volume selected. These advantages come at the expense of acquisition time (typically 15–30 min in human clinical systems).

Several technical challenges need to be overcome for successful application of ^1H MRS/MRSI. The range of metabolite frequencies are relatively narrow, requiring excellent B_0 homogeneity over the region from which data are obtained. This requirement is significantly more difficult to achieve in MRSI (collecting multiple spatially resolved spectra simultaneously over large brain regions) than localized MRS (single spectrum from a small region in the brain). While MRSI is in general used in research laboratories, localized ^1H MRS is most frequently used in clinical applications due to speed of acquisition and reduced technical challenges. ^1H MRS/MRSI requires the suppression of water, as the metabolite concentrations are in the millimolar (mM) concentration range while pure water is 53 M, each water molecule having two equivalent protons, produces a signal intensity of 110 M, five orders of magnitude greater than the signals from the metabolites.

Water suppression requires excellent system stability and the elimination of “stimulated echoes” from residual water after application of the suppression technique(s).

Most MRS/MRSI studies of brain use creatine as an internal concentration standard, as creatine has a long T_2 and has relatively constant concentration in most cell types. Advanced methods exist to allow quantitation of the metabolites measured using MRS/MRSI. These methods include the referencing of signal to the internal water signal and referencing in-vivo signal amplitude to signal amplitudes from chemical samples of known concentration. Quantitation requires the correction of signal amplitude for T_1 and T_2 of the metabolites for each acquisition technique. Ideal acquisition parameters for quantitative measurements employ minimum echo time (TE) and a long repetition time (TR) to minimize magnetic relaxation corrections and reduce the chance that changes in relaxation times with pathology could affect the determination of metabolite concentrations.

53.2.4 Computed Tomography Methods, X-ray Computed Tomography (CT), Single Photon Emission Computed Tomography (SPECT) and Positron Emission Tomography (PET)

Computed tomography techniques share a common mechanism for spatial encoding. A two dimensional projection of the object (similar to a standard X-ray or a single read out in MRI without phase encoding) at multiple angles can be reconstructed into two or three dimensional images. Data are acquired by rotation of a set of detectors either opposite a radiation source as in CT, or around the subject in the case of internal radiation sources as in SPECT and PET. This method can also be used with MRI by combining gradients to provide a series of readouts at multiple angles (typically from 64 to 256), however modern MRI acquisition techniques are more efficient, therefore this technique is rarely used in MRI.

Reconstruction of images from tomographic methods are performed using the reverse Radon transform (Herman 1980) which uses the series of angular projections to reconstruct images. The resulting data set can be displayed as a rotating three-dimensional movie or resliced in any direction to display a series of two-dimensional slices.

X-ray CT uses a high intensity X-ray beam and a series of detectors that rotate around the subject. Modern CT scanners use multiple sets of X-ray sources and detectors to simultaneously acquire multiple slices (currently, up to 64) to speed acquisition. This allows full brain coverage at high spatial resolution in a matter of seconds. High doses of X-ray (about 5 rads) are typical exposures for this type of exam.

SPECT detects emitted gamma rays from radioactively labeled molecules or cells. The position of the emitted

gamma ray is determined by an array of crystal detectors covered by a collimator, which limits the angles from which gamma rays can enter the detector. Collimators consist of either a lead shield with a pinhole for three-dimensional tomographic acquisitions or a series of slots for two dimensional, planar (2D) acquisitions. Sensitivity of SPECT is limited by the area of the collimators opening. This concept can be understood by thinking of a pinhole collimator placed at a distance from the center of the subject being scanned. For example, if a 1 mm diameter pinhole is placed 10 cm from a mouse, the area covered on the surface of the sphere prescribed by the opening of the collimator (radius = 10 cm) is 1257 cm^2 ($4\pi r^2$). The gamma ray capture area of a 1 mm pinhole (0.5 mm = 0.05 cm radius) is 0.008 cm^2 (πr^2). Emission of gamma rays from the radioactive tracers is isotropic (equal probability of travel in any direction) the fraction of total gamma rays captured by the pinhole will be $0.008/1257 = 6 \times 10^{-6}$. Sensitivity can be increased by increasing the size of the pinhole, at the expense of spatial resolution or increasing the number of detectors in order to increase the counts for each angular projection. Recent technical developments have introduced multiple pinhole collimators (9–12) per detector array and multiple detectors for a significant increase (factor of 40) in detection sensitivity. The relatively low sensitivity of SPECT is typically compensated by using high doses of radioactive label, typically 50–500 microcuries to allow detection of labeled molecules or cells down to the nanomolar (10^{-9} M) concentration range.

PET uses positron emission to increase sensitivity of detection over SPECT. Once a positron is emitted from the radioactive atom on the labeled molecule, it travels a short distance (2–5 mm, depending on the energy of the emitted positron) and undergoes an annihilation reaction with an electron. Annihilation, occurs because the positron is antimatter to the electron. The reaction creates two 0.511 MeV gamma rays which then travel 180° away from each other. PET scanners employ a ring of detectors (typically 1024) and coincidence detection (simultaneous detection of two gamma rays) to improve detection sensitivity and filter out background radiation. As a result of the high capture cross section of the ring of detectors and the virtual elimination of background radiation, sensitivity is enhanced approximately four orders of magnitude compared to SPECT. This allows detection of lower concentrations of tagged molecules and cells down to the picomolar (10^{-12} M) concentration range with lower doses of radioactivity injected into the subject (typically around 10 mCi).

Positron emitting nuclides have very short half lives, on the order of minutes to 2 h. This makes operation of a cyclotron and a radiochemistry laboratory essential to the use of PET scanners. ^{18}F is the longest radionuclide with a half-life of 1.87 h, making a central production facility within a city feasible for radiopharmaceuticals employing this nuclide.

Most clinical PET facilities have on-site cyclotrons and radiopharmaceutical laboratories to allow the use of short-lived isotopes in clinical studies.

One difficulty with some SPECT and PET images is the potential lack of anatomical detail, especially in studies of receptor distribution where widespread distribution of the label in tissue may not occur. Two methods are used to overcome this problem, coregistration to anatomical images and simultaneous acquisition of anatomical images. Coregistration is typically performed for images lacking anatomical detail using fiducial markers visible in both scan sets to allow spatial registration of the SPECT or PET images to anatomical images such as CT or MRI. A more robust and time efficient method is the use of SPECT/CT or PET/CT scanners in which anatomical and tracer studies are acquired simultaneously providing anatomical images acquired simultaneously with SPECT or PET image acquisition.

53.3 Information Content

Effective use of imaging methods requires the understanding of the type of information available from each scanning technique. As neuroscientists, it is necessary to consider pathophysiological correlates of disease when designing and interpreting imaging studies. Some current research is focusing on correlation of histopathology and neuroimaging in animal models of neuronal disease (Nelson et al. 2005; Boska et al. 2005) which will serve to validate and refine the interpretation of imaging results. Consideration of expected pathology is required for experimental design and application of the most appropriate imaging methods to specifically address a particular physiological question. In the previous section, the basic principles of imaging methods were presented to allow the neuroscience student to understand the signal source and methods used to obtain imaging information. We will build on this to obtain a broad view of the basic capabilities of each imaging method and examples of the information available by use of the method.

53.3.1 Magnetic Resonance Techniques

The wide variety of morphological, physiological, and biochemical information available from the multitude of MR techniques allow sophisticated combinations of methods to be applied to individual neurological studies. This is one reason that MRI is often the best choice for neuroimaging studies, as the combination of multiple methods in one study can provide a wide array of coregistered information on the effects of disease and experimental therapies on brain function and biochemistry.

53.3.1.1 T_1 , T_2 , and Proton Density Weighted MRI

The use of T_1 , T_2 and proton density weighted images comprise the bulk of clinical MRI scans due to the ability to detect cellular alterations in tissue and determine morphological changes with exquisite soft tissue contrast. As a result, brain lesions, especially white matter lesions, tumors, and damaged regions from vascular disease are visible typically as dark areas on T_1 and bright areas on T_2 weighted images.

While these imaging modalities are sensitive to cell type, the combination of T_1 , T_2 and proton density are not specific to cell type or the type of alteration in normal cell physiology. Improved specificity can be obtained by combining these MRI scans with other types of information including clinical manifestations of disease, other neuroimaging scans and advanced analytical methods to improve diagnostic accuracy.

Use of T_1 and T_2 weighted images can be used to determine grey matter, white matter, and CSF content in neuroimages and determine the volume of multiple brain structures. Reduced volumes of gray matter, basal ganglia, and subthalamic nuclei have been found in various neurological diseases, the details of which are beyond the scope of this chapter. Good reviews are available for findings from HIV dementia (Boska et al. 2004), Alzheimer's disease (Chong and Sahadevan 2005; Kantarci and Jack 2004; Masdeu et al. 2005), Parkinson's disease (Krabbe et al. 2005; Seppi and Schocke 2005) and the hippocampus in a wide range of neurological diseases (Geuze et al. 2005). Generally, decreased volumes of affected brain structures, such as the substantia nigra in Parkinson's disease and atrophy of the grey matter in dementia such as Alzheimer's disease have been found in these studies. Scientific investigation continues into this area and as imaging and analysis software improves and the definition of changes due to disease are better defined, the use of these data in clinical diagnosis and patient management will be put into practice.

Recent studies have also shown that neuronal depolarization will cause increases in T_1 and T_2 . T_1 was shown to increase by 13 % while T_2 increased by 88 % at 1.5 T in a rat model of spreading depression (Stanisz et al. 2002). While this effect has not been studied in great detail, it is possible that this effect could be a source of detecting abnormalities in neuronal polarization with careful quantitation of T_1 and T_2 in neuronal diseases.

It will be apparent to the neurosciences student that this field, even in the case of one of the best characterized and most studied imaging modalities, standard MRI, that new information is acquired rapidly, advanced through the acquisition of data in biologically characterized animal studies and translated to well characterized human disease states. This process is cyclical, as advances in imaging methods will improve the classification of human neurological disease, leading to improved characterization and more refined human study. Advances in data processing

methods through computer science advances, developments of imaging data correlated with biological data, and advanced statistical analyses will be primary areas of imaging research for the advancement of disease characterization and detection sensitivity and specificity of imaging methods for many years to come.

Contrast enhancement of brain lesions was an early method of analyzing lesions and remains an important method of lesion characterization (Figs. 53.15a and 53.16b) (Smirniotopoulos et al. 2007). Gadolinium based contrast agents for human MRI exams were first approved in 1988. The brain parenchymal enhancement (increased signal intensity) seen with MRI contrast agents on T_1 -weighted MRI sequences reflects rather simplistically blood-brain barrier (BBB) breakdown. This type of brain parenchymal enhancement is the "delayed static" appearance of the contrast in the brain and it is easily performed at even the most basic imaging centers. The relative ease of imaging brain parenchymal enhancement (inject the contrast and then obtained a T_1 -weighted image) continues to make this a powerful biomarker clinically used for analyzing disease processes. However, parenchymal enhancement is very non-specific and can be secondary to a number of etiologies. Enhancement helps to detect many pathological entities including primary tumors, secondary tumors, active demyelination, stroke, vasculitis, granulomatous disease and infection (viral, fungal or bacterial).

In primary brain tumors enhancement is useful for determining tumor grade and survival of patients with astrocytomas and oligodendrogliomas. These are the two largest categories of primary brain tumors. The presence of enhancement in these tumor types typically indicates higher grade tumor and shorter survival but this relationship isn't seen for all tumors. Some tumors will enhance, have longer survival and behave like true low grade tumors. Also, other low grade primary tumors such as pilocytic astrocytoma almost always enhance. Secondary tumors, e.g. metastatic disease, in the brain universally enhances making this an extremely useful characteristic for disease detection. The analysis for metastatic disease given the relative frequency of cancers that can metastasize to the brain (lung, breast, renal, ovarian, colon, testicular, etc.), the sensitivity for detecting even punctate (1–2 mm) metastatic disease, and the growing elderly population guarantees the continued analysis of "delayed static" contrast enhancement.

Active demyelination has a stage of BBB and contrast enhancement helps to detect these active lesions. There are short comings for using gadolinium enhancement to define active demyelinating disease. Demyelinating lesions that are active may not enhance. Demyelinating lesions may have only short time periods of enhancement and may not demonstrate enhancement on surveillance MRIs obtained at 6 month or 1 year intervals.

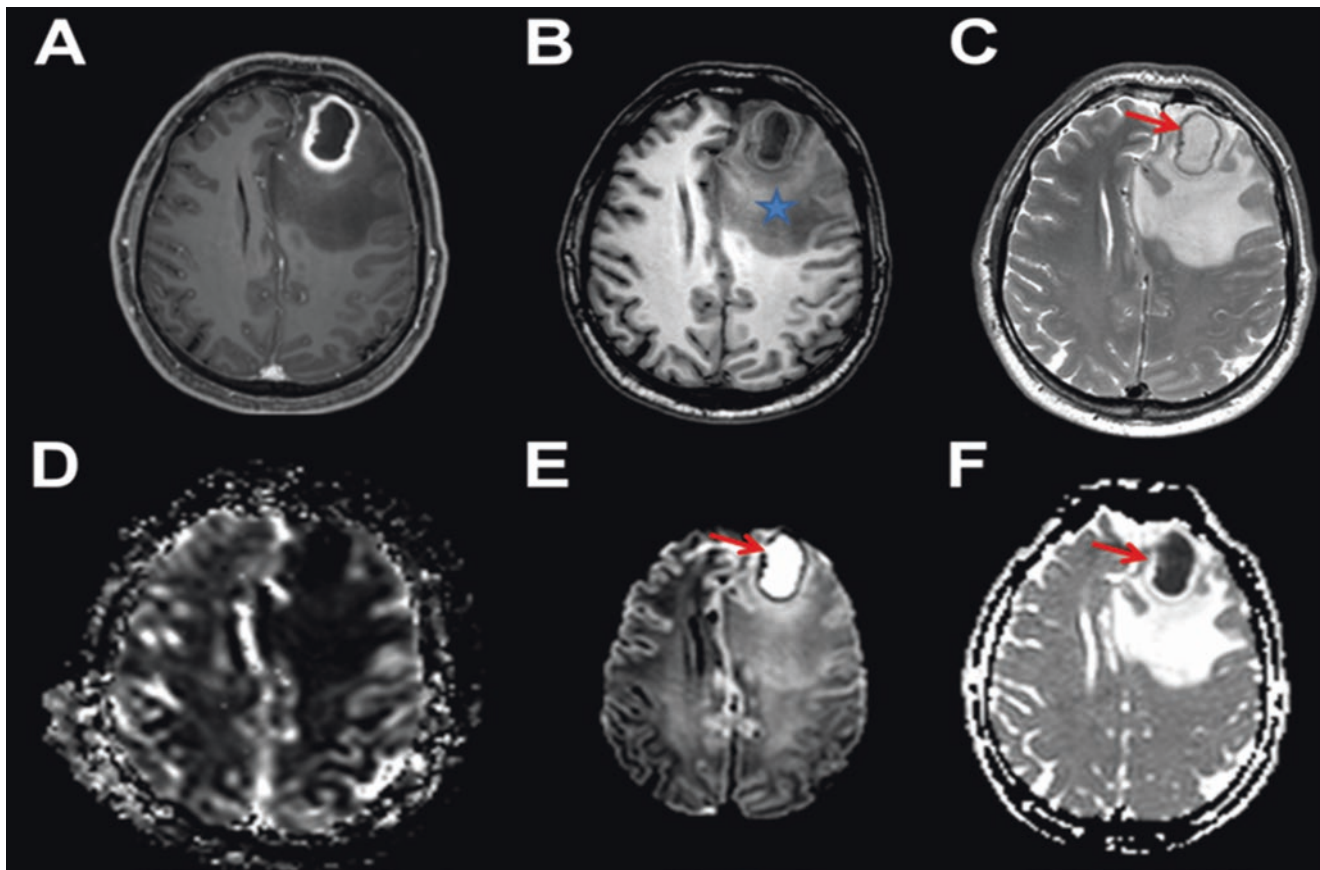


Fig. 53.15 Left frontal lobe abscess secondary to *Staphylococcus Aureus*. (a) Smooth thin rim enhancing lesion in the left frontal lobe on T1-weighted image after contrast administration. (b) T1-weighted image pre-contrast demonstrates the rim of the lesion to be grey and not hyperintense. Surrounding hypointensity is vasogenic edema (star). (c) The T2-weighted rim is hypointense likely secondary to the presence of free radicals (arrow). (d) A cerebral blood volume (CBV) map from

DSC sequence demonstrates low flow consistent with an abscess. Some abscess will have slightly increased CBV. (e) Diffusion weighted image demonstrates a hyperintense central fluid component and a hypointense rim (arrow). (f) The ADC map demonstrates that the central fluid is markedly restricted likely due to presence of high protein and cellular content. The rim is not restricted (arrow). The surround vasogenic edema has elevated ADC

The necessity for utilizing contrast enhancement of the brain in the analysis of stroke has decreased markedly over the last 15 years. Diffusion weighted imaging (DWI) has become the primary sequence for analyzing for acute and subacute strokes secondary to the rapidity by which DWI becomes positive and the wide availability of DWI on new MRI scanners. There are several characteristics of contrast enhancement in stroke that can be useful. Secondary to an occluded or nearly occluded artery vascular enhancement occurs immediately and this is diagnostically a more specific pattern of enhancement. The presence of leptomeningeal enhancement in stroke occurs around day 2/3 and parenchymal enhancement begins day 3/4 on MRI. These factors can help define the age of an ischemic stroke of unknown onset. The parenchymal pattern of enhancement of larger strokes is gyriform and this pattern can also guide one to the diagnosis of ischemic stroke in patients presenting at a time later than the acute or early subacute setting. Gyriform enhancement

can also be seen, but less commonly, with seizure activity, encephalitis or infiltrating tumor. Leptomeningeal enhancement is non-specific and can be seen after the placement of a carotid stent, secondary to tumor or due to infection/inflammation. The analysis of the MRI study findings needs to be done in context of the complete clinical setting of a patient and all MRI sequences need to be analyzed in aggregate in order to optimize the diagnosis.

Another pattern of enhancement that is diagnostically useful is the presence of a ring enhancing lesion (Figs. 53.15a and 53.16b). Ring enhancing lesions are a common abnormality patients might have and are associated with numerous pathologies that are undergoing current research for effects of treatment or changes in cognition. The main differential diagnosis for a ring enhancing lesion is a high grade primary tumor, metastatic disease, abscess or active multiple sclerosis lesion. Less common entities with ring enhancement are subacute ischemic stroke, resolving hematoma, lymphoma in an

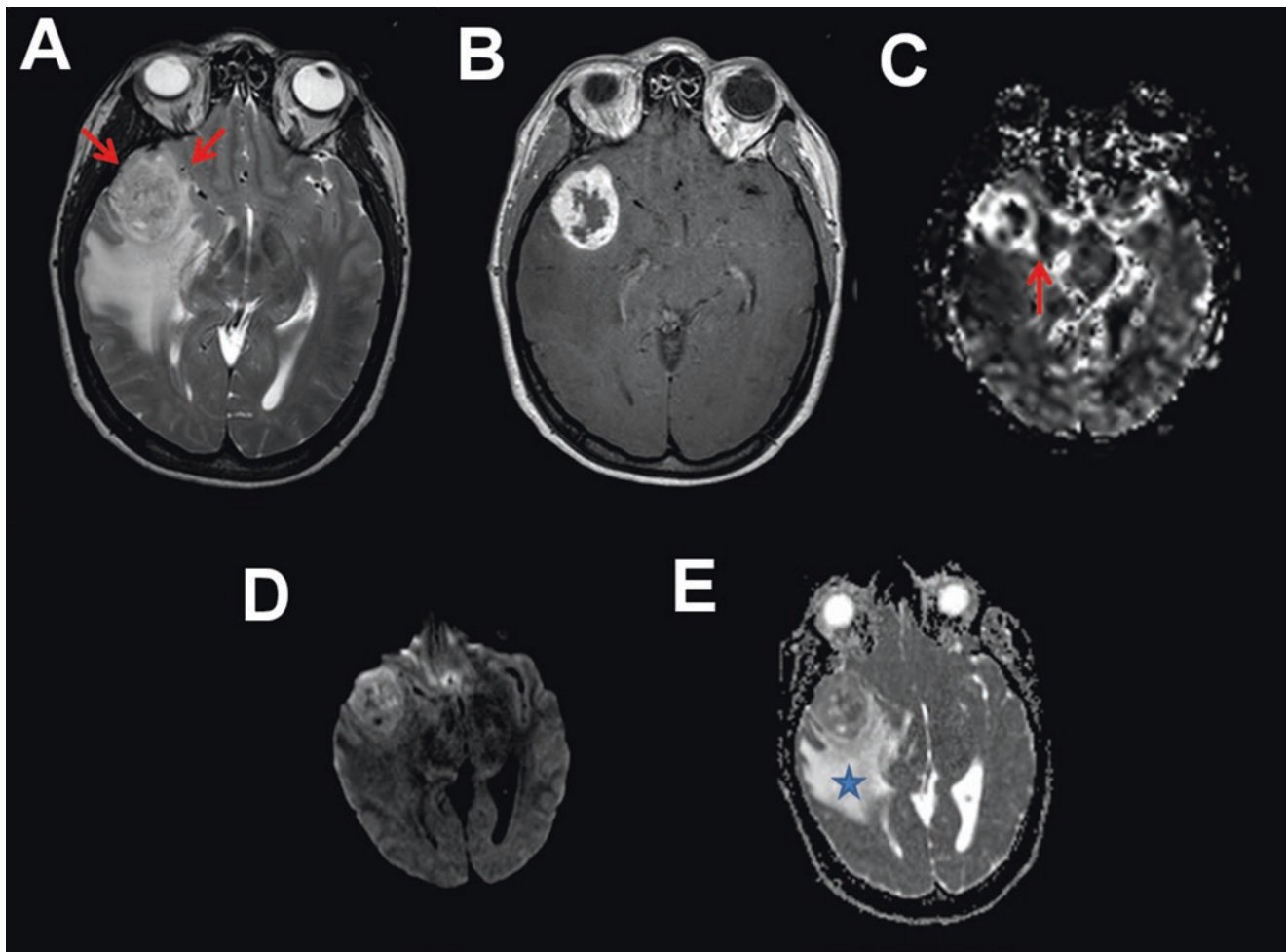


Fig. 53.16 Right anterior temporal lobe glioblastoma multiforme. (a) T2-weighted image demonstrates a mass (*arrows*) in the anterior right temporal lobe with vasogenic edema posterior. (b) The gadolinium enhanced image demonstrates a thick and irregularly rimmed enhancing mass. (c) The CBV map demonstrates high perfusion in the medial temporal lobe

where no enhancement is present in the peritumoral (perienhancement) region (*arrow*). (d) DWI image demonstrates the rim and most of the center of the mass to have higher signal. (e) The ADC map demonstrates predominantly mild restricted diffusion in the rim of the mass and in the center of the mass. The vasogenic edema has elevated ADC values (*star*)

immunoincompetent, and radiation necrosis. Enhancement characteristics can help improve the specificity of diagnosis such as the partial ring enhancement typically seen in demyelinating lesions. Abscesses have thin rims of enhancement whereas tumors usually have thickened and irregular rims of enhancement. The ring enhancement of a subacute stroke would likely have associated gyriform enhancement indicating ischemia as an underlying etiology. Advanced MRI imaging techniques such as perfusion imaging, diffusion imaging and spectroscopy have helped delineate the underlying etiological factors of ring enhancing lesions.

53.3.1.2 Diffusion Weighted MRI (DWI)

The primary use of DWI in clinical practice is detection of acute stroke lesions. Within minutes after a stroke, a low diffusion area of brain is visible in the majority of stroke cases. In a diffusion weighted image, this appears as a bright area,

as tissues with low diffusion lose less signal during the diffusion weighting. Signal abnormalities usually do not appear before 16 h on T₁ and 8 h on T₂ weighted images (Yuh et al. 1991). Diffusion weighting requires a long echo acquisition, so the appearance of bright areas on DWI can be either from low diffusion or increased T₂ signal which occurs in late acute to chronic infarcts. In order to eliminate confusion from this effect, two images are acquired, one with no diffusion weighting and one (or more) with high diffusion weighting. This allows the calculation of a parametric image of the apparent diffusion coefficient of water (ADC_w). Acute stroke lesions typically appear as bright on DWI and dark on ADC_w maps. As stroke lesions age, the cells in the lesion begin to break down after cell death and leave an area of edema. As the stroke lesion progresses towards cellular breakdown and edema, both diffusion and T₂ increase. After days to weeks, the diffusion “pseudonormalizes” to a value

equivalent to normal brain tissue, progressing to high diffusion. A combination of ADC_w , T_1 and T_2 maps can be used to characterize the age of the stroke lesion (Nagesh et al. 1998; Welch et al. 2001).

Diffusion is altered in other types of brain lesions and is not specific for stroke (Wang et al. 1998). The range and types of diffusion alterations are an area of active research. Combination of DWI, T_1 , T_2 , MT and perfusion may serve to more precisely characterize lesions from neurological diseases (Dijkhuizen and Nicolay 2003).

53.3.1.3 Diffusion Tensor Imaging (DTI)

Multiple metrics can be calculated from diffusion tensor imaging data, typically mean diffusivity (D_{av}) and fractional anisotropy (FA). Analysis of brain pathology using DTI metrics can be performed by lesion analysis, whole brain histograms of FA and D_{av} , tractography and analysis of otherwise normal appearing white matter. Fractional anisotropy has been studied in numerous diseases and detects abnormalities in otherwise normal appearing white matter by conventional MRI. This demonstrates the increased sensitivity to disease within the brain that DTI metrics can resolve. Abnormal FA in normal appearing white matter has been found to occur in multiple sclerosis, radiation injury, aging, schizophrenia, and amyotrophic lateral sclerosis (Cosottini et al. 2005; Foong et al. 2000; Filippi et al. 2001; Kitahara et al. 2005; Salat et al. 2005). Fractional anisotropy maps or DTI in general for acute stroke analysis has not been found to add significant clinical information in the setting of acute stroke therapy, although, active investigation in this area continues.

DTI can provide insight into microstructural changes in the brain in traumatic brain imaging (Cubon et al. 2011). The analysis of white matter changes by tract-based spatial statistics (TBSS) can even potentially quantitate how changes in different DTI parameters reflect mild (mean diffusivity) or severe injuries (fractional anisotropy).

Brain lesion analysis is also an active area of research. DTI metrics have been found to help differentiate between tumor types and promises to provide a sensitive method for abnormal tissue characterization. Fractional anisotropy has already been found to correlate with the cell density of brain tumors (Beppu et al. 2003; Beppu et al. 2005). The ability of FA to correlate with cell density likely accounts for the sensitivity of FA to tissue typing in brain lesions. Fractional anisotropy has been found to differentiate between low grade and anaplastic astrocytomas, to help differentiate between brain metastasis and high grade gliomas, and there is evidence that FA may help to detect tumor infiltration not visible by conventional MRI (Goebell et al. 2006; Tsuchiya et al. 2005; Holmes et al. 2004). Other DTI metrics have been utilized in brain lesion analysis and the early findings point to the breadth of future possibilities. Fractional anisotropy maps or DTI has not been found to add significant clinical information for determining the core or

penumbra of an infarct in the setting of acute stroke therapy, although, active investigation in this area continues. Fractional anisotropy has been found to be a measure that correlates with functional outcome in motor deficits related to stroke with rFA values in the corticospinal tract at day 30 correlating with the degree of motor deficit at 2 years ($P < 0.001$) (Puig et al. 2013). FA at day 30 was the only independent predictor of long-term motor outcome (Puig et al. 2013).

Apparent diffusion coefficients (ADCs) or mean diffusivity (MD), which are essentially the same parameter, are also useful measures of diffusion imaging (DI) analysis calculated from diffusion weighted imaging (ADC) or diffusion tensor imaging (ADC or MD). These parameters help delineate etiologies of brain lesions. ADC values typically in lymphoma are lower than in GBM (Toh et al. 2008). The decreased ADCs in lymphoma in general is the result of extensive areas of hypercellularity and GBMs usually have limited areas of restricted diffusion secondary to hypercellularity (Hakyemez et al. 2005). The distribution of high diffusion signal and relative restricted diffusion in a lesion can help differentiate abscess, glioblastoma multiforme and metastatic disease. Abscesses tend to have cavities of marked restricted ADC (Fig. 53.15e, f) and high diffusion signal whereas the necrotic cavities of tumors have higher ADC values (Fig. 53.16d, e) (Erdogan et al. 2005; Hakyemez et al. 2005). The opposite is true for the rims of these lesions and this is usually a better differential point between these two entities. This is due to the fact necrotic tumor cavities can have mild to moderate restricted diffusion mainly from cellular debris and hemorrhage. The rims of abscess are lower in signal with elevated ADC values (Fig. 53.15e, f) while the rims of GBMs and metastatic lesion tend to have lower ADC values (elevated DWI signal) due to cellularity (Fig. 53.16d, e) (Chang et al. 2002; Hakyemez et al. 2005). The peritumoral ADC/DWI analysis helps to differentiate GBM from metastatic disease secondary to the peri-enhancing region of tumor invasion that is seen with GBM and not mets (Jaeger et al. 2009; Wang et al. 2009).

Restricted diffusion (lower ADC values) in brain tumors has been shown to be associated with decreased survival and tumor grade (Zulfiqar et al. 2013; Kono et al. 2001). However, lower ADC values in high grade tumors have not universally been found to predict survival (Gupta et al. 2013). These disparate findings indicate that this is an active area of research. The restricted diffusion in brain tumors is thought to be primarily an effect of increased cellularity (hypercellularity) but measurement of the hypercellularity is confounded by presence of vasogenic edema, necrosis, hemorrhage, and calcifications.

The diffusion tensor data can also be utilized to perform white matter tractography (Jellison et al. 2004). Tractography is the virtual dissection of white matter tracts within the brain and provides a novel method to analyze the white matter tracts in disease states. Tractography in combination with

other DTI metrics and other MRI techniques such as perfusion and spectroscopy opens the door for currently uncharted areas of research. Previously there was nearly no indication of the intricate connections present in the white matter on conventional MRI. DTI tractography can be used to map out numerous fiber tracts including but not limited to the corticospinal tracts, superior occipitofrontal fasciculus, inferior occipitofrontal fasciculus, uncinate fasciculus, superior longitudinal fasciculus, arcuate fasciculus, and inferior longitudinal (occipitotemporal) fasciculus, (Jellison et al. 2004). With advancements and refinements in acquisition of DTI data, there likely will be improvements to tractography which will provide further advances in the “virtual dissection” of the white matter tracts.

A very exciting and innovative application of DTI and tractography that will have profound clinical application is analysis of brain lesions. There has already been early work defining tumor growth and metastases within major white matter tracts (Witwer et al. 2002; Hlatky et al. 2006; Yu et al. 2005). This application will provide measures of white matter tract displacement and tumor invasion that will be defined with great specificity. Previously estimates of white matter tract invasion could only be made by gross estimates of where the white matter tracts were expected to lie based on cross-sectional MRI or CT studies. However, current methods of DTI tractography cannot reproducibly demonstrate the true size of fiber bundles (Kinoshita et al. 2005).

53.3.1.4 Magnetization Transfer (MT) MRI

MT MRI is sensitive to areas of demyelination, as the MT effect from myelin is particularly strong. The use of MT MRI for the detection and characterization of lesions due to multiple sclerosis has been well studied (Atalay et al. 2005). Quantitative analysis of the magnetization transfer ratio (MTR) of gray matter in the earliest stages of the disease demonstrate statistically significant reduction in the basal ganglia of these patients (Audoin et al. 2004). In general multiple advanced MR techniques, including MTR maps, ^1H MRSI, quantitative T_1 and T_2 mapping, diffusion tensor imaging, and studies of blood flow have led to a more sophisticated understanding of the disease from the early view of simply an immune mediated demyelination process to the understanding of disease effects on neurons, axons, and oligodendrocytes (Minagar et al. 2005).

MT MRI has been shown to be sensitive to other minor structural abnormalities caused by neurological disease. MTR maps were analyzed with a statistical package to demonstrate that areas of epileptic activity which were not detected visually demonstrated statistically reduced MTR in the epileptic focus (Rugg-Gunn et al. 2003). When MT MRI was combined with DWI, reductions in both MTR and ADC_w were found in the epileptic focus of patients (Ferini-Strambi et al. 2000).

More advanced quantitation methods for MT MRI have been shown to be sensitive to white matter tracts and axonal density (Yarnykh and Yuan 2004) as well as being sensitive to the earliest events in stroke lesions (Jiang et al. 2001) including early detection of ruptures in the blood–brain barrier which may be susceptible to hemorrhagic transformation (Knight et al. 2005).

53.3.1.5 Perfusion MRI

Perfusion in cerebral tissue with normal vasculature is controlled by metabolic demand, allowing use of regional quantitative perfusion measures to detect neurodegeneration in a variety of diseases. Perfusion measures have been a cornerstone of nuclear medicine (SPECT and PET) studies for characterizing brain regions affected in neurological disease.

MRI perfusion studies have been used primarily to study perfusion deficits in stroke lesions. The mismatch between DWI and perfusion deficit may represent tissue at risk for damage (Neumann-Haefelin et al. 1999) which may be salvaged using thrombolytic therapy. However this interpretation of mismatch between DWI and perfusion MRI is controversial and study of this effect is an area of active research (Rowley 2005).

Brain tumor imaging is a growing area of clinical application of MRI perfusion studies. MRI perfusion studies have been found to provide valuable information when evaluating multiple facets of the imaging of gliomas, metastatic disease, abscesses, radiation necrosis, and meningiomas. The grading of gliomas pre-operatively has been found to be enhanced by utilizing MRI perfusion (Law et al. 2003; Law et al. 2004). Also, the determination if a lesion is a high-grade glioma or a metastatic lesion, the differentiation of which is markedly important clinically, can be facilitated utilizing MRI perfusion as well as MRS (Law et al. 2002). The differentiation of an abscess from a ring enhancing glioma is also potentially facilitated by MRI perfusion (Holmes et al. 2004). A significant problem that can occur with MRI perfusion is leakage of the contrast through the blood brain barrier on the first pass so that the appearance of the perfusion curve is distorted and a true evaluation of the tumor perfusion is not obtained. This is unfortunately a potentially frequent occurrence since blood brain barrier disruption is a hallmark of high-grade gliomas.

Studies of perfusion deficits in other neurological diseases have been hindered in MRI by the difficulties in quantitation of cerebral perfusion in bolus tracking perfusion measures. Arterial spin-labeling techniques have been successfully employed in recent studies of human disease including Alzheimer’s disease (Alsop et al. 2000; Johnson et al. 2005). Arterial spin-labeling perfusion measures are greatly enhanced at high field due to increased signal to noise and increased T_1 , improving reliability of the technique

(Wang et al. 2002). Increased availability of high field (3 T and above) clinical MRI systems will allow greater use of quantitative perfusion measures to assess neurodegenerative disease in clinical practice.

Arterial spin labeling (ASL) is a potentially beneficial methodology for measuring perfusion and given technical advances is becoming readily available in a time efficient scan on commercial available scanners. Previously ASL imaging had lengthy exams times and decreased spatial resolution. A great advantage for ASL is that no contrast agent needs to be administered so IV access isn't a problem and the exam can be safely performed in patients with poor renal function. This eliminates the risk of developing nephrogenic systemic fibrosis that can occur with gadolinium based contrast agents. Also, ASL doesn't suffer from the vascular leakage phenomena that plagues contrast based perfusion techniques (Boxerman et al. 2006). EPI techniques are routinely used for ASL to improve signal to noise ratios and temporal resolution but EPI introduces distortions in areas of magnetic field susceptibility at the base of the brain, around hemorrhages and increases them in areas of metal (Covarrubias et al. 2004; Petcharunpaisan et al. 2010). Fast 3-D sequences have been introduced that reduces image distortions and provide high signal to noise ratio (Wolf and Detre 2007).

Arterial spin labeling research has demonstrated that DSC perfusion exams can potentially be replaced by ASL (Lehmann et al. 2010; Warmuth et al. 2003). ASL exams have been found to correlate to the histopathology of brain tumors (Noguchi et al. 2008). Others have found ASL techniques useful for grading tumors but report challenges with inter-observer variability (Kim et al. 2008). ASL has been shown to be advantageous in determining glioma recurrence versus radiation necrosis (Ozsunar et al. 2010).

ASL techniques have been demonstrated to be useful in stroke, Alzheimer's disease, Parkinson's disease (PD) and MS. ASL using a 3D GRASE technique mapped changes of delayed bolus arrival time and reduced CBF in patients with ischemic stroke (Wolf et al. 2014). The findings match those of DSC imaging (Wolf et al. 2014). Used in isolation, CBF quantified with ASL was a good diagnostic marker for dementia (Bron et al. 2014). This was not surprising since hypoperfusion of brain tissue precedes atrophy in dementia. However, the study indicated only little added diagnostic value when combining ASL with the structural MRI data, which did not significantly improve over accuracy of structural MRI atrophy marker by itself (Bron et al. 2014). ASL indicated preserved perfusion in the degenerated brain regions of PD patients compared with healthy controls, and decreased perfusion in other regions, including the posterior parieto-occipital cortex, precuneus and cuneus, and middle frontal gyrus (Melzer et al. 2011). Studies have indicated that the neurovascular abnormalities observed in PD are dependent on the neurotransmitter

dopamine and this tight neurovascular coupling is unique (Chen et al. 2013; Choi et al. 2006). In patients with multiple sclerosis ASL provides insight into the disease pathophysiology with decreased CBF found in the bilateral thalami and right frontal lobe (Ota et al. 2013). Also, T1-hyperintense lesion volume was negatively correlated with regional CBF in areas such as both thalami (Ota et al. 2013).

These studies demonstrate the utility and robustness of ASL in research paradigms, applicability across the pathological spectrum and importance of correlating with other radiological modalities. An investigation of one technique compared to another radiological sequence can help confirm accuracy (Wolf et al. 2014). Multidisciplinary research teams and analysis utilizing clinical information strengthens the results of imaging research and provides for greater insight into disease pathogenesis. This is particularly well demonstrated by Chen et al. with the neurotransmitter dopamine (Chen et al. 2013).

Dynamic contrast enhancement (DCE) is a methodology that analyzes the T1 enhancement characteristics (blood-brain barrier (BBB breakdown)) of lesions temporally. Standard enhancement is a delayed static analysis of BBB breakdown. DCE is challenging due to having to image lesions with high temporal resolution, good contrast and appropriate coverage. The needed temporal resolution goal is <10 s but <5 s per imaging phase is often preferable. Quantitative Imaging Biomarker Alliance (QIBA) has put together a paper providing guidelines for standardization of DCE exams (RSNA, QIBA, Profile: DCE MRI Quantification, 2012). Limitations of these guidelines include that they are for 1.5 T MRI and do not take into account characterization differences of the available gadolinium contrast agents. The intrinsic relaxivity of a gadolinium agent affects the optimal imaging parameters (e.g. optimal flip angle for imaging contrast is partly determined by Ernst's angle).

The DCE technique has been widely applied to brain tumor analysis since DCE allows for evaluation of angiogenesis and blood vessel leakiness. DCE gives insight into brain lesion etiology, brain tumor grade, survival, tumor progression after treatment or pseudoprogression (Jain et al. 2011; Cha et al. 2006; Suh et al. 2013). Parameters analyzed from DCE data include:

K^{trans} Volume transfer constant between blood plasma and EES (transfer constant)

v_e Volume of extravascular extracellular space per unit volume of tissue

v_p Blood plasma volume per unit volume of tissue

However, numerous other parameters can be calculated (Bergamino et al. 2014). High-grade gliomas showed higher K^{trans} (0.050 vs. 0.010 in median value, $p=0.002$) and higher v_e (0.170 vs. 0.015 in median value, $p=0.001$) than low-grade gliomas. Receiver operating characteristic curve analysis showed significance in both K^{trans} and v_e for glioma

grading. However, there was no significant difference in diagnostic performance between K^{trans} and v_e (Choi et al. 2013). Analysis of DCE correlates with survival and it has been found patients with gliomas, survival was worse for groups of patients with high contrast transfer coefficient K^{trans} and plasma volume v_p obtained in tumor (Nguyen et al. 2014). DCE analysis can be applied to looking at tumor response to chemotherapy or radiation therapy. This application may in the future delineate what tumors are responding earlier to a treatment than standard imaging analysis of tumor volume on contrast enhanced images or T2 weighted images. Patients who are not responding to treatment then may have their therapy altered. DCE analysis has been found to be able to predict brain metastatic response with whole brain radiation therapy using principal component analysis (Farjam et al. 2014). Computing a couple of principal component coefficients is a much faster process than fitting PK modeling (Farjam et al. 2014). DCE is also being applied to study treatment responses in brain tumors. This is particularly important since anti-angiogenic drugs are of growing importance for brain tumor treatment and DCE MRI should help to measure effects of treatment on the BBB. BBB changes should give an early indication of tumor response to such treatments.

Analysis of DCE has been applied to other fields of work such as that related to stroke risk factors, ischemic strokes and multiple sclerosis plaques. It has been demonstrated that DCE analysis of carotid artery plaques can provide insight into the neovascularization (Truijman et al. 2013) and this data may be predictive of future risk of recurrent stroke. Also, the analysis of permeability may help predict what ischemic stroke lesions will result in symptomatic hemorrhagic transformation and/or malignant edema (Hoffmann et al. 2011). This work was based on CT but MRI would likely provide additional interesting analysis.

The pattern of enhancement of lesions such as seen with MS may give insight into potential different underlying pathophysiology (Absinta et al. 2013; Gaitan et al. 2011). This analysis then might allow for evaluation of treatment effects and prediction of treatment success in MS patients.

There is increasing interest in using DCE to identify more subtle BBB abnormalities, such as those which occur with normal ageing, Parkinson's disease, Alzheimer's disease, type II diabetes, HIV, cerebral microvascular disease, and non-enhancing multiple sclerosis lesions (Armitage et al. 2011). This work is particularly challenging given the subtle differences of signal change in these tissues in general with DCE and the difficulty of accurately tracking small signal changes overtime. To do these exacting studies work needs to be done to determine to what extent scanner noise, drift, intrinsic tissue properties and imaging sequence parameters affect the interpretation of post-contrast signal enhancement in different tissues and over the range of values relevant to

subtle BBB disorders (Armitage et al. 2011). If this is not done, systematic errors introduced by drift and intrinsic tissue parameters may be erroneously perceived as BBB differences between patients (Armitage et al. 2011). This type of analysis of subtle BBB changes has been applied to the study of normal appearing white matter in patients with MS in the periventricular location, a common location for MS lesions, demonstrated abnormal permeability that changed with treatment which indicated the level of permeability was correlating with the generalized MS inflammatory activity (Cramer et al. 2014).

53.3.1.6 Functional MRI

Functional MRI (fMRI) is extensively used in research for mapping task specific brain activation and continues to find greater clinical applications. Numerous paradigms have been used to test an expansive array of brain functions. A test may map areas of function for memory, motor function (hand, face, foot), sensory (pain, thermal, light touch, vibratory), multiple aspects of language and vision. Given the complexities of the brain there are numerous potential paradigms that are utilized for any one function. For instance, one might utilize passive listening or a reading paradigm to map language. It has been shown that more interactive paradigms such as word generation, verb generation, sentence completion or rhyming likely generate greater language activation (Pillai and Zaca 2011). Word generation might consist of the subject being presented letters and then the subject is to think of words that start with the letters being presented. Verb generation would consist of the subject being presented nouns and the subject is to think of actions for that noun.

A somewhat straightforward application of fMRI clinically is utilizing it to determine areas of brain function in relationship to a tumor that is going to be resected. The basis of performing fMRI in brain tumor patients is done in order to give guidance to achieve maximal tumor resection with preservation of function. However, the application of the technique for surgical guidance must be approached with caution. The tumor itself may alter the underlying physiology fMRI activation relies upon and give spurious results. The tumor may have infiltrated areas of function and the functional tissue may lie within the tumor itself and not be demonstrable by fMRI. People with brain tumors can have difficulties with performing even simple tasks and it can be difficult to determine if subjects are successfully performing tasks such as language and memory. Also, there is no accepted standard statistical thresholds to use for defining the activation blobs/regions (Chang et al. 2010). Despite these shortcomings fMRI performs robustly and provides useful guidance. Additionally, it is best to perform fMRI exams on MRI machines maximized for high signal (e.g. high tesla (at least 3 T) and high channel coils (32 channel preferred)).

The analysis of fMRI results in the setting of brain tumors gives a particular opportunity to validate fMRI results against the physical structure of the brain. The physical changes and how this affects patient function and fMRI results has provided validation for fMRI technique. Also, direct cortical mapping during operation on brain lesions provides another level of corroborating the validity of fMRI in general and more specifically the validity analyzing functional disruption in the setting of brain tumor. When fMRI was compared to direct cortical mapping, Spena et al. found mixed results (Spena et al. 2010). fMRI related by direct cortical mapping to sensorimotor correlated 92.3 % but to language was found to have a correlation of 42.8 % (Spena et al. 2010). It is not surprising that direct cortical mapping provided a better correlation to sensorimotor than language functions given the greater simplicity of measuring sensorimotor function. Language is a more complex function to test and differs to a greater extent between the test in the fMRI environment compared to mapping in the operating room. Some of the issues in this comparison may have also been the examinations were performed at only 1.5 T which would result in lower signal generation and only a fixed statistical threshold was utilized for the fMRI analysis. A fixed statistical threshold is a sound scientific approach but when analyzing fMRI in tumor patients this can be misleading given the variable functional response (Chang et al. 2010). However, Giussani et al. reviewed language fMRI studies in 2010 and had negative results (Giussani et al. 2010). They found fMRI compared to direct cortical stimulation gave contradictory results, no agreement in the literature on what task to use and fMRI maps capacity for function not what is required for function (Giussani et al. 2010). Pillai et al. and Bizzi et al. had much more encouraging results for language mapping (Pillai and Zaca 2011; Bizzi et al. 2008). Kapsalakis et al. in 2012 found highly encouraging results in tumor patients for fMRI with DCS correlating to fMRI results 91.9 % for sensory-motor cortex, 100 % for visual cortex and 85.4 % for language (Kapsalakis et al. 2012). Patients with better functional conditions performed better, as would be expected. DCS was felt to be particularly necessary for language mapping giving the lower correlation of the fMRI results (Kapsalakis et al. 2012). It should be kept in mind that despite the apparent intuitive strength of DCS for defining function via disruptive electrical signals in the brain DCS is surprisingly poorly understood (Borchers et al. 2012).

Resting state fMRI is an emerging technique that is broadly being applied to numerous neurological conditions. In addition to the brain networks associated with motor, sensory, vision, dorsal attention and language networks such as the executive network and default mode network can be seen (Fig. 53.17) (Guerra-Carrillo et al. 2014). Secondary to the ability with RS fMRI to demonstrate numerous brain networks

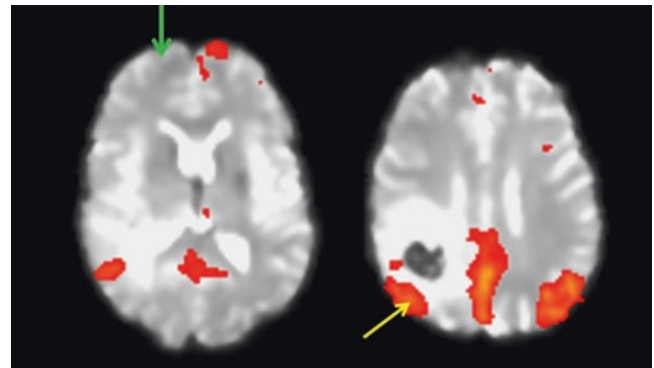


Fig. 53.17 Resting state fMRI with default mode network. There is decreased activation in the right hemisphere parietal lobe (yellow arrow) and frontal lobe (green arrow) due to the tumor in the right parietal lobe. Images calculated with MELODIC from FSL

via a single acquisition RS fMRI is providing exquisite insight into the functional brain changes with associated neurocognitive findings in many disease processes. RS fMRI has been successfully applied to analyzing brain function in multiple sclerosis (MS), Parkinson's disease, mild cognitive impairment and Alzheimer's disease. Numerous correlations can be utilized in combination with RS fMRI to enrich the data analysis and deepen the understanding of neurocognitive dysfunction. Such analysis can be focused on how neurological dysfunctions correlate with RS fMRI findings, how other studies such as nuclear medicine demonstrates physicochemical correlations to RS fMRI and RS fMRI can demonstrate changes overtime.

Clinical symptoms can be analyzed and correlated with cognitive changes within the brain. For instance fatigue is a common and possibly disabling symptom in MS patients. Fatigue severity in MS patients was negatively correlated utilizing RS fMRI with functional connectivity of basal ganglia nuclei with medial prefrontal cortex, precuneus and posterior cingulate cortex (Finke et al. 2014). Furthermore, fatigue severity was positively correlated with functional connectivity between caudate nucleus and motor cortex (Finke et al. 2014). No association of fatigue severity was observed with grey matter density, white matter integrity and basal ganglia volumes within patients (Finke et al. 2014). This study exemplifies how a specific symptom in a certain disease etiology can be clearly begun to be elucidated with RS fMRI when conventional imaging techniques fail. This then allows the opportunity for treatment interventions and measuring the RS fMRI initial state and RS fMRI response after treatment. This type of data could potentially then be useful to predict which patients might benefit and which patients might not benefit from a certain therapy. This type of analysis has been performed with aerobic exercise which was found to increase hippocampal volume and connectivity and improve memory in MS subjects (Leavitt et al. 2014). Aerobic exercise represents a cost-effective, widely available,

natural, and self-administered treatment with no adverse side effects that may be the first effective memory treatment for multiple sclerosis patients (Leavitt et al. 2014).

In Parkinson Disease (PD) RS fMRI has been analyzed overtime and in relationship to cognition and motor function (Olde Dubbelink et al. 2014). In an fMRI study in PD, a progressive loss of resting-state functional connectivity was demonstrated over a period of 3 years for multiple brain regions, especially in the posterior parts of the brain. The strong correlation with decreasing cognitive performance supports the pathophysiologic role of reduced functional connectivity in cognitive decline and the development of dementia in PD (Olde Dubbelink et al. 2014). Further analysis of RS fMRI combined with DATSCAN and neuropsychological testing was performed to demonstrate how the physiochemical changes with PD effects brain networks and how these findings are reflected in cognition (Lebedev et al. 2014). For the range of deficits studied, better executive performance was associated with increased dorsal fronto-parietal cortical processing and inhibited subcortical and primary sensory involvement. This profile was also characterized by a relative preservation of nigrostriatal dopaminergic function. The profile associated with better memory performance correlated with increased prefronto-limbic processing, and was not associated with presynaptic striatal dopamine uptake. SBR ratios were negatively correlated with modularity of the “cognitive network,” suggesting integrative effects of the preserved nigrostriatal dopamine system on this circuitry (Lebedev et al. 2014). This research demonstrates the complexities and layers of evaluation which are available to a neuroscience researcher and how multiple techniques can be utilized to gain a better understanding of a disease process rather than any singular technique itself. It is likely the complex interaction of multiple systems at different levels interacting that produce the clinical presentation and response in a particular patient.

fMRI analysis of the default mode has been utilized to demonstrate in subjects with Alzheimer’s disease that there is associated decreased activity in the posterior cingulate gyrus and in the hippocampi (Greicius et al. 2004). This suggests that hypometabolism seen in the posterior cingulate gyrus on PET studies in Alzheimer’s disease may be explained by hippocampal dysfunction (Greicius et al. 2004). Resting state fMRI has further been utilized to analyze behavioral effects on the hippocampi, e.g. how exercise training in the elderly benefits their brain (Burdette et al. 2010). Network analysis of a group undergoing exercise training demonstrated increased connectivity of the hippocampus and the hippocampus was consistently shown to be within the same network group as the anterior cingulate gyrus in the subjects undergoing exercise training (Burdette et al. 2010). Additionally this was coupled with demonstration of increased blood flow in the hippocam-

pus in the exercise training group using arterial spin labeling technique (Burdette et al. 2010).

53.3.1.7 ¹H MRSI

Proton MRS detectable metabolites are primarily N-acetyl aspartate (NAA), choline (Cho), creatine (Cre), glutamate (Glu), glutamine (Gln), and myoinositol (mI). NAA is found predominately in the neurons and the typical physiological intraneuronal concentrations is 9–12 mM (Urenjak et al. 1992), and as such is widely used as a marker of neuronal density. However, biochemical studies suggest the possibility of multiple roles for the metabolite NAA including an energy metabolism reserve (Bates et al. 1996; Heales et al. 1995), a supply of acetyl-Coenzyme-A for the tricarboxylic acid cycle in mitochondria (Mehta and Namboodiri 1995; Miller et al. 1996), a source of N-acetyl-aspartylglutamate (a neuropeptide that is involved in neurotransmission), a source of glutamate (a neuroexcitatory molecule which itself is a source for gamma-amino butyric acid) (Miller et al. 1996) and a role as an osmotic regulator (Taylor et al. 1995). NAA is synthesized exclusively in the mitochondria of neurons and decreases in NAA may be due, at least in part, to mitochondrial dysfunction (Goldstein 1969; Heales et al. 1995; Mehta and Namboodiri 1995). Neuronal cell death induces an irreversible loss of NAA due to localization of NAA in the neuron (Urenjak et al. 1992), while reversible loss of NAA is likely associated with reversal of mitochondrial dysfunction or replacement of damaged mitochondria within the neurons. The choline peak (Cho) is due to total levels of mobile choline containing compounds. Cho mainly consists of glycerophosphocholine and phosphocholine, compounds involved in phospholipid metabolism in brain tissue. Choline concentrations are known to be elevated in multiple sclerosis (MS) during periods of active demyelination (Simone et al. 2001). Total creatine (Cre) concentration [Cre] is the amount of creatine plus phosphocreatine in brain tissue. Cre is part of the creatine kinase energy metabolism buffer system used to maintain ATP levels in times of acute mismatch between oxidative adenosine triphosphate (ATP) supply and ATP demand. The [Cre] reflects the health of systematic energy use and storage (Miller 1991). Total [Cre] tends to remain relatively constant in most cell types, although the [Cre] in glial cells is four times that in neuronal cells (Petroff et al. 1995) and is known to be elevated in multiple sclerosis and epilepsy where gliosis is significant. mI may also act as a marker of glial cell numbers (Chang et al. 1999) and as an osmoregulator or intracellular messenger (Ross 1991).

Lactate [Lac] is seen as a result of numerous etiologies such as secondary to anaerobic glycolysis in tumors. Hypoxic insult can also cause an elevation of Lac. Lipid occurs in the same spectral region as Lac and can obscure Lac. It is often difficult to separate these two peaks from each other but lactate has a classic doublet appearance. The presence of lactate

can be confirmed by utilizing a TE of 136 ms causing the lactate doublet to invert (appear upside down in the spectrum) due to j-coupling (Yablonskiy et al. 1998). Lipids indicate the presence of necrosis or disruption of myelin sheaths. A higher level of lipid in a brain tumor generally indicates a high-grade malignancy. Lipids are best seen on spectra obtained with short TEs. However, contamination of the spectrum with scalp fat or bone marrow in the skull can result in a lipid peak, so care must be taken to interpret spectra with an understanding of the technical details of the acquisition.

¹H MRS and MRSI have been demonstrated to be a sensitive method for early detection of neuronal dysfunction or loss, reflected by loss of NAA. The application of MRS adds specificity to the diagnosis of a lesion relative to MR imaging alone (Ross and Michaelis 1994; Howe and Opstad 2003; Nelson 2003).

MR spectroscopy is used extensively in clinical practice to help differentiate tumor from non-tumor lesions in the brain. The Cho/Cr and NAA/Cho ratios can be utilized to help accomplish this task (Poptani et al. 1995; Moller-Hartmann et al. 2002). Increased Cho/Cr and decreased NAA/Cho are positive indications of a brain tumor (Poptani et al. 1995; Moller-Hartmann et al. 2002). The lipid and lactate peaks are more variable in tumor and non-tumor lesions but can also aid in tumor diagnosis. There are a number of disease processes, such as multiple sclerosis plaques, that cannot always be differentiated by spectroscopy from a brain tumor.

Tumor grading can be challenging with MR spectroscopy and there are overlaps between the appearance of the spectra from different grades of astrocytomas and oligodendrogliomas. The Cho peak and the Lip/Lac peaks tend to demonstrate increases as an astrocytoma progresses from low-grade to glioblastoma multiforme (GBM) (Howe et al. 2003; Nelson et al. 2002). Due to the complexity and necrosis in GBMs the peak values are somewhat variable (Howe et al. 2003). The Cr and the NAA peaks are decreased in low-grade GBM (Nelson et al. 2002). Myo-inositol is increased in low grade astrocytomas and is decreased in high grade astrocytomas (Castillo et al. 2000). The Lip and Lac peaks can be used to separate low-grade from high-grade (anaplastic) oligodendrogliomas in which they have been found to be significantly elevated (Rijpkema et al. 2003). In low grade oligodendrogliomas, glutamate and glutamine have been found to be elevated and this may help to differentiate oligodendrogliomas from other brain tumors such as astrocytomas (Rijpkema et al. 2003).

Gliomatosis cerebri can demonstrate subtle changes on MR spectroscopy. The subtle changes likely result in part from the infiltrative nature of the tumor and that a component of the spectrum represents normal brain. It has been described that the Cho/Cr levels may be normal and that only an elevated MI level will be detected in gliomatosis cerebri (Mohana-Borges et al. 2004). Also, there may be normal

Cho/Cr levels, associated decreased NAA and Lip/Lac present (Pyhtinen 2000).

It can be critical to obtain a MRSI study over the entire complex heterogeneous areas of a brain lesion in order to institute appropriate therapy, plan surgeries and accurately diagnose the brain lesion (Nelson 2003). MRSI, rather than the single voxel techniques, helps to define the spatial heterogeneity that is present in brain lesions and also contributes to defining the spatial extent of brain lesions. Two-dimensional MRSI performed at multiple levels or a single three-dimensional MRSI sequence can be performed to accomplish this task. MRSI can be used to help differentiate infiltrating primary brain tumors from metastatic disease. With metastatic tumors in the brain the spectral abnormality will not extend beyond the area of enhancement but with primary infiltrating brain tumors the spectral abnormality will persist outside of the area of enhancement.

Diagnosing radiation necrosis versus tumor recurrence is also a useful and accurate application of intracranial MR spectroscopy (Nelson 2003; Schlemmer et al. 2001). Radiation necrosis will have large Lip and Lac peaks without elevated Cho whereas tumor would have elevated Cho. MRSI is particularly useful for distinguishing recurrent tumor versus radiation necrosis given its ability to spatially sample multiple areas of a lesion (Chernov et al. 2005).

53.3.2 CT

Computed tomography of the brain revolutionized the care of neurological patients and was instrumental in helping to elucidate many disease processes after its introduction. However, the advantages of utilizing CT to image the brain have been greatly reduced by MRI's markedly better CNS contrast, MRI's physiologic imaging abilities and the metabolic information obtainable from PET or SPECT imaging. CT still plays an important role in the setting of trauma due to its proven ability to detect hemorrhages that require surgical intervention. CT also is advantageous in the trauma setting since it is often too difficult to adequately screen a patient for an emergent MRI and to monitor the acute trauma patient in the MRI scanner. Hemorrhages appear hyperdense acutely on CT and CT can accurately demonstrate subdural, epidural, parenchymal, intraventricular, and subarachnoid hemorrhages. CT exquisitely demonstrates the skull and skull base and is unsurpassed in detecting fractures.

CT has limited ability to differentiate the type of insult occurring in the brain and usually a brain lesion results in a lower density than the surrounding tissues. Lesions with calcification, hemorrhage, or high cellularity (such as lymphoma, primitive neural ectodermal tumors, medulloblastomas or small cell lung cancer metastases) will result in increased density. Iodinated contrast agents administered intravenously help evaluate lesions in the brain for blood brain barrier break-

down and the contrast helps improve lesion conspicuity. However, an intravenous gadolinium contrast enhanced MRI is more sensitive to detecting evidence of blood brain barrier breakdown than a contrast enhanced CT (Mihara et al. 1989).

CT has been shown to be able to detect early changes in the development of Alzheimer's disease (de Leon et al. 1993), although it has been found that other technologies, such as MRI and PET imaging, show changes better than CT. Also, in the analysis of seizure patients CT can demonstrate numerous parenchymal pathologies but a MRI examination is often a better evaluation (Heinz et al. 1989). CT is particularly useful when evaluating seizure patients for small calcified lesions such as in neurocysticercosis, periventricular calcifications of tuberous sclerosis, or the cortical calcifications present in Sturge-Weber syndrome.

A non-contrasted CT is often performed as the initial evaluation of the brain in the stroke patient secondary to CTs availability and CTs ability to exclude hemorrhage. The non-contrasted brain CT is also the neuroimaging study that was utilized in major stroke treatment studies (Furlan et al. 1999; Tissue plasminogen activator for acute ischemic stroke. The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group 1995). The non-contrasted brain CT offers limited information compared to MRI but recently with the development of CT perfusion of the brain additional information can now be obtained. CT perfusion moves CT imaging from static morphological imaging to providing physiological data that has already been shown to be useful for stroke imaging (Lev et al. 2001; Wintermark et al. 2006).

Multiple parameters can be calculated with CT perfusion imaging of the brain and include cerebral blood flow, cerebral blood volume, time-to-peak, and mean transit time (Wintermark et al. 2006). The information obtainable with CT perfusion is very powerful and when combined with computed tomographic angiography (CTA) the studies can be used as a substitute for MRI when evaluating stroke patients (Schramm et al. 2004).

CTA is a method for relatively non-invasively imaging the arteries and veins of the brain. The ability to create a CTA examination of the circle-of-Willis became possible with the advent of spiral CT, however, the quality of the examinations and the robustness of the techniques were greatly improved with the advent of multislice CT. The speed of acquisition continues to advance at a remarkable rate. With a 64-slice CT scanner the whole brain can be imaged in seconds with submillimeter slice thickness. The importance of being able to scan fast when performing CTA of the brain cannot be overstated given that a main application is evaluating patients with subarachnoid hemorrhage for the presence of cerebral aneurysms. Patients with subarachnoid hemorrhage often have mental status changes that make it difficult or impossible for them to follow directions and to lie still for an extended period of time. CTA is now often used to screen

patients with subarachnoid hemorrhage for cerebral aneurysms in order to determine if an aneurysm is present and what type of therapy (surgical clipping or intravascular coiling) is appropriate. Performing a CTA examination of the neck and circle-of-Willis fast is also optimal for decreasing the amount of venous contamination present. Venous enhancement on a CTA examination can obscure fine details such as the presence of small aneurysms.

The evaluation of acute stroke patients with CTA is being performed to evaluate for the underlying vascular etiology (Smith et al. 2006). CTA can nicely demonstrate areas of stenosis or thrombosis in the arteries. The presence of a large vessel intracranial occlusion in an acute stroke patient has been found to even be an independent predictor of poor outcome (Smith et al. 2006). Also, computed tomographic venography has been found to be beneficial for evaluating patients for the presence of venous thrombosis.

53.3.3 Single Photon Emission Computed Tomography (SPECT)

SPECT in general has a greater clinical availability compared to Positron Emission Tomography (PET). Secondary to SPECT's greater availability, proven capabilities and its lower cost it is an often used clinical and research imaging modality. SPECT is very useful for evaluating brain perfusion and has demonstrated utility for evaluating the brain perfusion of many disease processes including stroke, Alzheimer's disease and other dementias, Parkinson's disease, seizure patients, and brain tumors. Perfusion is just one parameter that can be measured with SPECT and there are many different radiotracers that can be used with SPECT for neurotransmitter system imaging (Kegeles and Mann 1997). The Ioflupane I-123 SPECT scan (DaTscan) can help differentiate between parkinsonian syndromes and essential tremor. An analysis of the utility of this scan demonstrated therapies were changed 63 % of the time secondary to the scan results (Sadasivan and Friedman 2015).

Thallium-201 SPECT and fluoro-deoxyglucose (FDG) PET are both used to help differentiate residual brain tumor and brain tumor recurrence from radiation necrosis. Thallium-201 SPECT has been found to be just as good or even better for tumor detection than FDG PET (Stokkel et al. 1999; Kahn et al. 1994; Maria et al. 1998). Thallium-201 SPECT can also be utilized to help differentiate lymphoma from toxoplasmosis which is an important question in immunocompromised patients particularly patients with human immunodeficiency virus (Young et al. 2005). Difficulties have been found when evaluating immunocompromised patients in the clinical setting and this may in part be due to the difficulty of evaluating small lesions (Licho et al. 2002).

There are numerous psychiatric applications of SPECT and these include evaluation of major depressive disorder, obsessive compulsive disorder, and schizophrenia (Alves et al. 2006; Topcuoglu et al. 2005; Novak et al. 2005). Studies have been performed evaluating these diseases and other psychiatric disorders at their initial presentations, during disease progression, and what the associated imaging changes are with medical intervention. There are many continued opportunities for investigation utilizing SPECT in psychiatry particularly whether it is utilizing a newly formulated radiotracer, utilizing advanced analytical techniques or combining SPECT data with MRI data.

SPECT has been found to be quite useful when evaluating patients with seizures (McNally et al. 2005; Tae et al. 2005). SPECT imaging is commonly used for the ictal study of seizure patients in order to define areas that are the seizure focus. It has in general been much easier to perform ictal SPECT than ictal PET. Advanced analytical techniques of SPECT imaging have helped to improve the data analysis and utilization of the SPECT data in the evaluation of seizure patients but further improvements and validation of the techniques are required (Knowlton et al. 2004).

53.3.4 Positron Emission Tomography (PET)

The information obtained from a PET scan depends on the radiotracer used and there is a wide array of radiotracers that can be used by the neuroscientist when analyzing the brain. PET using fluoro-deoxyglucose (PET-FDG) has wide clinical and research availability and has many applications in the neurosciences. PET-FDG can be used to study numerous pathologies including tumors, strokes, dementia, addiction disorders, schizophrenia, depression, seizures, and developmental abnormalities. There are a greater number of tracers that have been developed for PET than SPECT that allow in-vivo mapping of numerous neurotransmitter systems in humans (Kegeles and Mann 1997).

PET-FDG has been used extensively to investigate brain tumors including astrocytomas, oligodendrogliomas, meningiomas, lymphomas, metastatic disease, and the differentiation of tumor necrosis from tumor recurrence. In the evaluation of brain tumors PET-FDG has been found to help determine the grade of malignancy and can provide predictive prognostic information (De Witte et al. 2000; Borgwardt et al. 2005; Kaschten et al. 1998). Carbon-11-methionine has also been found useful, if not better, than PET-FDG for predicting histological grade and the prognosis of gliomas (Kaschten et al. 1998). Carbon-11-methionine is felt to be superior to FDG secondary to its sensitivity and clearer delineation of tumors (Van Laere et al. 2005; Pirotte et al. 2004), although, it is not as readily available for clinical use and investigation as FDG. Thallium-201 SPECT as mentioned in the previously section also has been shown to perform as

good if not better than PET-FDG in brain tumor analysis (Kahn et al. 1994; Maria et al. 1998; Stokkel et al. 1999).

Evaluating dementias with PET imaging is another area that has generated a lot of attention as well as clinical and research applications. The ability of PET scanning to help differentiate whether a patient has Alzheimer's disease has generated great interest due to the large number of individuals affected. PET-FDG is the radiotracer that is routinely utilized in the evaluation of dementias. PET-FDG can help to determine if a patient has Alzheimer's disease, mild cognitive impairment, and if a patient with mild cognitive impairment is likely to convert to Alzheimer's disease (Mosconi et al. 2004; Anchisi et al. 2005). Frontal-temporal dementia, vascular dementia and lewy body dementia diagnosis can also be facilitated with PET-FDG (Mirzaei et al. 2003; Higdon et al. 2004; Kerrouche et al. 2006).

Amyloid and Tau PET imaging are both adding significant new opportunities for evaluating patients who have memory dysfunction. The ability to have tracers that can define these abnormal proteins within the brain in-vivo makes it possible to detect and quantitate the disease processes leading to dementia at stages where therapy is potentially more effective. Pittsburgh compound B (PIB) was the first and is the most extensively studied of such compounds for detecting AB plaques (Shokouhi et al. 2014). PIB has been demonstrated to have increased uptake in patients with Alzheimer's and has been confirmed to be present in AB plaques post-mortem. Tau imaging has advanced rapidly and can now help to further refine the diagnostic biomarkers in-vivo also being able to detect the neurofibrillary tangles appearing in the transentorhinal cortex, followed by the entorhinal cortex and the hippocampus, and subsequently the neocortex in Alzheimer's disease (Okamura et al. 2013). Also, other tauopathies, such as chronic traumatic encephalopathy, can be detected and diagnosis facilitated (Mitsis et al. 2014).

The utility of imaging patients who have or potentially have Parkinson's disease can be valuable in multiple ways including early confirmation of the diagnosis, predicting clinical course, and to evaluate the results of therapies. When evaluating Parkinson's disease both PET-FDG and 6-[(18)F] fluoro-L-dopa (FDOPA), a common presynaptic dopaminergic radiotracer, can be effectively utilized (Kaasinen et al. 2006). FDG and FDOPA PET has been shown to be an indicator of the severity of Parkinson's disease and at patient's initial evaluations can be used to predict the clinical prognosis (Kaasinen et al. 2006). The differentiation by PET-FDG of Parkinson's disease, progressive supranuclear palsy and multiple system atrophy may also be facilitated given differences of metabolism that have been found (Juh et al. 2004).

Therapeutic induced changes of brain metabolism can be evaluated for both surgical and medical interventions (Zhao et al. 2004; Hershey et al. 2003; Hilker et al. 2002). These type of inquiries provide knowledge that can be used to judge

an intervention's success, how an intervention's success may have been produced, and potentially to predict early on in a treatment paradigm what long term success may be obtained.

There are several possible applications of PET imaging for seizure evaluation. PET-FDG again has been the predominant radiotracer that has been utilized for seizure evaluation, although, numerous radiotracers have been studied. Pre-operative evaluation of temporal lobe epilepsy is a frequently performed application of PET-FDG and PET-FDG is able to help predict the success of surgical resection (Theodore et al. 1997; Kim et al. 2003). PET as already mentioned usually studies the seizure patient in the interictal state since it is difficult to perform ictal PET studies. PET-FDG can also be a predictor of verbal decline after anterior temporal lobectomy—a surgery performed to help control complex partial seizures (Griffith et al. 2000). It has also been shown that PET-FDG is beneficial for evaluating for seizure foci in a general epilepsy population (Swartz et al. 2002).

53.4 Summary

This chapter has presented an overview of the mechanisms and applications of neuroimaging for the student of neuroscience with a view towards providing an understanding of the potential of neuroimaging techniques in neuroscience research. As the field progresses, methods for whole brain analyses, development of new detection methods and new radiotracers combined and coregistered will provide whole brain non-invasive histology for diagnosis of disease and tracking the effects of new therapies in animal models of neurological disease and, eventually, human subjects. Research techniques being developed will combine multiple coregistered techniques combined with molecular and histological analyses to provide sensitive and specific characterization of the gamut of combined neuroimaging methods for diagnosis and monitoring of neurological diseases.

53.5 Review Questions

- Diffusion weighted images become positive in acute stroke in:
 - 16 h
 - Minutes
 - 8 h
 - 1 Day
- Advantages of diffusion tensor imaging include:
 - Demonstrates abnormalities when conventional MRI is normal.
 - Provides functional maps of brain activity.
 - It is indispensable for planning stroke therapy.
 - Can be used to calculate perfusion metrics.
- Perfusion imaging data can be used to create maps that:
 - allow for “virtual dissection” of white matter tracts.
 - are used for creating MR angiograms.
 - can be utilized to determine tissue at risk for damage in patients with stroke.
 - show peaks of lactate in tissue damaged by stroke.
- Multivoxel magnetic resonance spectroscopic imaging (MRSI) does **not**:
 - help define the spatial heterogeneity of brain lesions.
 - provide less useful information than single voxel spectroscopy for distinguishing radiation necrosis versus recurrent tumor.
 - allow for accurate diagnosis of brain lesions.
 - demonstrate the same metabolic peaks as single voxel spectroscopy.
- Computed tomographic brain imaging:
 - continues to have great utility when evaluating trauma patients.
 - demonstrates acute hemorrhages as hypodense.
 - has replaced MRI for many imaging applications.
 - provides better metabolic information than PET or SPECT.
- Computed tomographic perfusion imaging:
 - has already been utilized in major stroke treatment studies.
 - can be utilized to evaluate brain diffusion.
 - has limited utility for evaluating stroke patients.
 - allows multiple parameters such as cerebral blood volume and mean transit time to be calculated.
- When evaluating seizure patients CT is particularly useful for:
 - demonstrating the periventricular calcifications of tuberous sclerosis.
 - demonstrating small soft tissue cortical lesions not detected by MRI.
 - demonstrating the cortical tubers in patients with tuberous sclerosis.
 - demonstrating the brainstem calcifications of Sturge-Weber syndrome.
- SPECT imaging of the brain:
 - is more expensive than PET imaging.
 - has been nearly replaced with PET imaging.
 - availability is a major problem in the United States.
 - is very useful for evaluating brain perfusion.
- SPECT imaging does not have:
 - many radiotracers that can be utilized.
 - many brain perfusion applications.
 - the ability to analyze brain tumors with Thallium-201.
 - undisputed ability to evaluate for lymphoma in patients with human immunodeficiency virus.

10. PET imaging:
 - (a) has fewer radiotracers that can be utilized than SPECT.
 - (b) has few imaging applications for which FDG is used.
 - (c) *has the ability to differentiate tumor necrosis from tumor recurrence.*
 - (d) lacks clear ability to differentiate Alzheimer's disease from mild cognitive impairment.
11. Which of these statements is true?
 - (a) T2-weighted images become positive in a stroke at around 24 h.
 - (b) Diffusion images allow for an estimation of the mean transit time.
 - (c) MR spectroscopy utilizes radiotracers.
 - (d) *CTA is used to screen for cerebral aneurysms.*
12. MR spectroscopy does not:
 - (a) *demonstrate increased myo-inositol in high grade astrocytomas.*
 - (b) potentially only demonstrates subtle findings for gliomatosis cerebri.
 - (c) demonstrate increased myo-inositol in low grade astrocytomas.
 - (d) show glutamate and glutamine elevations in low-grade oligodendrogliomas.

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Abstract

Proteomic and genomic technologies, particularly when combined as functional genomics, are promising experimental approaches to creating expression profiles of proteins and their connection to disease specific changes, starting with transcription and ending at the level of posttranslational modifications. It seems that molecular mechanisms underlying many neurodegenerative disorders may have common features. In a short amount of time, proteomics underwent unprecedented development from its early stages of collecting high numbers of protein identifications in a single experiment. Quantitative mass spectrometry of proteins is now a standard approach in all laboratories. However, analytical techniques based on principles of liquid chromatography are dominating and advancing the field. Further technological developments such as single cell analysis, will further facilitate a more precise view of changes occurring in proteomes resulting from alterations in biological systems. Another big step will be to go beyond slowing down or stopping neurodegenerative processes, and push the immune system to repair damage and promote regeneration. To what extent this is possible and how soon it can be accomplished remains an open question. It is certain, however, that understanding functions of complex biological systems, such as duality of neurotoxic/neurotrophic function of mononuclear phagocytes in the brain during inflammation, will require coordinated monitoring of a large number of parameters simultaneously and well-designed proteomic, genomic, and neuro-imaging experiments.

Keywords

2 dimensional electrophoresis • Bioinformatics • Biomarker • Bottom-up analysis • Cerebrospinal fluid • Gene arrays • Genomics • HIV-1 associated dementia • Hyphenated techniques • Liquid chromatography • Mass spectrometry • Neurodegeneration • Neuroproteomics • Protein fingerprinting • Proteomics • SELDI-TOF • SWATH-MS • Systems biology • Tissue profiling • Top-down analysis

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54.1 Introduction

Upon successfully sequencing the human genome, it was shown that it consists of a considerably smaller number of genes (20,000–25,000 protein coding genes) (International Human Genome Sequencing Consortium et al. 2004) than expected, with the number of proteins greatly exceeding the number of genes. This had a big impact on how we envision the human genome, along with that of other species, and how

we approach proteomic analysis. Isoforms and proteoforms, which are products of one gene modified by one or more of 354 posttranslational modifications (<http://www.abrf.org/index.cfm/dm.home?AvgMass=all>) or alternative splicing events, are only one part of the complexity because function and biological role can also depend on cytoplasmic, sub-cellular compartments, and/or extracellular localization. Interactions with other proteins and non-proteinaceous molecules add yet another layer of complexity. At the same time, we observed unprecedented development of mass analyzers with increased sensitivity, resolution, mass accuracy and dynamic range. Considering this, it is clear that the recent 15 years have had a tremendous impact on shaping the proteomic field.

54.2 Proteomics and Genomics: Omics Technologies for Global Oversight of Complex Biological Systems

While the word *genomics* was first coined by Dr. Thomas H. Roderick, a geneticist at the Jackson Laboratory in Bar Harbor, ME, in 1986 (Yadav 2007), proteomics as a scientific field emerged in mid-1990 along with technological advances in analysis of proteins and peptides. Two-dimensional polyacrylamide gel electrophoresis (2D SDS-PAGE), originally developed in the late 1970s (Barritault et al. 1976), was rapidly advanced to the next level with immobilized pH gradient (IPG) strips (Corbett et al. 1994). This improvement allowed for the analysis of gene products containing over 2000 proteins in a mixture (Gianazza 1995) with increasing reproducibility. Mass spectrometry of proteins and peptides has developed quickly, providing new methods and reagents for quantitative analysis such as Isotope Coded Affinity Tags (ICAT), isobaric Tags for Relative and Absolute Quantitation (iTRAQ) (Ross et al. 2004), Stable Isotope Labeling with Amino acids in Culture (SILAC) (Oda et al. 1999) and Sequential Window Acquisition of all Theoretical Mass Spectra (SWATH) mass spectrometry, which was first introduced in the ABSciex qTOF (TripleTOF) system (Sturm et al. 2008). Development of user friendly kits for protein sample preparation and extraction has enabled non-protein chemists to isolate proteins, fractionate complex samples, and develop their own protocols in a similar way to the development of expression vectors and systems (Feuerstein et al. 2005). Growth of various databases followed by their integration, along with new and improved algorithms for database searches, has opened the door for high throughput experiments with substantially increased quality of protein identification and quantification (Ku and Yona 2005). Thus it became a reality to identify fingerprints of change induced by infection, malignant transformation, exposure to toxic agents, or differentiation (Pang et al. 2006). As a result of this advancement, substantial progress in prognosis and monitoring of disease progression and the discovery of new biomarkers and drug targets was and

is widely expected. Concurrently, gene sequencing and gene arrays, which can be used to measure changes in expression levels to detect single nucleotide polymorphisms (SNPs) or to genotype or target re-sequencing, were rapidly advancing technologies that provide fast and low cost mapping of whole genomes. Gene array and gene sequencing techniques, which have been used in studying biological systems for many years, provided an enormous amount of valuable information and led to many discoveries and advancements, including completion of the Human Genome Project (International Human Genome Sequencing Consortium et al. 2004). Due to the development of these technologies, we are able to generate unprecedented amounts of data containing information about the function of biological systems under investigation. This created a bottleneck of data analysis and a demand for better statistical and bioinformatics tools for data analysis.

Reproducibility and orthogonal validation, as well as data integration, remain a substantial challenge to be tackled in years to come. Figure 54.1 shows a general overview of top-down and bottom-up experimental approaches. The top-down approach deals with system modeling leading to hypothesis, prediction, and simulation of behavior of an organism under various physiological conditions. The bottom-up approach studies individual pieces on a discovery basis and combines all the information in an effort to understand how the system in question works as a whole (Fig. 54.1). In proteomics, a third approach known as middle-down is based on generating peptides that are longer than in bottom-up proteomics but shorter than intact proteins in top-down proteomics are analyzed by mass spectrometry (Forbes et al. 2001). Proteomics offers a number of additional and unique benefits. For example, proteins, which are the functional products of an expressed gene, can be posttranslational modified and their sub-cellular localization can be altered in a different physiological state. This important information cannot be obtained using gene arrays but is available from proteomic studies.

Results from studies of biological systems led to the conclusion that individualized diagnosis, clinical evaluation, and treatment is inevitable. In many instances, the amount of sample available for diagnostic purposes is limited, resulting in the demand for more sensitive techniques and instrumentation and the development of single cell analysis. Automated and simplified steps of sample processing, combined with highly sensitive and specific measurements, will improve what currently contributes to the loss of important markers in a limited sample.

54.3 Proteomics Technologies

A variety of technologies are being developed to support qualitative and quantitative measurements of protein changes in proteomes and to address important issues in proteomics as a global overview of protein makeup (Petricoin and Liotta

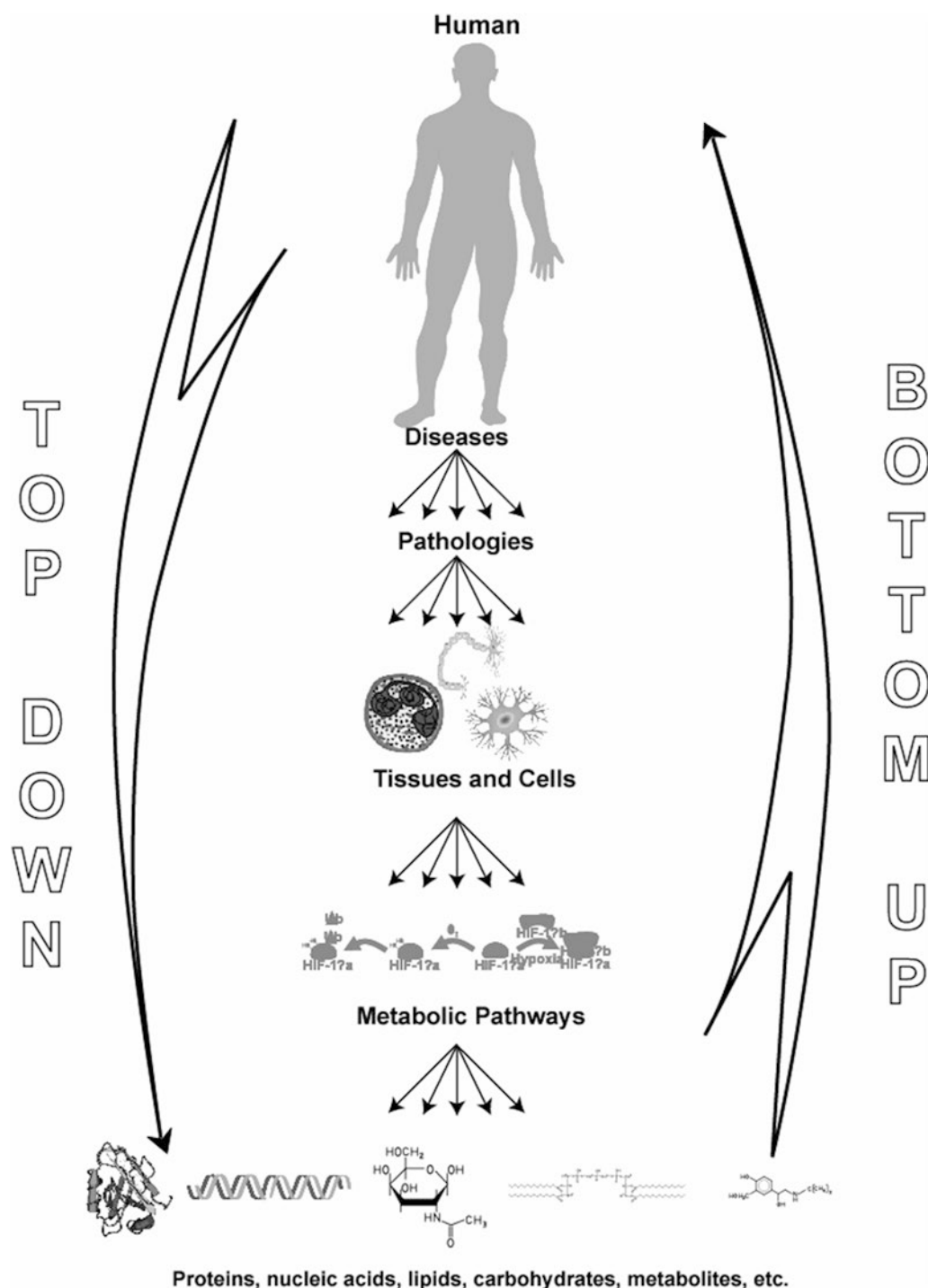


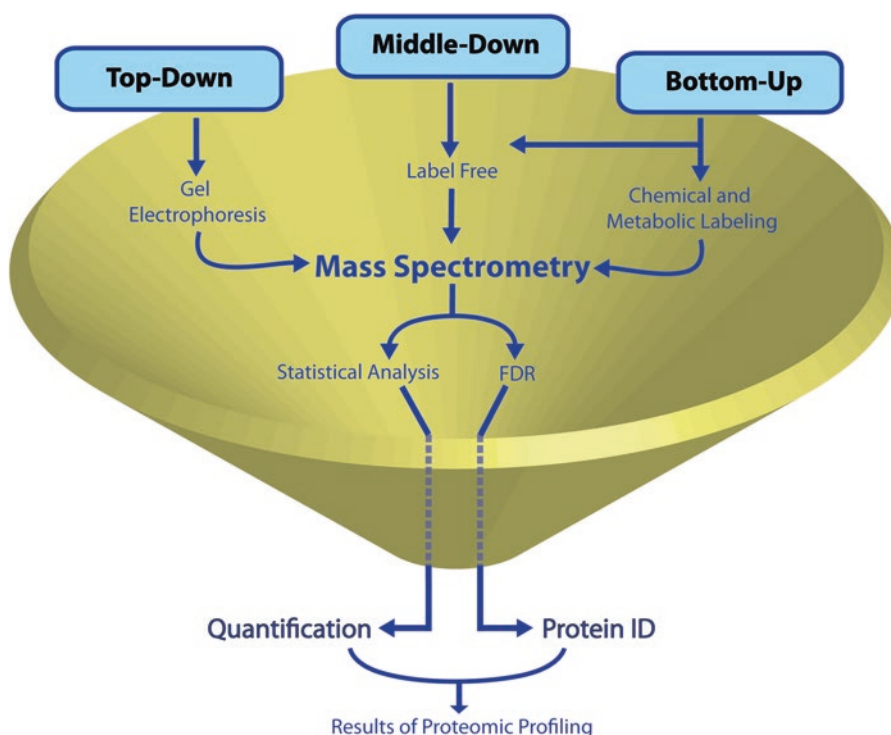
Fig. 54.1 Top-down and bottom-up approach in studying systems biology. There are three components contributing equally to study biology systems: theoretical, conceptual, and experimental approaches. All of

these approaches more and more heavily depend on implementation of effective bioinformatics tools. Hypothesis driven and discovery driven projects equally participate in studying systems biology

2003; Rohlf and Southan 2002; Woods et al. 2014; Patel 2014). Therefore, selection of the proteomic platform to be used in addressing any particular question is of utmost importance. A summary of proteomic workflows is presented in Fig. 54.2, which is divided into three major groups: top-

down, middle-down and bottom-up. Top-down analysis is based on isolation, analysis, and characterization of intact protein to reveal its function. Fourier transformed ion cyclotron resonance mass spectrometry (FT-ICR) (Marshall et al. 1998) facilitates such approach in protein identification as a

Fig. 54.2 A summary of proteomic platforms. The three primary methods of proteomic analysis involving mass spectrometry are top-down, middle-down, and bottom-up. While each requires different methods of sample preparation, they all have the same outcome; identification and quantification of samples, which leads to complete proteomic profiling



result of the random fragmentation of an intact molecule. In contrary, the bottom-up approach is based on up-front fragmentation of the protein in question using various proteolytic enzymes with known specificity (Millea et al. 2006; Chalmers et al. 2005). In these experiments, trypsin is most commonly used. The important question remains to be whether more information will be obtained from the bottom-up approach, which is protein identification on a cost of protein characterization, or from the top-down approach, which is distinguishing differences between two similar proteins/proteoforms. Of course, the ideal situation is to obtain both answers from one type of analysis. Handling mixtures of peptides generated from complex protein mixture (bottom-up approach) will be very different from sample processing at the protein level prior to analysis (top-down approach). Therefore, integration of these two will be a technological challenge in coming years because in order to make significant contributions, both approaches will require collaboration of scientists with diverse expertise with cross-disciplinary nature. Methods representing top-down approaches are used for separation of intact proteins using either gel electrophoresis or liquid chromatography. Chemical or metabolic labeling, as well as label free techniques, are widely used for bottom-up studies. With the middle-down approach, larger size protein fragments are generated and analyzed by tandem mass spectrometry with front-end liquid chromatography separation. In these experiments, label free techniques are used. A summary of technologies most commonly used in proteomics is presented in Table 54.1. Our intension here is that this summary not only

presents past experience, but also serves as guidance for the design of proteomic workflow in future studies.

The most commonly used proteomic platform is the combination of liquid chromatography and tandem mass spectrometry. In first dimension, tryptic digested peptides are fractionated on a strong cation exchange column and collected fractions are further fractionated on Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) with direct infusion to the mass spectrometer. Alternatively to SCX, an OFFGEL fractionation based on isoelectric point is quite often used. OFFGEL platform was introduced by Agilent, Inc. in 2006.

The majority of analytical techniques currently used for proteomic profiling have a liquid chromatography based step of fractionation. This will be RP-HPLC directly interfaced with mass spectrometer and off-line fractionations such as strong cation exchange (SCX) chromatography, IEF (OFFGEL technology), immunodepletion or all configurations of pull-down and enrichment steps. Multidimensional fractionation is an essential step but we have to keep in mind that extending pre-profiling fractionation may not always work towards our advantage, since every analytical step will introduce some degree of variability.

Enrichment of specific proteins or group of proteins is certainly the main goal of pre-fractionation. For example, immunodepletion of the most abundant proteins from serum or cerebrospinal fluid samples will facilitate detection of low abundant proteins in complex samples (Ramstrom et al. 2005; Chromy et al. 2004; Fountoulakis et al. 2004). However, the

Table 54.1 Summary of analytical and preparative methods most commonly used in proteomic studies

Method	Description	Applications
1D SDS PAGE	Fractionation based on approximate molecular weight (m.w.)	Preparative/analytical
IEF	Fractionation based on isoelectric point (pI). In addition to IPG strips used for first dimension separation as part of 2DE, includes OFFGEL technology	Preparative/analytical
2 D electrophoresis (IEF/SDS PAGE)	High resolution separation/fractionation based on pI and m.w., long and complex procedure. IPG strips with variety ranges of pH are available. 2DE DIGE using differential labeling of proteins with fluorescent Cy dyes	Preparative/analytical
LC-MS/MS	Chromatographic one or two dimensional fractionation followed by mass spectrometric peptide sequencing and protein identification	Analytical
Capillary Zone Electrophoresis (CZE)	Fractionation based on m.w. or pI, directly connected to tandem mass spectrometry	Analytical/preparative
HPLC/FPLC	Fractionation based on physico-chemical features of protein (m.w., pI, hydrophobic profile)	Preparative/analytical
SELDI-TOF	Fractionation based on selective binding to activated surfaces followed by MS determination of m.w., automatic fast sample processing combined with mass spectrometry	Analytical
MALDI-TOF	Requires high resolution mass spectrometry for protein identification based on peptide fingerprinting. High density plate spotters combined with micro-capillary RP-HPLC fractionation are available	Analytical
Protein Arrays	Identification/separation techniques based on protein—protein interactions. Requires high quality and well characterized antibodies.	Analytical

remaining amount of proteins will be quite different between samples. A published report of the Human Proteome Organization (HUPO) Human Plasma Protein Project (HPPP) showed that almost all laboratories participating in this international effort used some mean of sample pre-fractionation. Eventually, 3020 proteins were included in a database set from 15,677 proteins reported (Omenn et al. 2005). Enrichment using TiO₂ columns for phosphoproteins and phosphopeptides or affinity columns to selectively pull down ubiquitination is to facilitate identification and quantification of low abundant modified proteins. If enrichment is at the

protein level, samples can be used for top-down profiling. In many instances, proteins are proteolytically digested and enrichment is performed at the level of peptides. Such samples are used for bottom-up analyses.

2D SDS-PAGE, which has been used for about 40 years, combines two modes of separation: isoelectric focusing and polyacrylamide gel electrophoresis (O'Farrell 1975; Barritault et al. 1976). Development of immobilized pH gradient (IPG) strips made this method much more user friendly and, most importantly, much more reproducible. This allowed construction of computerized protein databases based on two-dimensional separation (Celis et al. 1990). Although the resolution, thus reproducibility, of 2D SDS-PAGE remains a limitation, especially in high molecular mass ranges or for hydrophobic proteins, advancements in protein sequencing by electrospray ionization (ESI-MS/MS) or Matrix Assisted Laser Desorption Ionization Time-of-Flight/Time-of-Flight (MALDI-TOF/TOF) mass spectrometry made it possible to identify proteins directly from the protein "spots" in 2D SDS-PAGE. This method is used for top-down proteomics. Difference Gel Electrophoresis (DIGE) compares two samples, one labeled with Cy 3 and the other labeled with Cy 5 fluorescent dye. These two samples are mixed together and analyzed in one 2D SDS-PAGE experiment. Common proteins are represented by yellow spots while different colored spots, which are detected with a laser-based scanner, represent differentially expressed proteins. This is another example of further technological advancement.

Another tool for protein profiling is Surface Enhanced Laser Desorption Ionization Time-of-Flight (SELDI-TOF) (Hutchens and Yip 1993). This technology utilizes a matrix-assisted laser desorption ionization time of flight mass spectrometer for detection, along with one one step chromatography to reduce complexity of the analyzed sample. The latter involves the use of proprietary Protein Chips initially commercialized by Ciphergen, Inc. and now offered by BioRad Laboratories. This is an analytical tool with relatively low resolution and mass accuracy in addition to high variability of intensities. Nevertheless, the big advantage of this method is ease of use for fast screening and collection of information regarding properties and conditions associated with binding of the proteins of interest to ion-exchange, hydrophobic, Immobilized Metal Affinity Chromatography (IMAC), and/or normal phases, which can be utilized with success in translation to preparative methods using corresponding HPLC column (Enose et al. 2005). This technology is also susceptible to relatively high variability in the analyses of multiple SELDI-TOF spectra. Jeffries et al. (Jeffries 2005) developed an algorithm, which greatly improved spectra alignment and thus improved reproducibility. MALDI-TOF instruments with higher resolution than SELDI-TOF, combined with various chromatographic techniques, have

also been used but did not produce convincingly better results than SELDI-TOF (Coombes et al. 2005).

A separate field of so-called “hyphenated methods” has been developed and deals with combining two or more methods together in succession. The first component of the combined methods will deal with separation/fractionation of an analyzed sample, followed by an in-line connected detection system. Hyphenated techniques were developed and became popular in the 1970s and 1980s—more than 30 years ago, and remain a vital part of current technological advancement (Murray et al. 1975; Lee et al. 1976). Examples of the most commonly used hyphenated methods in proteomics are LC-MS (liquid chromatography combined with mass spectrometry), GC-MS (gas chromatography combined with mass spectrometry) (Kazakevich et al. 2005) and LC-UV-SPE-NMR (liquid chromatography, UV detection, solid phase extraction, and nuclear magnetic resonance) (Exarchou et al. 2005). Sample fractionation prior to detection and/or analysis usually leads to enhanced analytical outcome. Hyphenated techniques are also meeting a big challenge in proteomic analyses; dealing with an extremely large and dynamic range of protein levels (Julka and Regnier 2005; Kusnezow et al. 2006).

Protein arrays attempt to occupy another space in proteomic technologies. While this approach is similar in concept to gene arrays, protein arrays are based on antigen-antibody interaction rather than hybridization of complementary strands of nucleic acid. As much as antigen—antibody interaction is very specific, it also depends on the quality of well standardized antibodies. This is much harder to accomplish. Therefore, accuracy and reproducibility of protein array data highly depends on proper experimental design, normalization procedures, eliminating systematic bias, and appropriate statistical analysis. Eckel-Passow and co-authors made several excellent suggestions about how experiments should be designed and analyzed to avoid false results (Eckel-Passow et al. 2005). For example, the authors propose to use several different clustering tools instead of a single cluster analysis for verification of results in discovering differences in a class of molecules, e.g. cytokines. Another obstacle in working with protein microarrays is maintaining stability of protein structures, which is critical for proper folding and thus interaction with the binding partner. Another side of this problem is exposure of the interacting surface/epitope while immobilized on the chip's surface. This technology, although not as popular now, is slowly gaining ground in biomedical research with increasing potential of applications in clinical diagnosis. Similar to DNA microarrays, protein arrays are commercially available as user-friendly kits, made specific for various microorganisms and diseases, with improved and sophisticated fluorescent tags. For those working in the area of drug development, protein microarrays are vehicles of accelerated discoveries. Protein arrays also make it possible to screen functions such as protein-antibody, protein-protein, protein-ligand, protein-

drug, and enzyme-substrate interactions. They can also be used to perform multi-analyte diagnostic assays. For others, protein microarrays are envisioned as tools for the exploration and understanding of pathological processes, kinetics of changes at the molecular level, drug candidates, and interactions between molecules such as lipids, nucleic acids, carbohydrates, etc. Protein microarrays are a powerful tool when proteomic analysis is narrowed to a group of proteins such as growth factors, chemokines, cytokines etc. These classes of proteins already have a great number of pre-required, well-characterized and highly specific antibodies reacting with high affinity. Such protein arrays (chips) were immediately developed and are successfully used in monitoring markers of immune responses (Kastenbauer et al. 2005) complementing DNA arrays (Ho et al. 2005).

In summary, one should decide which platform to use based on questions that are asked. The experimental design and sample processing should align with the type of biological material being investigated. Investigation of proteomes of body fluids and culture supernatants or lysates of whole cells, tissues or cellular compartments/organelles would all require a different platform to be used and this can be determined from reviewing already published studies. Along with improvements of sensitivity, resolution, mass accuracy, dynamic range and speed of data acquisition of new generations of mass spectrometers, proteomic data are becoming massive and reduction of sample complexity is inevitable. Otherwise analysis of such massive data and interpretation becomes very complicated.

54.4 Biomarkers

Biomarkers in disease can be defined as quantifiable analytes or factors reflecting changes in an individual's physiological state. These changes, or biological responses, can be found at the molecular, cellular, and/or whole organism level. They play a critical role in assessing disease outcome, particularly in patient treatment, how these treatments will be individualized and/or modified in future clinical trials and how they will affect directions in drug development. Therefore, biomarkers must provide objective measures of normal versus pathogenic processes and responses to treatment whether this leads to cures, improvement, or only maintenance of a patient's health. Thus, biomarkers are absolutely essential for early diagnosis of any disease, whether it be a slowly progressing pathological process or acutely developing infection.

HIV associated neurocognitive disorders (HAND) and in its extreme form, HIV associated dementia (HAD), is a good example in discussing the importance and qualities of reliable biomarkers. HAND is a brain pathology that develops over a long period of HIV-1 infection, ranging in progression

from subclinical to end stage. Once started, this slowly progressing disease ultimately leads to death. Currently, HAND is diagnosed by a battery of neuropsychiatric and psychological tests and it is impossible to predict and measure the time of onset and progression. The main reason for this is a lack of appropriate molecular biomarkers, which inform us about mechanisms of brain injury triggered by HIV-1 in the brain.

Given this information, what would constitute a good and reliable biomarker of HAND? We expect that regardless of whether a biomarker is found in the CSF or plasma of diseased people, it will give us an insight into the cellular mechanisms of an ongoing infection. This insight is important for those who are designing new clinical trials or regimes of existing treatments. Whether or not a biomarker enters clinical trial is based on vague measures, which might be influenced by the subjective impact of the examiner, causing an additional risk of inaccurate results. Thus, an ideal biomarker should inform us as precisely and as objectively as possible about the mechanism, status, and prediction of therapy and disease. The ideal biomarker should also help in the design of “individualized” therapy for the most effective clinical result. In our example of HAND, an activation of mononuclear phagocytes recruited in high numbers due to on-going inflammation led to conclusions that up- or down regulated proteins originating from these cells due to HIV-1 infection might be good candidates for biomarkers. Such approach, however, should be considered with caution. Our studies (Ciborowski et al. 2004) showed that although HIV-1 down-regulates MMP-9 expression in monocyte derived macrophages in *in vitro* studies, the net level of this protein in the CSF of HAD individuals as well as its net proteolytic activity is increased due to recruitment of high numbers of these cells to the inflamed brain.

Recent and quick development of nanotechnology, not only in drug delivery but also in diagnostics and monitoring, together with the technological boom of miniaturization, made it possible to use lower amounts of sample for analysis while sustaining the same level of sensitivity and specificity. Single cell analysis and tissue laser dissections are becoming a reality, although we have to be aware that not all proteins or their modified forms can be effectively detected at this level. Unlike in genomics, the proteomics approach does not have the luxury of *in vitro* amplification of proteins. Besides improving the yield of proteins of interest during sample preparation, limitations of detection in laboratory tests still exist, creating an analytical challenge. Nevertheless, there are numerous examples in which proteomics was successfully used to discover new biomarkers. Commercial tests, on the other hand, are almost always based on assays utilizing different principles, e.g. ELISA (Sandhu et al. 1991; Hampel et al. 2004).

It is also important to note that an effective biomarker can be a specific fragment of protein that is present in the diseased state but not under normal conditions. Currently, many biomarkers of this nature have been identified to be measures

of neurodegenerative disease. In several cases, the biomarker is a misfolded protein, showing a slightly different molecular weight from its corresponding wild type. In Alzheimer’s disease (AD), an existing biomarker of this type is the A β ₄₂ fragment of α -amyloid protein (Olsson et al. 2003). The accumulation of internalized misfolded Tau protein has also been found to correlate with the progression of AD, as well as frontotemporal dementia (FTD) (Wu et al. 2013; Rademakers et al. 2012b). Likewise, the aggregation of α -synuclein into Lewy Bodies has emerged as a biomarker for Parkinson’s disease (PD) (Luk et al. 2012).

54.5 Proteomics in Biomarker Discovery

There are four necessary key components for performing proteomic (global) experiments: (1) identification of differences generated within a system under various physiological conditions, (2) fractionation of complex mixtures of proteins and other molecules, (3) identification of differentially expressed proteins and their interacting partners, and (4) data analysis, which includes algorithms for database searches, tools for statistical analysis, and tools for bioinformatics analysis. The choice of method, instrumentation, and experimental approach should be selected based on the questions being asked and answers being sought.

As we collectively accumulate knowledge and experience on how proteomic experiments should be conducted in order to yield the most information, a shift from full unbiased profiling to targeted proteomics has been observed. Therefore, the majority of studies are now focused on specific groups of proteins or even single proteins and their interacting partners whose function(s) and/or interaction(s) are significant in biological processes. The expectation is that these studies will generate results that will be translated to the development of commercial diagnostic tests. The major bottleneck in this process at this time is the processing of increasingly complex data generated by proteomic studies.

Proteomics cannot exist without interdisciplinary research, grouping experts from a wide variety of specialties who understand and are able to communicate about issues related to protein chemistry and bring proteomics to the level of functional studies. Currently, research teams combining a relatively narrow group of scientists including protein chemists, mass spectrometrists, and bioinformaticians are pushing the levels of sensitivity and accuracy of analytical instrumentation to unprecedented levels, but experiments still remain at the stage of comparing two conditions only. Therefore, to benefit more from proteomics we need to organize international groups/consortia to tackle prioritized problems. One example is global proteomic analysis such as the Human Plasma Proteome Project (Omenn et al. 2005), which can be accomplished only through international collaborations of laboratories equipped

with various types of mass spectrometers and capable of performing complex analyses. Undoubtedly, efforts of this and other currently on-going international initiatives, e.g. Human Brain Proteome Project (<http://www.hbpp.org/>), will build a foundation for new discoveries and functional studies. Everyone in the broad research community will benefit from this work, particularly a rapidly growing number of smaller proteomics laboratories and programs with research goals focused on specific question(s) of biological importance. The most exciting potential lies in functional studies combining proteomic and genomic approaches.

54.6 Proteomics of Cerebrospinal Fluid

The total volume of cerebrospinal fluid (CSF, Liquor cerebrospinalis) in an adult human is about 130–150 mL and production is relatively constant under normal physiological conditions at the rate of approximately 0.4 mL/min (more than 500 mL daily). This means that turnover of CSF is at the rate of 4 volumes daily. A significant decrease in CSF secretion and turnover is associated with neurological diseases such as Alzheimer disease and chronic hydrocephalus (Silverberg et al. 2004; Tsunoda et al. 2002). Thus, CSF levels are highly reflective of the physiological status of the CNS.

Cerebrospinal fluid, which surrounds the brain and spinal cord, acts as an intermediate between blood and nervous tissues. Functions of the CSF include buoyancy, acid base buffering and delivery of electrolytes, signaling molecules, transport molecules and micronutrients to the brain parenchyma (Silverberg et al. 2004). CSF is produced predominantly by choroids plexus, specialized vascular tissue located in the brain ventricles (Serot et al. 2003). Choroid plexus tissues are intraventricular structures composed of villi covered by a single layer of ciliated, cuboid epithelium. The plexuses secrete cerebrospinal fluid, synthesize numerous molecules, carry nutrients from the blood to the CSF, reabsorb brain metabolism by-products and participate in brain immunosurveillance (Serot et al. 2003). Active transport across the choroid plexus is bidirectional, which allows for macromolecular transport out of the CSF. Blood flow to the choroid plexus is approximately six–times greater than that of the equal volume of brain tissue, suggesting that the choroid plexus is very metabolically active (Silverberg et al. 2004).

Changes in the biochemical composition of CSF could serve as a useful tool for investigations of pathological processes in the central nervous system (CNS). CSF is also in contact with blood plasma through the blood–brain barrier and thus resembles an ultra-filtrate of plasma in its protein constituents. CSF contains sugars, lipids, electrolytes and proteins. The concentration of proteins in CSF ranges from 0.2 to 0.8 mg/mL (0.3–1% of serum protein concentration) but more than 70% of the proteins in CSF are isoforms of albumin, transferrin and immunoglobulins (Ogata et al. 2005;

Wittke et al. 2005). The proteome of CSF contains proteins that are expressed in and secreted from circumventricular CNS structures. Therefore, the CSF proteome could provide unique biomarkers for early-stage diagnosis or the staging of a neuronal disease. This could offer potential insight into the biochemical characterization of affected neuronal populations and clarify the molecular basis of CNS pathologies (Yuan and Desiderio 2003; Yuan and Desiderio 2005).

Previous studies have used proteome comparisons to study neurological disorders such as Alzheimer's disease, Parkinson's disease, frontotemporal dementia, schizophrenia, and Creutzfeldt-Jacob disease. Some examples of utilizing proteomics approaches for discovery of biomarkers for neurological disorders in human CSF are given in Table 54.2.

Although insightful, the investigation of biomarkers in CSF has had some important limitations. Availability of larger volumes of CSF samples is limited by the need for invasive lumbar punctures. The large dynamic range of protein concentrations similar to this in plasma, which can be up to 12 orders of magnitude between the highest and the lowest expressed proteins, makes the analysis difficult (Maccarrone et al. 2004). Various schemes of sample pre-fractionation have been used to circumvent these problems. The widely used albumin removal method is affinity chromatography on various resins. However, this method is not specific only to albumin and it is known to bind numerous other proteins (Maccarrone et al. 2004; Gianazza and Arnaud 1982). A newer approach is to use immunoaffinity methods utilizing monoclonal or polyclonal antibodies to deplete not only albumin, but also other highly abundant proteins from biological samples. Resins coupled with antibodies could be packed in HPLC columns for depletion of the most abundant proteins for simultaneous depletion of 6, 12, 14 or more proteins (PMID: 17950469, 17929958). The application of HPLC columns for CSF preparation before 2D electrophoresis and shotgun mass spectrometry was compared with Cibacron Blue and protein G resins by Maccarrone et al. (Maccarrone et al. 2004). Immunoaffinity columns showed less nonspecific binding of CSF proteins. Another example of pre-treating CSF samples before proteomic analysis is the use of immunoglobulin Y (IgY) antibody microbeads and spin filters for depletion of the most abundant proteins (Seppro Mixed 6, GenWay, San Diego, CA). Comparison of IgY microbeads and immunoaffinity columns for pretreatment of CSF samples was performed by Ogata et al. (Ogata et al. 2005). The column method removed major proteins more effectively and approximately 50% more spots were visualized when compared to the 2D gel of CSF without protein depletion. Two other commercially available kits for removal of Human Serum Albumin (has) and HSA/IgG were used by Ramstrom et al. (Ramstrom et al. 2005) for CSF preparation prior to Liquid Chromatography Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (LC-FTICR MS). Both depletion methods provided a significant reduction of HSA, and the identification of lower abundant components was clearly facilitated.

Table 54.2 Summary of protein biomarkers of neurodegenerative disorders discovered using proteomics approach

Disease	Biomarker (protein)	Applied method	Lit. reference
Alzheimer's disease	Cystatin C, B-2 microglobulin isoforms, 4.8 kDa VGF polypeptide, unknown 7.7 kDa polypeptide	SELDI TOF and LC/MS	Carrette et al. (2003)
	Apolipoprotein A1, apolipoprotein E, apolipoprotein J, B-trace, retinol binding protein, kininogen, alpha-1 antitrypsin, cellcycle progression 8 protein, alpha 1B	2D electrophoresis and MALDI-TOF	Puchades et al. (2003)
	Glycoprotein, alpha-2—HS glycoprotein	MALDI-TOF	Wildsmith et al. (2014)
	Tau, hyperphosphorylated tau (p-tau), A β_{42}		
Frontotemporal Dementia	Transthyretin, fragment of VGF S-cysteinylated transthyretin, truncated cystatin C, fragment of chromogranin B	SELDI-TOF and MS/MS	Ruetschi et al. (2005)
Creutzfeldt-Jacob Disease	14-3-3 protein	2 D electrophoresis	Harrington et al. (1986)
	Cystatin C	SELDI-TOF, and LC-MS/MS	Sanchez et al. (2004)
	Ubiquitin	SELDI-TOF	Steinacker et al. (2010)
Schizophrenia	Apolipoprotein A-IV	2D electrophoresis and LC/MS	Jiang et al. (2003)
	Fibrinopeptide A	MALDI-TOF	Zhou et al. (2014)
	A-defensins	SELDI-TOF	Craddock et al. (2008)
Multiple Sclerosis	Cartilage acidic protein, tetranectin, SPARC-like protein, autotaxin t	2 D electrophoresis and LC -MS/MS	Hammack et al. (2004)
	Atg16L2	MALDI-TOF	Yin et al. (2014)
	Alpha-1-antichymotrypsin, contactin-1, apolipoprotein D, clusterin, kallikrein-6	ITRAQ	Kroksveen et al. (2013)

Immunodepletion of most abundant proteins, also called albuminome because albumin is most abundant protein in plasma, is based on interaction of proteinaceous antigen with immunoglobulin. Although it is a highly specific interaction, it still remains to be protein—protein interaction that might be non-specific to some extent. On the other hand, some proteins are partially removed from plasma samples because of their interactions with proteins that are being removed. Partial removal of other proteins may affect quantification and should be taken under consideration. More information about proteins removed along with albumin can be found in (Ciborowski 2013)

Every method of removing highly abundant proteins from CSF samples before proteomic analysis facilitates identification of discrete proteins, which could be important as a biomarker for classifying physiological stages of the CNS. However, the possibility of nonspecific removal of important peptides (through nonspecific binding to resins or forming complexes with target proteins) should be taken into account.

54.7 Neuroproteomics

Proteomic studies of the brain (central nervous system) pose significant challenges. Brain tissue collection, dissection, preservation, and maintenance of biochemical and molecular integrity are all critical aspects of neuroproteomics and

neurogenomics. Despite the fact that many proteins and nucleic acids are stable post-mortem (Yates et al. 1990) rapid degradation of other proteins may result in changes in overall protein composition (Choudhary and Grant 2004; Abbott 2003). Protein profile can change rapidly within minutes after death, making it very difficult to sort out changes related to disease from those related to death itself. In many studies done so far, protein extraction of the entire brain was used without distinguishing the specific region of origin (Wang et al. 2005). To address these issues, a variety of brain dissection techniques have been developed (Sanna et al. 2005). Manual dissection methods are still very useful and yield enough material for microarray analysis with somewhat limited “contamination” of the sample of interest with neighboring regions, which has a chance of affecting reproducibility of genomic and proteomic analyses. Ultimately, the laser dissection of brain tissues technique, which is a quickly emerging technique (He et al. 2013; Ly et al. 2011), further facilitated by increasing sensitivity of mass spectrometers, will be the method of choice for retrieving small regions, or even single cells of interest, from brain tissue to improve anatomical accuracy (Evans et al. 2003). Post-mortem collection of CSF has been shown to be a valuable source of information; however, it has to be analyzed with caution because of brain necrosis following death (Lescuyer et al. 2004). Lescuyer et al. found in post-mortem collected CSF samples three proteins that have been reported

as potential markers of various neurodegenerative disorders: prostaglandin D synthase, glial fibrillary acidic protein, and cathepsin D. Other proteins include ubiquitin C-terminal hydrolase L1, peroxiredoxin 5 (Lescuyer et al. 2004).

Some neurological disorders such as AD, PD, HAND, MS, schizophrenia or ALS are slowly developing diseases and will require extended longitudinal studies to search for biomarkers. In contrary, brain injury due to stroke, trauma, exposure to toxic substances, hypoxia or microbial infections are fast acting and require immediate intervention. Nevertheless, in all these cases the central nervous system is affected and damaged irreversibly to various extents. Thus, quick diagnosis and medical intervention is absolutely critical for the outcome of CNS diseases. Slowly developing neurodegenerative disorders are diagnosed by the observation of clinical symptoms after an already existing disease has been established. Thus, as much as biomarkers to measure disease progression are important, we should not stop in exploring prognostic biomarkers, which will inform us who might be highly susceptible to the development of neurodegenerative diseases. Although genetic tests help us in such prognosis, they are only applicable to a portion of cases. For example, in PD about 15 % of cases are correlated with a genetic predisposition due to having a first-degree relative with this disease. Therefore, when genetic tests cannot be used as predictors, proteomics appears to be an indispensable approach in the quest for predictive biomarkers.

Aggregated and modified proteins (α -synuclein in PD and tau in AD), along with deregulation of mitochondrial functions leading to overproduction of toxic ROS and glutamate, are factors responsible for neuronal death that are observed in many neurodegenerative disorders (Sultana et al. 2005). Nevertheless, specific triggers of neurotoxic mechanisms are vastly unknown and various proteomic techniques are employed to address these questions. High-throughput shotgun analysis used by Soreghan et al. (Soreghan et al. 2005) to identify targets of protein carbonylation in a PS1+AbPP transgenic mouse model pointed to iNOS-integrin signaling, CRE/CBP transcription regulation, and rab-lyst vesicular trafficking pathways as future research directions on neurodegeneration.

Significant progress has been made in CSF proteomic profiling in search of biomarkers for MS, which was reviewed by Kroksveen et al. (Kroksveen et al. 2013). Fourteen studies reviewed in this paper showed ten verified proteins, out of which six can be linked to immune response. Although neuroinflammation is a hallmark of MS, it is not specific for MS and its signatures have been shown in the CSF and plasma of individuals suffering from other neurodegenerative disorders. Haptoglobin, alpha-1-antichymotrypsin, Ig μ -chain C region, alpha-2-HS-glycoprotein/fetuin A, kallikrein-6, apolipoprotein D (ApoD) and chitinase 3-like protein 1 (CHI3L1) are almost uniformly detected in the CSF and plasma of individuals with all kinds of diseases, including

cancer. CHI3L1 is secreted by activated macrophages from viral infections (Ciborowski et al. 2007).

Another neurodegenerative disorder is HIV-1 Associated Neurocognitive Disorder (HAND). Despite the dramatic effect of antiretroviral treatment (ART) on slowing disease progression, HAND continues to be a major cause of HIV associated pathologies. This suggests that ART does not provide complete protection against neurological damage in HIV-infected brains. Laboratory measures of HIV-1 associated inflammation of the brain to determine HAND progression have been developed. Although valuable, these measures are not specific enough to be diagnostic at this time. Studies performed in non-human primate models with SIV infection did not lead to the discovery of much sought after biomarkers and only changes in relatively abundant proteins representing processes associated with many diseases have been observed (Wiederin et al. 2012). Taken together, results of this study provide an explanation as to why, despite convincing quantitative measures seen during biomarker discovery, protein candidates often fail during the subsequent validation phase. The authors propose that in chronic conditions, biological but not technical variance is a major obstacle in biomarker discoveries. Therefore significant changes of proteins with medium to high concentrations can be measured if such differences are high enough to exceed levels of natural fluctuations. The authors also envision that to overcome such a hurdle better correlates need to be made in improving experimental models, in system biologic statistical and computational approaches and in using better defined and more relevant clinical endpoints. It was encouraging to see that the same group showed changes in some of the same proteins such as geloslin and ceruloplasmin between humans and non-human primates (Pottiez et al. 2012).

Considering all obstacles related to neuroproteomic proteomic studies, questions arise as to which models are preferred. While there is no doubt that studies involving the intact nervous system are more relevant for elucidating mechanisms underlying neurodegenerative disorders, the use of proteomic profiling with in vitro culture models has many advantages. Ciborowski et al. proposed that proteomic profiling of secretome in in vitro HIV infected human macrophages may provide biomarker candidates to be measured directly in the CSF or plasma, omitting full unbiased profiling (Ciborowski et al. 2007).

In summary, the integration of proteomic profiling studies with in vitro, in vivo and ex vivo models can provide key insights on mechanisms of underlying pathologies.

54.8 Gene Arrays

Due to the established principle of making protein based on transcription of genomic DNA using mRNA as a messenger between chromosomes and ribosomes, gene arrays appear to

Table 54.3 Gene mutations associated with neurodegenerative diseases discovered using next generation sequencing approach

Disease	Gene	Mutation(s)	Effect on disease	References
Parkinson's disease	<i>VPS35</i>	Heterozygous p.Asp620Asn	Causative	Zimprich et al. (2011) and Vilarino-Guell et al. (2011)
Alzheimer's disease	<i>TREM2</i>	Heterozygous p.R47H	Increased risk (OR > 3)	Guerreiro et al. (2013) and Jonsson et al. (2013)
	<i>SORL1</i>	Heterozygous missense and nonsense	Potentially causative	Pottier et al. (2012)
Hereditary diffuse leukoencephalopathy with spheroids	<i>CSF1R</i>	Heterozygous missense, insertions and deletions all affecting the protein tyrosine kinase domain	Causative	Rademakers et al. (2012a)
Amyotrophic lateral sclerosis	<i>PFN1</i>	Heterozygous missense	Causative	Wu et al. (2012)
Autosomal-recessive cerebellar ataxia with spasticity	<i>GBA2</i>	Homozygous missense and nonsense	Causative	Hammer et al. (2013)

Source: Guerreiro R, Brás J, Hardy J, Singleton A. Next generation sequencing techniques in neurological diseases: redefining clinical and molecular associations. *Hum Mol Genet.* 2014, 23:R47–R53

be an indispensable link between genetic information and the functional product of genes—proteins. It was a surprise to discover that the human genome codes for only ~25,000 genes, that only approximately 75 % of the genome is transcribed into RNA, and that the majority of these RNA transcripts lack protein coding potential (Djebali et al. 2012), making them non-coding or ncRNAs. The relatively recent development of next-generation sequencing (Margulies et al. 2005; Shendure et al. 2005) revolutionized and advanced our knowledge about many diseases and allowed for the identification of new genes and genetic factors associated with diseases of the CNS. Data presented by Guerreiro et al. (Guerreiro et al. 2014) in Table 54.3 below shows examples of the impact next generation sequencing had on the identification of novel genes associated with neurodegenerative diseases.

Regardless of the fundamental importance of genomic experiments, this technology platform provides only initial and far from completed results. Proteomics, on the other hand, is a follow-up approach, and when results acquired from both technology platforms are combined, a more comprehensive picture of functional significance arises. Integration of these two experimental platforms is another challenge because of the complexity of systems biology. *First*, a bottom-up or a top-down strategy has to be chosen as an experimental strategy. *Second*, which part will be a driving force, genomics or proteomics? *Third*, an integrated bioinformatics platform has to be secured for complementary and coherent transformation of data into information. The ability to produce custom made DNA microarrays designed and manufactured by commercial entities and/or individual investigators to address specific biological question(s) would provide enormous support to such integrated strategies. Extensive literature can be found reviewing technical issues related to gene arrays including hybridization, post-hybridization quality control, normalization and data processing (Hartmann 2005; Boes and Neuhauser 2005; Reimers 2005). These specific tools allow for the performance of genomic experiments

based on results generated in initial proteomic studies (Soumelis et al. 2015) and our experience shows that experimental design and depth of data mining are critical.

Many more comprehensive or more focused studies have been performed to establish links between gene expression and neurodegenerative diseases in search of biomarkers. A thorough review of all these efforts would require a separate book, and therefore we will highlight here those of which we found to have an impact on our collective “-omics” knowledge. One such interesting study was performed by Tian et al. (Tian et al. 2014). The authors made very interesting comparisons between microarray data from patients with Sporadic Creutzfeldt-Jakob Disease (sCJD), Fatal Familial Insomnia (FFI), and Alzheimer's disease (AD). Based on principal component analysis, they identified commonalities between global gene expressions, thus supporting the idea that all neurodegenerative diseases have, to some extent, similar underlying mechanisms. Further bioinformatics analysis showed that signal transduction, synaptic transmission, and neuropeptide signaling pathways representing essential functions of the CNS are commonly affected in all three aforementioned diseases. A major weakness of this study was the limited number of samples; nevertheless, these data show the proof of principle that —omics technologies are indispensable in understanding the complexity of pathogenesis mechanisms and in searching for diagnostic and therapeutic biomarkers.

Recently, an interesting study of gene expression by microarray technology has been published by Durrenberger et al. (Durrenberger et al. 2015). The authors used 113 samples of post-mortem brains to create six study groups and controls of the following neurodegenerative disorders (NDs): Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD), multiple sclerosis (MS), and amyotrophic lateral sclerosis (ALS) and schizophrenia (SZ). The minimum number of samples in the diseased group was nine in the case of ALS. Bioinformatics data analysis showed that antigen

processing and presentation, cell adhesion, inflammatory response, regulation of cell proliferation, pattern specification process (referring to a developmental process), and response to drugs and nutrients and skeletal development were common for all six neurodegenerative disorders. The remaining genes, which were not included into these categories, were further grouped as signal transduction, angiogenesis, nervous system development, oxidation-reduction, apoptosis, and synaptic transmission ranging from 2 to 5 genes per category. Interestingly, the authors came to a conclusion that the molecular basis of the shared features in neurodegenerative diseases may not be as common as initially thought, which reinforced their view that each disease microenvironment is unique. Although the authors' assumption is experimentally substantiated, such a generalized conclusion should be taken with caution due to a lack of complementary proteomic studies.

In summary, it is now quite evident that all aspects of functional systems biology studies, including gene expression, mRNA and proteins with all PTMs, are essential for understanding molecular mechanisms underlying not only pathological processes, but developmental as well.

54.9 Proteomics and Tissue Profiling

With the current state of proteomic technology, tissue can be examined for global protein expression directly without time-consuming sample preparation. However, time required for data acquisition by scanning tissue section reminds significant. Can such profiling be used effectively in rapid clinical diagnostics to aid pathologists in their work? Can sections of tissue be placed on target, covered with matrix and profiled directly in mass spectrometers? Recent advancements indicate that regardless of limitations and challenges, this idea could lead to a breakthrough in modern clinical diagnosis in the near future (Jimenez and Verheul 2014; M'Koma et al. 2011).

Early attempts to develop patterns of specific compounds and proteins were based on tissue homogenates (Burns 1982; Jimenez et al. 1997; Mitchell et al. 1997). Tissue profiling, or more accurately called tissue imaging, based on direct mass spectrometry measurements is a younger area of research that began its development in the late 1990s when MALDI-MS measurements were performed on intact tissue sections (Chaurand et al. 1999). Techniques already well-established in proteomic workflows, such as microwave assisted and multi-enzyme digestion, have been adapted to tissue proteomics including FFPE and fresh tissue samples (Turiak et al. 2014).

MALDI-TOF mass spectrometers with high resolution and sensitivity reaching the attomolar range are the choice class of instrumentation for direct proteomic tissue analyses. In addition to proteins and peptides, this method allows for the detection of lipids, carbohydrates or glycoconjugates and

small molecules. Formalin-fixed paraffin-embedded (FFPE) tissue can also be investigated, making this approach an ideal tool for disease diagnostics as well as biomarker discovery. Since spectra are generated very fast, a wide range of masses are acquired. Hundreds and even thousands of spots can be analyzed using one tissue section, which is further processed by computers to create an image. Besides the high throughput of this type of analysis, a major advantage is that collected spectra can be linked to a location on the tissue section. Thus, molecules in question can be localized to tissue structures supplementing diagnosis based on histology, with immunohistology adding a molecular pathology component to histopathological diagnostics (Kriegsmann et al. 2014). This, in turn, is important for monitoring processes such as distribution of therapeutics, which can be small molecules as well as peptides and/or proteins. The remaining question to be addressed in tissue imaging is how important is structural analysis (peptide sequence, drug structure) of identified species since downstream processing of spectra is based on the presence, intensity, and signal-to-noise ratio data. Scattered presence of another molecule(s) with close molecular m/z (mass-to-charge ratio) can lead to misleading results. If the analyte's m/z is within 2000, identification based on sequencing can be performed in instruments equipped with post-source decay mode (Stoeckli et al. 2002). Another question deals with absolute quantitation, which is the weakness of MALDI-TOF comparing to other types of mass spectrometers. Nevertheless, meaningful information can be acquired if the system is properly validated. Despite the challenges discussed above, application of this advancing technology has resulted in reports on subjects such as classification of tumor samples using this technology platform (Chaurand et al. 1999) or single cell analysis (Li et al. 1999). More thorough reviews of this field that go beyond the scope of this chapter can be found on PubMed. Although certainly not limited to these, the following references give examples of such review papers published to date (Espina et al. 2009; Rodrigo et al. 2014; Lagarrigue et al. 2012).

54.10 Databases and Search Engines

Expression of proteins and genes on a global scale requires new and efficient tools for database searches and data analysis. Advancements in proteomics include the development of multiple search engines to determine how likely (or unlikely) a peptide sequence is represented by a particular mass list containing both precursor and fragment masses (Yates et al. 1995; Mann and Wilm 1994). Each search engine has inherent strengths and weaknesses and these must be considered when choosing which search engine(s) to use in processing data. We highly recommend using more than one search engine, particularly when data are search for PTMs. MASCOT, SEQUEST, Paragon used

by ProteinPilot™ and the more recently produced Andromeda, as part of MaxQuant, are the most widely used for database searches. Other search engines used less often include Protein Prospector, !XTandem or Spectrum Mill.

After an initial effort by many to create a universal protein database, an international consortium created the Universal Protein Resource (UniProt), which provides a comprehensive and freely accessible central resource of protein sequences and functional annotation (www.ebi.ac.uk/uni-prot/). UniProt consists of two sections: UniProtKB/Swiss-Prot, which is manually annotated and reviewed and UniProtKB/TrEMBL, which is automatically annotated and not reviewed. Despite this coordinated international effort, information included in protein databases is not complete and it is impossible to even estimate when, if ever, we will be able to claim victory of completeness.

Posttranslational modifications (PTM) occur in different biological contexts and therefore, complex protein mixtures extracted from biological samples invariably contain proteins carrying PTMs. The association of Biomolecular Resource Facilities lists 355 PTMs (<http://www.abrf.org/index.cfm/dm.home?AvgMass=all>). An additional level of complication is that PTMs can be heterogeneous, and therefore consist of various modifications present on the same peptide core. A good example of this are histones, which have a mosaic of PTMs dynamically changing in time as a result of their regulatory role (Burlingame et al. 2005). Variable modifications may or may not be present, e.g. phosphorylation of serine, threonine and tyrosine occurring in a percentage of the population of protein molecules. While searching for variable modifications is a powerful tool for identifying the PTMs, one needs to be cautious when specifying the number of variable modifications since adding even a single variable modification will generate more possible peptides to be searched against. If there are multiple modifiable residues within a single peptide, the workload for searching all the possible modification permutations could be exponentially increased.

Cells, their compartments and organelles (nucleus, mitochondria, endosomes etc.), as well as body fluids, are complex and dynamic systems comprising of multiple networks of interacting molecules: proteins, glycoproteins, glycolipids, nucleic acids, metabolites etc. Forming a better understanding of these systems and their mutual interactions and communications requires specific bioinformatics tools. This issue is further amplified by the amount of data/information accumulated in every experiment of high throughput analyses. A full understanding of interactions between different types of molecules such as DNA, RNA, and proteins in any biological system will require coordination of multiple experimental techniques at the same time, which will either require more sample or reduction of sample size for each individual test. Developing an application of micro fluidics in new analytical instrumentation will definitely facilitate miniaturization efforts in proteomics.

54.11 Review Questions/Problems

- Mathematical models of the molecular pathways in cells will facilitate:
 - (a) prediction of previously unknown interactions
 - (b) combining genomic and proteomic data
 - (c) discover novel proteins, cellular functions, and pathways
 - (d) *all of the above*
- Successful translation of results from laboratory to clinic depends on:
 - (a) exploratory research studies
 - (b) *validation*
 - (c) prospective screening studies on large-scale population
 - (d) *all of the above*
- Weakness of 2-dimensional SDS-PAGE is:
 - (a) *lack of superior reproducibility*
 - (b) lack of protection of proteins from structural de-stabilization
 - (c) inability of obtaining protein sequencing data
 - (d) lack of superior methods of protein labeling
- Major difficulty in proteomic studies of certain type of samples such as cerebrospinal fluid is:
 - (a) contamination with drugs penetrating through blood brain barrier
 - (b) *size and availability of the sample*
 - (c) lack of correlation with clinical diagnosis
 - (d) contamination with red blood cells during spinal tap
- Top-down approach in studies of systems biology is preferential over bottom-up approach because:
 - (a) it provides more global view
 - (b) it allows for faster conclusions which will lead to direct development of clinical tests
 - (c) requires less sophisticated technology and instrumentation
 - (d) *both approaches are equal*
- Major question to be addressed when considering application of genomics and proteomics to screening and testing include:
 - (a) selection of the mouse species
 - (b) selection of the non-radioactive reagents
 - (c) selection of the reputable provider of proteomics and genomics technologies
 - (d) *selection of conditions which are true reflections of in vivo biological function*
- Commercially available antibody arrays technology has several weaknesses. Major weakness is:
 - (a) problems associated with data analysis, interpretation, storage, and retrieval
 - (b) problems associated with high background created by not well developed reagents

- (c) problems associated with necessity of using radioactive materials
- (d) *problems associated with quality and standardization of antibodies*
- 8. What is the best approach to determine the sensitivity and specificity of the discussed methodologies, and to validate and standardize these technologies?
 - (a) to reduce “biological noise” in the samples and reduce number of replicates
 - (b) to link data obtained from database searches to biological effects via fast computers
 - (c) *to develop a standard approach to understanding and communicating variability in experimental data*
 - (d) none of the above
- 9. While planning proteomics experiments, a researcher should:
 - (a) have available all types of mass spectrometers
 - (b) mandatory access to protein microarrays
 - (c) *be aware of strength and weaknesses of each technology*
 - (d) always be an expert in statistics and computer programming for solving problems associated with bioinformatics
- 10. Neuroproteomics analysis is most conclusive when
 - (a) experiments are done using entire brain so that all cell types are used in one assay
 - (b) only in vitro models are conclusive
 - (c) *selected types of cell from brain are obtained and manipulated*
 - (d) two or more cell types are mixed together to facilitate interaction mimicking in vivo situation

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Abstract

Pharmacogenomics is the study of the role of genetic variations in drug response. In the study of pharmacogenomics, one can relate variations in drug exposure and/or drug response (pharmacology) to the variations in genes (genomics). An administered drug may fail to work for one patient, but show the expected result for another. In some cases a drug may work well for a larger group of patients, while causing a subset of patients to experience unexpected or severe side effects. These adverse drug reactions can be harmful and sometimes deadly. Factors such as the environment, diet, age, lifestyle and co-morbid conditions all may influence the individual's response to drugs. Each is covered in this chapter with an eye towards how best to administer drug to individual patients.

Keywords

Gene variants • Metabolizers • Neurodegenerative • Pharmacogenomics

55.1 Introduction

Since the beginning of medical practice, different individuals respond differently to the same drug (Roden and George 2002). In line with this idea, several studies have shown that genetic variability clearly has implications for both drug safety and efficacy (Li et al. 2011). Neurodegenerative diseases are complex disorders of neurons, that include genetic, epigenetic, and environmental factors, and are characterized by the deterioration of higher activities of the central nervous system. Over 60 % of Alzheimer disease (AD) risk is estimated to be a result of genetic factors; several genes have been associated

with AD pathogenesis and neurodegeneration (Gatz et al. 2006; Eisenstein 2011). Studies have shown that patients with AD respond to drug treatment depending on which of the three genetic variants of the apolipoprotein E gene a person carries (Guerreiro et al. 2012; Cacabelos 2008; Choi et al. 2008). Genes involved in the absorption, distribution, metabolism and excretion of drugs, and various drug target genes make up the most important genetic determinants (Ma et al. 2002). The pharmacologic treatment of neurodegenerative diseases has seen some progress, but more effort needs to be made to understand the genetic components and the genome-drug interactions. In this chapter, we will review the roles of several defective genes involved in neurodegenerative diseases and correlate them with the clinical outcomes.

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55.2 Classifications of Anti- Neurodegenerative Drugs in Various Disease Phenotypes

Neurodegenerative diseases include AD, multiple sclerosis (MS), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS).

55.2.1 Alzheimer's Disease

Alzheimer's disease (AD) is one of the most common causes of dementia among the aging population, and is characterized by declining cognitive failure, loss of communication and thinking capabilities. AD has become a major health challenge in both developed and developing countries (Gatz et al. 2006). Memory impairment is an integral feature of AD and is often the initial symptom. Deficits in other cognitive domains may occur with or after the onset of memory impairment. Language function and visual-spatial skills tend to be affected relatively early on, while loss of executive function and changes in personality and behavior generally occur later in the disease course. The pattern of memory impairment in AD is distinctive; deterioration in ability to remember facts and events is a cardinal feature. Neuropathologic findings include extracellular amyloid plaques, intracellular neurofibrillary tangles, synaptic deterioration, and neuronal death (Mathisen 2003). Amyloid plaques result from accumulation of β -amyloid peptides (A β). A β is an amino acid peptide formed by proteolytic cleavage of amyloid precursor protein (APP) by beta- and gamma—secretase (Mathisen 2003). Tau, a protein involved in microtubule assembly, is critical for normal axonal growth and neuronal development. Hyper-phosphorylated tau protein aggregates into helical filamentous neurofibrillary tangles that are deposited within neurons. Death of neurons in critical locations in the brain leads to a deficiency of acetylcholine, a neurotransmitter important for memory (Hellstrom-Lindahl 2000). Familial AD occurs earlier in life and involves mutations in genes involved in the processing of APP including *presenilin 1* and *presenilin 2*, whereas the risk for late onset AD is mostly influenced by apolipoprotein E (*APOE*) gene (Guerreiro et al. 2012). According to the Alzgene database, among hundreds of genes associated with AD, the top ten are *APOE* (19q13.2), *BINI* (2q14), *CLU* (8p21–p12), *ABCA7* (19p13.3), *CRI* (1q32), *PICALM* (11q14), *MS4A6A* (11q12.1), *CD33* (19q13.3), *MS4A4E* (11q12.2), and *CD2AP* (6p12) (Cacabelos et al. 2014). Other genes associated with AD are included in Table 55.1.

The US Food and Drug Administration (FDA) has approved four drugs for the treatment of AD: donepezil, rivastigmine, galantamine and memantine. Donepezil, rivastigmine and galantamine are cholinesterase inhibitors which are therapeutically active in ameliorating the clinical pathologies of mild to moderate AD (Loveman et al. 2006). Inhibition of acetylcholinesterase, the enzyme responsible for the destruction of acetylcholine, increases the availability of acetylcholine. Memantine is a noncompetitive N-methyl-D-aspartate receptor antagonist that is approved for the treatment of moderate to severe AD. Glutamate activation at the NMDA receptor is needed for learning and memory processes in the brain. Glutamate is the dominant excitatory amino acid in the brain, and stimulates NMDA receptors. This in turn leads to increased intraneuronal concentrations of calcium. Excessive

Table 55.1 Some Alzheimer's disease associated genes and their genomic locations. Table adapted from Calero et al. (2015) and Cacabelos et al. (2014)

Genes	Genomic location
<i>Familial genes</i>	
<i>APP</i>	21q21.3
<i>PSEN1</i>	14q24.3
<i>PSEN2</i>	1q31–q42
<i>Sporadic genes</i>	
<i>APOE</i>	19q13.2
<i>TREM2</i>	6p21.1
<i>PLD3</i>	19q13.2
<i>AKAP9</i>	7q21–q22
<i>GRN</i>	17q21.32
<i>MAPT</i>	17q21.1
<i>PRNP</i>	20p13
<i>ADAM10</i>	15q22
<i>TOMM40</i>	19q13
<i>BINI</i>	2q14
<i>CRI</i>	1q32
<i>ABCA7</i>	19p13.3
<i>FERMT2</i>	14q22.1
<i>HLA-DRB5-DRB1</i>	6p21.3
<i>CD2AP</i>	6p12
<i>PTK2B</i>	8p21.1
<i>INPP5D</i>	2q37.1
<i>CUGBP-CELF1</i>	11P11
<i>S100B</i>	21q22.3
<i>CD33</i>	19q13.3
<i>SLC24A4-RIN3</i>	14q32.12
<i>ZCWPW1</i>	7q22.1
<i>MEF2C</i>	5q14.3
<i>NME8</i>	7p14.1
<i>MS4A cluster</i>	11q12.2
<i>EPHA1</i>	7q34
<i>PICALM</i>	11q14
<i>CLU</i>	8p21–p12
<i>CASS4</i>	20q13.31
<i>SORLI</i>	11q23.2–q24.2
<i>DSG2</i>	18q12.1

glutamate, and therefore calcium, in the CNS may cause neuronal damage. Blockade of NMDA receptors by memantine slows the intracellular calcium accumulation and helps to prevent further nerve damage (Danysz and Parsons 2003). A higher dose of donepezil, with or without memantine was approved to treat moderate to severe AD (Cummings et al. 2013). However, these drugs have different pharmacokinetic and pharmacodynamics profiles with donepezil having a relatively longer half-life ($t_{1/2}$) of 70 h than galantamine ($t_{1/2}$ =6–8 h) and rivastigmine ($t_{1/2}$ =1–2 h), whereas memantine has a ($t_{1/2}$ =60–70 h) (Noetzli and Eap 2013). Studies have shown that the combination therapy of memantine and acetylcholinesterase inhibitors in patients with AD may be associated with benefits of slowing cognitive impairment and preventing the onset of agitation and aggression (Shao 2015; Gareri et al. 2014). Hepatic CYP2D6 and CYP3A4 are

mainly responsible for the metabolism of donepezil and galantamine, whereas rivastigmine, a potent, selective inhibitor of brain acetylcholinesterase (AChE) and butylcholinesterase (BChE), is metabolized in liver and intestine independent of CYP450 system. Reports show that polymorphisms in genes involved in the cholinergic system including acetylcholinesterase, butylcholinesterase, choline acetyltransferase and paraoxonase are involved in the clinical response to AChE inhibitor (Noetzli and Eap 2013).

55.2.2 Multiple Sclerosis

Multiple Sclerosis (MS) is a common neurodegenerative disease that typically affects young adults to mid age group. It is a **demyelinating disease** in which the **insulating covers** of **nerve cells** of the **brain** and **spinal cord** are damaged. It is mediated by T-cell autoimmunity whereby these cells migrate across the blood brain barrier, and subsequently induce inflammatory responses resulting in myelin destruction, oligodendrocyte destruction, axonal breakdown, and gliosis in the central nervous system (Tsareva et al. 2016). MS is characterized by clinical features including fatigue, paralysis, visual field impairments, chronic pain and cognitive changes. Most MS patients experience a relapsing-remitting disease form characterized by varying periods of disease intensity and with time progress to a steady progression of symptoms known as secondary progressive MS. When disease is characterized by rapid increase in disability rate, it is known as primary progressive MS. Over 100 genetic risk factors have been associated to MS, but the class II region of the human leukocyte antigen (HLA)-DRB1*15:01, encoded within the major histocompatibility complex has been shown to be the largest contributing genetic risk component (Hollenbach and Oksenberg 2015). Both HLA and non-HLA genes have been associated to the genetic susceptibility of MS. Treatment options for MS are very wide because the FDA has approved several disease modifying therapies (DMTs) with proven efficacies compared with placebo in preventing relapses associated with MS (English and Aloï 2015). The list of DMTs include interferon beta (IFN β), glatiramer acetate, tumor necrosis factor inhibitors etanercept and adalimumab; fingolimod, mitoxantrone, natalizumab, teriflunomide, dimethyl fumarate, peginterferon- β -1a and alemtuzumab. Interferon injectables and glatiramer acetate were the first FDA approved DMTs that specifically decrease disease activity and are still considered first-line treatments for relapsing-remitting forms of MS due to their desired long term safety and efficacy profiles (English and Aloï 2015). Studies have identified some gene variants that are associated with therapy response. However, HLA class II genes which are the major MS susceptibility genes, showed associations with glatiramer acetate but not with IFN β . Some of those results for IFN β and glatiramer acetate are highlighted in Table 55.2.

Table 55.2 Genes and their polymorphisms associated with Multiple Sclerosis therapy. Table adapted from Tsareva et al. (2016)

Drug	Gene	Polymorphism
Interferon β	<i>IFNAR1</i>	rs1012334, rs55884088
	<i>LMP7</i>	rs2071543
	<i>CTSS</i>	rs1136774
	<i>MXA</i>	rs2071430, s17000900
	<i>IRF5</i>	rs2004640
	<i>IRF8</i>	rs17445836
	<i>USP18</i>	rs2542109
	<i>IFNG</i>	Polymorphic microsatellites in the first intron
	<i>IL10</i>	rs1800896/rs1800871/rs1800872
	<i>CCR5</i>	rs333
	<i>TGFB1</i>	rs1800469
	<i>TRAILR1</i>	rs20576
	<i>CD58</i>	rs12044852
	<i>CD46</i>	rs2724385
	<i>GPC5</i>	rs10492503, rs1411751
Glatiramer acetate	<i>HLA-DRB1</i>	rs3135388
	<i>CCR5</i>	rs333
	<i>TCRB</i>	rs71878
	<i>IL12RB2</i>	rs946685
	<i>MBP</i>	rs470929
	<i>ILIR1</i>	rs956730
	<i>CD86</i>	rs1129055
	<i>CTSS</i>	rs2275235, rs1415148
	<i>FAS</i>	rs982764

55.2.3 Parkinson's Disease

Parkinson's disease (PD) is a neurodegenerative disorder that results in selective loss of dopamine neurons in the substantia nigra. Dopamine is a catecholamine formed in the body by the decarboxylation of dopa; it is an intermediate product in the synthesis of norepinephrine, and acts as a neurotransmitter in the central nervous system. PD is characterized by several clinical extrapyramidal features: resting tremor, rigidity, bradykinesia, postural instability, and freezing. PD has a low incidence among Asians and Africans as opposed to Caucasians. The various targets of anti-parkinson drugs are dopamine receptors (DR), dopamine transporter (DAT), catechol-O-methyltransferase (COMT) and monoamine oxidase B (Table 55.3). Levodopa (*L*-dopa) is the gold standard for Parkinson disease therapy and is highly effective when given in combination with a dopa-decarboxylase inhibitor (carbidopa). This combination reduces the decarboxylation of levodopa to dopamine outside of the blood-brain barrier, thereby allowing more efficient dosing of levodopa. Dopamine receptors are proteins on the surfaces of certain cells that bind specifically to the neurotransmitter dopamine. Stimulation of these receptors on vascular epithelial cells in the brain by dopamine cause the cerebral arteries to dilate, resulting in increased flow of blood. The dopaminergic system consists of the vital dopamine receptors (DR)

Table 55.3 Genes and their polymorphisms associated with Parkinson's disease. Table adapted from Drozdik et al. (2013)

Gene	Polymorphism
Levodopa	<i>DRD2</i> rs1800497(<i>TaqIA</i>)
	<i>DRD3</i> rs6280(<i>Ball</i>), rs4646996(<i>MspI</i>)
	<i>DRD2</i> (CA) _n STR in intron 2
	<i>DRD3</i> rs6280(Ser9Gly)
	<i>GRIN2B</i> rs1806201, rs7301328, rs1019385
	<i>SLC6A4</i> VNTR
	<i>DRD2</i> various (9)
	<i>DRD3</i> various (2)
	<i>DRD4</i> various (3)
	<i>DAT</i> 40-bp VNTR
	<i>OPRM1</i> rs1799971 (118A>G, Asn40Asp)
	<i>COMT</i> rs4680(Val158Met)
	<i>MAOB</i> rs1799836 intron 13
	<i>BDNF</i> rs6265 (Val66Met)
	<i>GBA</i> various mutations
	<i>ACE</i> rs4646994 (I/D-ins/del)
	<i>APOE</i> rs429358 rs7412 (e2, e3, e4)
	<i>CCK</i> rs1799923 (−45 C>T)
	<i>CCKAR</i> rs1800857 (779 T>C)
	<i>CCKBR</i> rs1805002 (rs1550G>A)
	<i>SLC22A1 (OCT1)</i> rs622342
COMT inhibitors	<i>UGT1A</i> A528G
	<i>COMT</i> rs4680(Val158Met)
Pyridoxine	<i>COMT</i> rs4680(Val158Met)

which are classified in five categories, namely D1–D5, which are co-expressed at various levels in the CNS and peripheral tissues and are located at chromosome 5, 11, 3, 11 and 4, respectively. The following polymorphisms may affect anti-parkinson drug therapy. *DRD1* genetic polymorphism includes −48 A>G (D1.1; B1/B2) and 1403 T>C (D1.7; C1/C2), *DRD2* with *TaqIA* located in kinase *ANKK1* (ankyrin repeat and kinase domain containing 1), *DRD3* associated with Ser9Gly (or *MscI* or *Ball*), which in turn is responsible for the insertion of *DRD3* receptor membrane and subsequent intracellular signal transduction. The different susceptible loci are the familial atypical forms of PD including *SNCA* (*PARK1*), *LRRK2*, *PRKN* (*PARK2*), *PINK1* (*PARK6*), *DJ-1* (*PARK7*), and recently *ATP13A2* (*PARK9*), *PLA2G6* (*PARK14*) and *FBX07* (*PARK15*). Sporadic PD is associated with common genetic variants such as N-acetyltransferase 2 (*NAT2*), monoamine oxidase B (*MAOB*), glutathione transferase (*GST*), mitochondrial tRNA, S18Y variant of ubiquitin carboxy-terminal hydrolase L1 (*UCHL1*), Rep variant of alpha-synuclein (*SNCA*) and tau (*MAPT*)H1 haplotype and leucine-rich repeat kinase-2 (*LRRK2*). Other newly recognized loci have been identified using GWAS namely; *BST1*, *GAK*, *HLA-DR*, *ACMSD*, *STK39*, *MCCC1/LAMP3*, *SYT11*, *PARK16*, *FGF20* and *GPNMB*. However, the anti-PD drugs targeting the motor systems lose their efficacy over time due to the emergence of side effects such as dyskinesias and psychosis. The genes and their SNPs that induced dyskinesias are *DRD2* with 9 polymorphisms (*TaqIA*, *TaqIB*, *TaqID*,

Table 55.4 Lists of some genes and their genomic locations associated with ALS. Table adapted from Cady et al. (2015) and Renton et al. (2014)

Gene name	Genomic location
<i>Familial genes</i>	
<i>SOD1</i>	21q22
<i>VCP</i>	9p13
<i>TARDBP</i>	1p36
<i>C9ORF72</i>	9p21
<i>FUS</i>	16p11
<i>Sporadic genes</i>	
<i>ATXN2</i>	12q24
<i>FIG4</i>	6q21
<i>OPTN</i>	10p13
<i>SETX</i>	9q34
<i>ANG</i>	14q11
<i>DCTN1</i>	2p13
<i>SQSTM1</i>	5q35
<i>VAPB</i>	20q13

Val96Ala, Leu141Leu, Pro310Ser, Ser311Cys, A>G231, −141C ins/del), *DRD3* with 2 (Ser9Gly, *MspI*), *DRD4* with 3 (48-bp VNTR, 13-bp repeat, 13-bp deletion) and 40-bp VNTR in *DET* gene. Among the *COMT* gene polymorphism Val158Met; (rs4680) is the most elaborately studied one and correlation with dyskinesia has been found (Payami and Factor 2014; Drozdik et al. 2013).

55.2.4 Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is a rapidly progressive, invariably fatal neurological disease that attacks the nerve cells responsible for controlling voluntary muscles, and belongs to a group of disorders known as motor neuron diseases, which are characterized by the gradual degeneration and death of motor neurons. In the vast majority of all ALS cases (more than 90%), the disease occurs apparently at random with no clearly associated risk factors. The remainder is hereditary. The familial form of ALS usually results from a pattern of inheritance that requires only one parent to carry the gene responsible for the disease. Mutations in superoxide dismutase 1(*SOD1*) have been identified as the highest genetic risk factor to cause familial ALS. Other genes that have been linked to familial cases of ALS include *TARDBP*, *VCP*, *C9ORF72*, and *FUS*. It is important to note that these genes are associated with familial ALS and also to a smaller degree to sporadic ALS (Renton et al. 2014). Other genes such as *IMPG2*, *TBK1*, *ANO9*, *LGALS1*, *CRLF3*, *DNMT3A*, *GPR158*, *GREB1L* with their coding variants are among the top ALS associated genes (Cirulli et al. 2015; Cady et al. 2015). Prevalence of variants in other ALS-associated genes are listed in Table 55.4.

Although more than 20 genes have been identified to be associated with ALS, effective treatment is still under inves-

tigation. The only licensed drug for ALS is riluzole. Riluzole has several pharmacological properties, but its precise mode of action is unclear. Riluzole seems to interfere with the damaging effects of excitatory amino acids, mainly glutamate, caused by excessive stimulation motor neurons nerve cells. Postulated mechanisms for this effect include the inhibition of glutamate release, blockade or inactivation of voltage-dependent sodium channels, and/or an inactivation of a G-protein mediated pathway (Cifra et al. 2013).

55.2.5 Huntington's Disease

Huntington's disease (HD) results from genetically programmed degeneration of neurons in certain areas of the brain. This degeneration causes uncontrolled movements, loss of intellectual faculties, and emotional disturbance. HD is a familial disease, passed from parents to children through a mutation in the huntingtin gene (*Htt*). Each child of an HD parent has a 50–50 chance of inheriting the HD gene. The involuntary movements associated with this disorder have been described as “chorea”. A 2006 systematic review of clinical studies evaluating pharmacologic treatments for HD concluded that the typical neuroleptics, haloperidol and fluphenazine, were possibly useful for treating chorea in patients with HD (Bonelli and Wenning 2006). Neuroleptics (anti-psychotic medications) act by blocking dopamine transmission. Tetrabenazine is a selective, reversible, centrally-acting dopamine depleting drug that works by inhibiting vesicular monoamine transporter 2 (VMAT2). Tetrabenazine depletes presynaptic dopamine, norepinephrine, and serotonin storage and antagonizes post-synaptic dopamine receptors. In vitro data indicate that tetrabenazine exhibits a weak binding affinity at the dopamine-2 receptor. Clinically, tetrabenazine improves the hyperkinetic movement disorder symptoms associated with HD (Chen et al. 2012). One of the earlier theories of the pathology of the disease is the formation of intracellular inclusion bodies due to the aggregation of mutant Huntington protein, which in turn binds and sequesters proteins and transcription factors that dysregulate the normal transcriptional activities (Fig. 55.1). Different pathways and the concerned genes such as neurotransmitter receptors, G-protein coupled receptor signaling, calcium signaling and homeostasis, neurotrophin receptor signaling, ubiquitin-proteasome system that are dysregulated in the HD has been listed in Table 55.5. Other systems that influence the genetic pathways of the disease are retinoid signaling, lipid metabolism, transcription and chromatin remodeling, and synaptic transmission. Genes of these pathways have altered expression levels that lead disease pathologies. Researchers have also found that histone deacetylase (HDAC) inhibitors might have a positive effect in the reversal of the Huntington-related gene (Chen et al. 2012; Seredenina and Luthi-Carter 2012).

55.3 Role of Genetic Variations in Cytochrome P450 Drug Metabolizing Enzymes

Most of the CNS drugs are metabolized by enzymes of the cytochrome P450 (CYP) family. The genes encoding *CYP2D6* and *CYP3A4* isoenzymes have a high degree of polymorphism leading to marked variability in the activity of the encoded enzymes. There is a high level of allelic variation in different ethnic groups that have been linked to inter-individual differences in drug response (Seripa et al. 2010).

55.3.1 CYP2D6

The *CYP2D6* gene (cytochrome P450, family 2, subfamily D, polypeptide 6) lies at locus 22q13.1 (Gough et al. 1993) and is involved in the metabolism of an estimated 25 % of all drugs (Wang et al. 2009). The CYP2D6 enzyme catalyzes the oxidative metabolism of over 100 clinically important and commonly prescribed drugs, such as cholinesterase inhibitors (tacrine, donepezil, galantamine), antidepressants, neuroleptics, opioids, class I anti-arrhythmics, analgesics and many other drug categories, acting as substrates, inhibitors or inducers with which cholinesterase inhibitors may potentially interact (Cacabelos 2008). Cholinesterase inhibitors are the first line symptomatic therapy for AD which increase active levels of acetylcholine in the synaptic cleft by inhibiting the enzymes that degrade acetylcholine (Mayeux 2010). Tetrabenazine, the first FDA-approved drug for the management of HD-associated chorea, is a monoamine storage inhibitor extensively metabolized by the hepatic CYP2D6 to its primary active metabolite, alpha-dihydroxytetrabenazine (Mehanna et al. 2013). More than 100 allelic variants of CYP2D6 have been identified with the enzyme activities ranging from deficient (poor metabolizers, PM), intermediate (intermediate metabolizers, IM), normal (extensive metabolizers, EM) to increased enzymatic activity (ultrarapid metabolizers, UM) with frequencies ranging from 5–10 %, 10–17 %, 70–80 %, 3–5 % respectively in Caucasian populations. PM have little or no CYP2D6 function because they have two null alleles and therefore unable to use the CYP2D6-dependent metabolic pathway for drug elimination. The most common variants associated with PM phenotype are *CYP2D6*3*, *CYP2D6*4*, *CYP2D6*5*, and *CYP2D6*6* in Caucasians, *CYP2D6*10* in Asian, and *CYP2D6*17* in Africans (Bradford 2002; Zhou 2009). The IMs metabolize drugs at a rate somewhere between the poor and extensive metabolizers whereas those classified as EMs have one or two alleles with normal CYP2D6 function. Expression of UM genes leads to greater than normal CYP2D6 function. Consequently, drugs are metabolized rapidly in UMs, causing decreased efficacy,

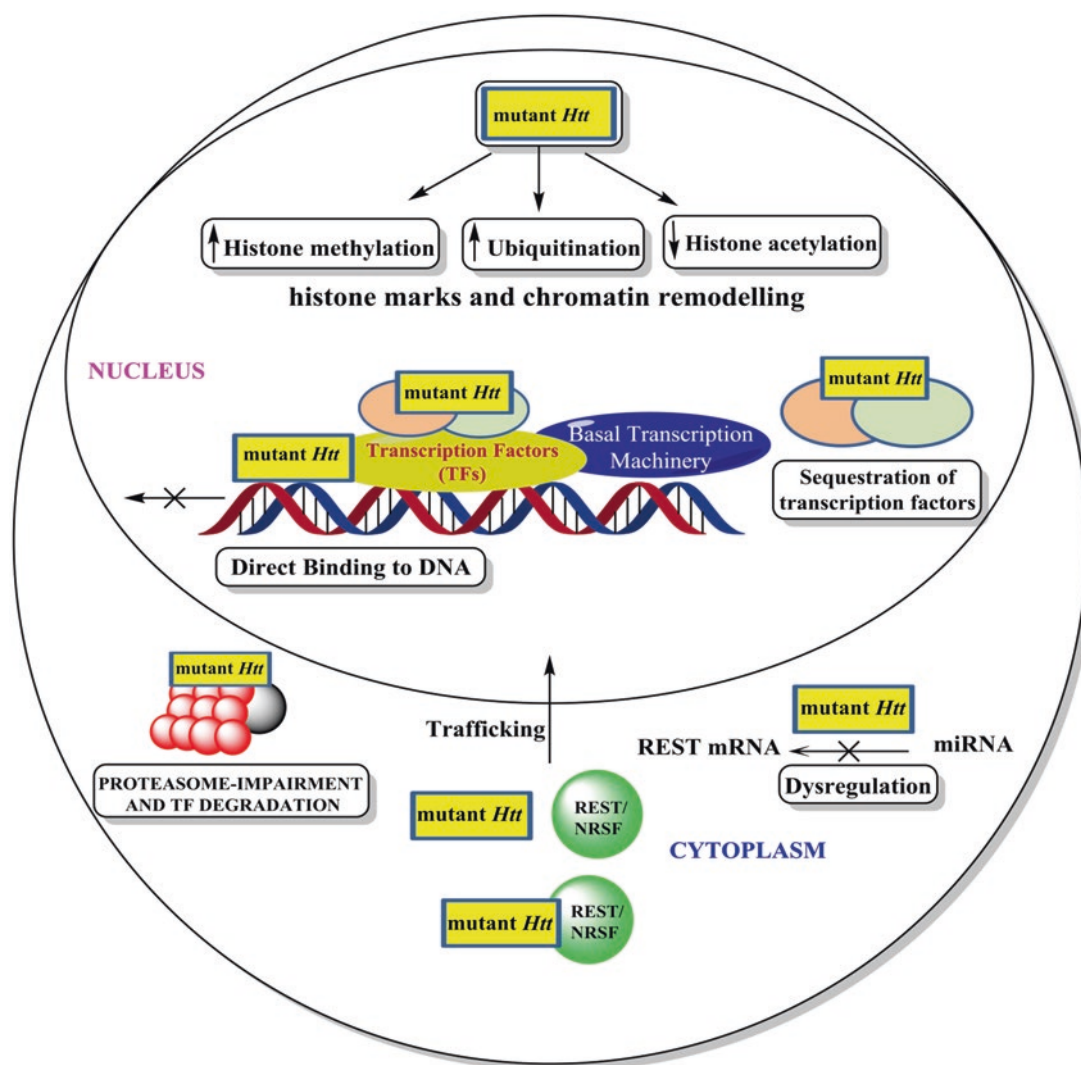


Fig. 55.1 Schematic representation of genetic dysregulation in Huntington's disease (Seredenina and Luthi-Carter 2012). *Htt* Huntingtin gene, *TFs* transcription factors, *REST/NRSF* RE1-silencing transcription factor/neural-restrictive silencer factor

Table 55.5 Lists of genes dysregulated in HD and their associated genetic pathways. Table adapted from Seredenina and Luthi-Carter (2012)

Genetic pathways	Genes dysregulated in HD
Neurotransmitter receptors	<i>DRD1A</i> , <i>DRD2</i> , <i>TH</i> , <i>CNR1</i> , <i>ADORA2A</i> , <i>GABRA5</i> , <i>GABRA4</i> , <i>GABRG2</i> , <i>GABRD</i> , <i>GAD1</i> , <i>GLRB</i> , <i>GRIA1</i> , <i>GRIA3</i> , <i>GRM1</i> , <i>GRM2</i> , <i>GRM3</i> , <i>GRIN2A</i> , <i>GRIN2B</i> , <i>SLC1A4</i> , <i>HEH3</i> , <i>OPRM1</i> , <i>OPRK1</i> , <i>OPRD1</i> , <i>HRH3</i> .
G-protein receptor signaling	<i>PPP1R1B</i> , <i>ARPP21</i> , <i>GNAL</i> , <i>ADCY5</i> , <i>GNG3</i> , <i>GPR88</i> , <i>GPR6</i> , <i>PDE10A</i> , <i>PDE2A</i> , <i>PDE1B</i> , <i>RGS2</i> , <i>RGS4</i> , <i>RGS9</i> , <i>RASD2</i> , <i>RAS</i> , <i>RAPGEF3</i> , <i>PTPN5</i> , <i>GUCY1B3</i> , <i>PRKCB1</i> , <i>NGEF</i> , <i>PAK1</i>
Calcium signaling and homeostasis	<i>RYR1</i> , <i>CALB1</i> , <i>PPP3R1</i> , <i>PCP4/PEP-19</i> , <i>HPCA</i> , <i>RASGRP2</i> , <i>CPNE5</i> , <i>STRN</i> , <i>KCNIP2</i> , <i>KCNA1</i> , <i>SLC24A3</i> , <i>PLCB1</i> , <i>ATP2A2</i> , <i>ITPR1</i> , <i>CAMKK2</i> , <i>CABP1</i> , <i>SYNJ1</i> , <i>TESC</i>
Neurotrophin receptor signaling	<i>BDNF</i> , <i>NTRKB</i> , <i>NTRKA</i>
Neuropeptides	<i>PENK</i> , <i>NPY</i> , <i>PDYN</i> , <i>OXT</i> , <i>AVP</i> , <i>CART</i> , <i>POMC</i> , <i>SST</i> , <i>NTS</i> , <i>CRH</i>

whereas slow metabolism of drugs in PMs causes toxicities owing to drug accumulation (Noetzli and Eap 2013).

The CYP2D6 enzyme is the major enzyme involved in the hepatic metabolism of donepezil to its active metabolite 6-O-desmethyldonepezil (Varsaldi et al. 2006). Donepezil is an inhibitor of acetyl cholinesterase, and is currently used for the symptomatic treatment of mild-to-moderate AD. Studies show that when administered with or without food, donepezil is well absorbed with a bioavailability of almost 100% (Cacabelos 2008; Seripa et al. 2011; Benjamin and Burns 2007; Varsaldi et al. 2006). Studies have also shown that functional polymorphisms in the CYP2D6 gene may affect enzyme activity and thus, the metabolism of donepezil, indicating that the plasma concentration of donepezil is dependent on CYP2D6 polymorphism (Pilotto et al. 2009; Varsaldi et al. 2006). In the general population, EMs account for 55.71%, IMs for 34.7%, PMs 2.28%, and UMs 7.31% whereas in AD, EMs, IMs, PMs, and UMs account for 56.38%, 27.66%, 7.45%, and 8.51%, respec-

Table 55.6 Summary of pharmacogenetic studies on anti-dementia drugs. Table adapted from Noetzli and Eap (2013)

Drug	Gene, variant	Number of patients	Results
Donepezil	CYP2D6	108	Significantly decreased clearance in PMs, increased clearance in UM compared with EMs
	Predicted phenotype		
	CYP2D6	94	EMs and IMs best responders in a combination therapy of donepezil with citicoline paracetamol and nicergoline
	Predicted phenotype		
	CYP2D6	127	Significant higher frequency of the G-allele, associated with a higher enzyme activity, in non-responders than in responders
	rs1080985 C>G		
	CYP2D6	57	Significant higher frequency of alleles conferring decreased or absent enzyme activity in responders than in non-responders
	16 functional SNPs		
	CYP3A	54	Genetic variants have no influence on plasma concentration and treatment outcome
	CYP3A4/CYP3A5 alleles		
	APOE	286	No significant influence of the APOE genotypes on treatment response
	ϵ 2, ϵ 3, ϵ 4		
	APOE	40	APOE ϵ 4 carriers demonstrated a poorer response on the AD Assessment Scale-Jcog score after 3 years of therapy
	ϵ 2, ϵ 3, ϵ 4		
	APOE	81	Better response in APOE ϵ 4 carriers in specific cognitive domains and on mini-mental state examination score
	ϵ 2, ϵ 3, ϵ 4		
Galantamine	CYP2D6	356	The population pharmacokinetic model indicates a reduced clearance in PMs compared with EMs
	Predicted phenotype		
	CYP2D6	27	Significantly increased plasma concentrations in PMs compared with EMs
	Predicted phenotype		
	APOE	202	No significant influence of the APOE genotypes on treatment response
	ϵ 2, ϵ 3, ϵ 4		
Rivastigmine	APOE	214	No significant influence of the APOE genotypes on treatment response
	ϵ 2, ϵ 3, ϵ 4		
Donepezil, rivastigmine	BCHE	114	Wild-type carriers showed better response to rivastigmine than to donepezil, whereas the treatment response was similar within carriers of the variant allele
	rs1803274		
Menantine	SNPs in SLC22A1/2/5,	108	NR1I2 rs1523130 significantly affected memantine clearance, with carriers of the CT/TT genotypes presenting a slower elimination than carriers of the CC genotype
	SLC47A1, ABCB1, NR1I2,		
	NR1I3, PPAR		

tively. The therapeutic response to conventional drugs in patients with AD is genotype-specific, with *CYP2D6*-PMs, and *CYP2D6*-UMs having the worst drug response (Cacabelos et al. 2012). Studies investigating the relation between clinical response to donepezil and a common variant (rs1080985) of *CYP2D6*, confirmed that rs1080985 is associated with poor response to the drug (Albani et al. 2012). Recently, studies have shown that *CYP2D6* is present in neurons in numerous human brain areas, including the thalamus, hypothalamus, hippocampus, substantia nigra, cerebellum, and in several layers of the frontal neocortex (Dutheil et al. 2009). The enzyme was implicated in metabolism of the endogenous compounds 5-methoxytryptamine, anandamide, progesterone and tyramine and in generation of serotonin and dopamine from trace amines; these findings raise questions about its potential role in the function of these neurons (Miksys and Tyndale 2013). The hypothesis that *CYP2D6* may have a role in the brain was strengthened by

a transgenic mouse model study: *CYP2D6* expression in the brain was associated with higher levels of serotonin measured in several brain regions, including the cerebellum and hippocampus (Cheng et al. 2013) (Table 55.6).

55.3.2 The Cytochrome P450 (CYP) 3A4 Gene

The *CYP 3A4* gene (cytochrome P450, family 3, subfamily A, polypeptide 4) lies at locus 7q21.1. The *CYP3A* gene includes a cluster of *CYP3A4*, *CYP3A5*, *CYP3A7*, *CYP3A43* and two pseudogenes named *CYP3A5P1* and *CYP3A5P2* (Seripa et al. 2010). These genes encode monooxygenases that catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids. The *CYP3A4* is the predominant hepatic form that demonstrates a large inter-individual variability in expression and activity,

and is involved in the metabolism of acetylcholinesterase inhibitors donepezil, and galantamine (Lamba et al. 2002; Noetzli et al. 2013). In a small study that evaluated the impact of CYP3A4 and CYP3A5 allele variants on donepezil plasma concentration and clinical outcome, there was no association.

55.4 Role of Defective Genes in Apolipoprotein E

Apolipoprotein E (*APOE*) gene mediates cholesterol metabolism in an isoform-dependent manner and is involved in lipid transport and injury repair in the brain involving beta-amyloid proteins (Eisenstein 2011). The human *APOE* gene is polymorphic, and has three allelic variants namely $\epsilon 2$, $\epsilon 3$ and $\epsilon 4$ at a frequency of 11 %, 72 % and 17 % respectively, and five common genotypes 2/3, 3/3, 2/4, 3/4, and 4/4 (Farrer et al. 1997; Guerreiro et al. 2012). The *APOE* gene has been shown to be the main genetic determinant of late-onset AD risk (Saunders et al. 1993; Bertram et al. 2007). Genome-wide association studies (GWAS) have repeatedly shown that the $\epsilon 4$ allele is the strongest known genetic risk factor for AD. Patients were found to respond to drug treatment depending on which of the $\epsilon 2$, $\epsilon 3$ or $\epsilon 4$ genetic variants of the *APOE* gene a person carried. Studies showed that individuals with the *APOE* $\epsilon 4$ allele relative to those without the *APOE* $\epsilon 4$ allele have at least a twofold risk for developing AD (Tang et al. 1998). Generally, those carrying the $\epsilon 4$ allele are at increased risk of AD compared with those carrying the more common $\epsilon 3$ allele, whereas the $\epsilon 2$ allele has been shown to decrease the risk (Liu et al. 2013; Eisenstein 2011). The *APOE* $\epsilon 4$ allele frequency was found to be significantly higher in the AD groups compared with the control group (Tsuang et al. 2013). Studies further indicate that *APOE* $\epsilon 4$ carriers preferentially benefit from receiving acetylcholinesterase inhibitors (tacrine, donepezil, galantamine, and rivastigmine) as AD treatment that improve their cholinergic deficit (Hanson et al. 2015). Other studies indicate that the presence of the *APOE* $\epsilon 4$ allele differentially affects the beneficial outcome and level of drug sensitivity in AD patients treated with neuroprotective compounds, nootropics (“smart drugs” or “memory enhancers”), endogenous nucleotides (CDP-choline), immunotrophins (such as Polypodium leucotomos: anapsos), neurotrophic factors (cerebrolysin), rosiglitazone, or combination therapies (Cacabelos et al. 2012; Cacabelos 2009). The presence or absence of *APOE* $\epsilon 4$ allele modifies treatment response to a wide variety of therapeutics therefore its role is very important and should be carefully considered when clinical studies are being conducted for future drug optimization.

AD is a complex disorder involving many different gene clusters. However several studies have shown that the *APOE*

gene seems to be a major risk factor for both degenerative and vascular dementia. *APOE*-4/4 carriers show a faster disease progression and a poorer therapeutic response to all available treatments than any other polymorphic variant. Studies analyzing the therapeutic response of AD patients with both the *APOE* and *CYP2D6* genes showed that it is genotype specific, with *CYP2D6*-PMs, *CYP2D6*-UMs, and *APOE*-4/4 carriers categorized as the worst responders. This indicates that *APOE* and *CYP2D6* may influence each other, as pleiotropic genes, in the metabolism of drugs and hepatic function. It has been observed that liver metabolism and transaminase activity were affected differently depending on whether patients were *APOE*-4/4 carriers or not. Therefore *APOE* may influence liver function and drug metabolism by modifying hepatic steatosis and transaminase activity (Cacabelos 2008, 2009).

55.5 Role of Acetylcholinesterase Inhibitors

Acetylcholinesterase (AChE) is an enzyme found mainly in neuromuscular junctions and cholinergic brain synapses that is involved in hydrolyzing the neurotransmitter acetylcholine into acetate and choline, thereby terminating neurotransmission. In humans, AChE and butyrylcholinesterase (BChE) are the two major forms of cholinesterase enzymes. Donepezil, galantamine, and rivastigmine are AChE inhibitors that prevent hydrolysis of acetylcholine, and are commonly used for the treatment of AD (Noetzli and Eap 2013). Donepezil is a potent non-competitive and reversible AChE inhibitor and functions by increasing the acetylcholine levels that are available for synaptic transmission in the central nervous system. Donepezil is the most widely used drug for mild to moderate to severe AD treatment. When donepezil is administered orally, over 90 % undergoes first-pass metabolism by the *CYP2D6* enzyme leading to one active metabolite, 6-O-desmethyldonepezil and several inactive metabolites. The active metabolite of donepezil 6-O-desmethyldonepezil has a half-life of about 70 h, easily crosses the blood brain barrier and is slowly excreted from the body (Noetzli and Eap 2013; Benjamin and Burns 2007; Seripa et al. 2011; Cummings et al. 2013). Studies analyzing an effect of *CYP2D6* gene variants on treatment response show that it is genotype specific, and patients with *CYP2D6*-PMs and *CYP2D6*-UMs were categorized as the worst responders. The pharmacokinetic profiles of these patients showed significantly decreased drug clearance in PMs and increased clearance in UM when compared with EMs (Noetzli and Eap 2013; Benjamin and Burns 2007; Seripa et al. 2011; Varsaldi et al. 2006; Pilotto et al. 2009). A study investigating the effect of *CYP2D6**10 and *APOE* polymorphisms on both steady state plasma concentrations and clinical response of donepezil in patients with mild-to-moderate AD found that patients with mutant allele (*10)

in *CYP2D6* gene may respond better to donepezil than those with wild allele (*1) (Zhong et al. 2013). However they did not find any relationship between *APOE* ϵ 4 status and the efficacy of donepezil. Recently, studies analyzing the influence of *CYP2D6* and *APOE* polymorphisms on the therapeutic effect of donepezil in patients with mild-to-moderate AD showed no significant differences between responders and nonresponders (Klimkowicz-Mrowiec et al. 2013; Liu et al. 2014). Other studies looking at the *APOE* gene variants showed that *APOE* ϵ 4 carriers displayed worst treatment response (Cacabelos et al. 2012), whereas the *CYP3A* genetic variants showed no influence on plasma concentration and treatment outcome (Noetzli and Eap 2013). These results encourage a continued evaluation of the role of various polymorphisms in response to donepezil treatment in different ethnic groups, and to assess other genetic variants of *CYP2D6*.

Galantamine is a competitive, rapidly-reversible AChE inhibitor that functions by increasing the acetylcholine levels at cholinergic synapses. It also interacts with the nicotinic acetylcholine receptor at binding sites separate from those for AChE, and may increase the levels of glutamate and serotonin. Galantamine is metabolized primarily by O-demethylation by *CYP2D6*, then O-oxidation by *CYP3A4* and glucuronidation. Studies investigating the clinical effectiveness and tolerability of galantamine with respect to plasma concentrations and *CYP2D6* polymorphisms showed that *CYP2D6* PMs displayed 45 % and 61 % higher dose-adjusted plasma concentrations than *CYP2D6* hetEMs and homEMs, respectively (Noetzli et al. 2013). However, other studies have failed to find any significant influence of the *CYP2D6* or *APOE* genotypes on galantamine treatment response (Clarke et al. 2011) (Noetzli and Eap 2013).

Rivastigmine is a non-competitive and very slowly reversible AChE inhibitor. The metabolism of rivastigmine is primarily by esterases in both the liver and the intestinal tract to an inactive and non-toxic metabolite NAP226-90. It is the only AChEI that inhibits both BuChE and AChE. This is an important aspect for selectivity since the activity of AChE is higher in the CNS, and the BChE activity is higher in the periphery; additional inhibition of BChE might be beneficial (Noetzli and Eap 2013). It has a bioavailability of 40 % and a half-life of about 2 h. In order to investigate the clinical efficacy and safety of rivastigmine for patients with AD, a database of double-blinded, randomized, controlled trials were analyzed and results point that rivastigmine improved the rate of decline of cognitive function, activities of daily living, and severity of dementia for people with mild to moderate AD in comparison with placebo (Birks and Grimley Evans 2015).

55.6 Personalized Medicine in Neurodegenerative Diseases

55.6.1 Potential Therapeutic Benefits

Personalized medicine in pharmacogenomics focuses on the ability to predict how a patient would respond when a drug is administered. In recent years, the US Food and Drug Administration (FDA) has aggressively pursued drug-label modification when studies have shown that excess risk can be convincingly linked to a genetic marker. Several examples are listed in the FDA's Table of Pharmacogenomic Biomarkers in Drug Labels <http://www.fda.gov/Drugs/ScienceResearch/ResearchAreas/Pharmacogenetics/ucm083378.htm>. The US FDA granted market approval for the first pharmacogenetic test known as The AmpliChip *CYP450* test that uses DNA microarray technology to identify genotypes in cytochrome P450 *CYP2D6* and *CYP2C19*. The test uses software to predict phenotypes, and tests for 27 *CYP2D6* alleles, including the deletions and duplications, and three *CYP2C19* alleles. The AmpliChip *CYP450* test classifies individuals into the EM and PM *CYP2C19* phenotypes by testing three alleles, and into four *CYP2D6* phenotypes namely UMs, EMs, IMs, and PMs (de Leon et al. 2006a, b). Most of the treatments so far have been symptomatic but with the understanding of genetic components to the disease, personalized therapeutics based on individual genomic profile will improve treatment and reduce adverse side effects. Examples of potential therapeutic benefits are shown in a study with pharmacogenomic testing whereby a patient who was highly sensitive to medications showed slow action of the Cytochrome P450 enzyme encoded by the *CYP2C19* gene, meaning the patient was a PM of most antidepressants. With these results, doctors were able to improve the therapeutic outcome by reducing her medication dose of venlafaxine (25 mg BID) and giving her a low-dose short-acting atypical antipsychotic (quetiapine IR, 25 mg/day). Another patient's pharmacogenomic profile showed increased function of enzymes encoded by *CYP2D6* and *CYP2C19* genes indicating an UM phenotype that called for adjusting treatment with escitalopram at 30 mg/day, higher than the recommended 20 mg/day (Dinama et al. 2014). As more genome wide association studies are performed, scientists will be able to assign specific genomic patterns to EM, IM, PM or UM leading to a more studies geared toward optimized drug therapy. This is very important for the future of personalized medicine as targeted therapies are designed for patients with specific gene variations (Pacanowski et al. 2014).

55.6.2 Current Clinical Trials of Neurodegenerative Diseases

In order to understand the preclinical and clinical course of AD, a multinational collaborative project also known as The Dominantly Inherited Alzheimer Network (DIAN) was initiated where cohorts who are at risk for autosomal dominant Alzheimer disease (ADAD) with mutations in either of the *APP*, *PSEN1* or *PSEN2* genes are studied. The outcome of the study concluded there was a difference in overall pattern among younger DIAN cohorts compared to late-onset AD population (Storandt et al. 2014). A study comparing changes in brain imaging and fluid biomarkers for *PSEN1* E280A mutation carriers versus non-carriers showed that mutation carriers had lower precuneus cerebral metabolic rates for glucose, smaller hippocampal volume, lower cerebrospinal fluid (CSF) A β 1-42, higher CSF total tau and phosphorylated tau, and significantly higher plasma A β 1-42 that was not affected by age (Fleisher et al. 2015). Other clinical trials investigated Bapineuzumab, a humanized anti-amyloid-beta monoclonal antibody against A β (1–6) to treat mild-to-moderate AD patients categorized as *APOE* ϵ 4 allele carriers and non-carriers. Results showed reduction in brain amyloid plaques and phosphorylated tau in CSF but no improvement in cognitive or functional outcome between the *APOE* ϵ 4 allele carriers compared to non-carriers was observed (Folch et al. 2016). These results are in agreement to the view that AD pathology begins before symptoms of dementia develop and largely influenced by amyloid plaques. However, more work needs to be done to incorporate the genetic aspect of neurodegenerative diseases.

55.6.3 Challenges to Overcome

Several challenges still need to be addressed for personalized medicine to become more effective. One is the use of combination therapy, which makes it difficult to study a large enough sample of patients treated with a single agent. In addition, the dosage of the drug may vary by regimen, further complicating efforts to study the pharmacogenomics of a specific drug of interest. Furthermore, replication of findings made in genome wide association studies from large randomized clinical trial is often difficult, because high costs and ethical considerations may mean that a second identical trial is not feasible. Other challenges to overcome involve environmental factors, the interactions between different genetic loci and between different genes. When data from multiple studies are combined, there is increased potential for confounding variables that may affect how results are interpreted. Influence of sex, race or inadequate sample size may lead to the lack of inclusion of a causal genetic variation leading to an erroneous interpretation.

55.7 Review Questions

1. What is pharmacogenomics?
2. Identify the four CYP2D6 phenotypes.
3. Explain the role of acetylcholine in the AD pathology.
4. What is the goal of personalized medicine?

55.8 Answers

1. Pharmacogenomics is the study of how specific genetic variations influence the therapeutic outcome.
2. Normal activity (extensive metabolizers, EM); low or no activity (poor metabolizers, PM); intermediate activity (intermediate metabolizers, IM); and high enzymatic activity (ultra-rapid metabolizers, UM).
3. Neuronal death in the brain at key locations leads to the deficiency of the neurotransmitter, acetylcholine thus causing memory impairment, hence leads to AD.
4. To combine clinical observations with information gotten from GWAS and candidate gene studies to predict how an individual will respond to a particular drug so that the problem of disease etiology is approached based on individual genomic profile. With this, clinicians can optimize drug dosage at beginning of treatment, find effective ways to diagnose, treat and prevent complex disorders with personalized therapeutics.

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Abstract

Immunology, neuroscience and pharmacology are each independent broad multidisciplinary fields that are well developed for the biomedical sciences. The evolution of each at the molecular, biochemical and cellular levels have been, by any measure, enormous in impact by its collective abilities to change the world as we know for biomedical research. This book was fashioned to share with students and trained professionals a vision and integration for the discipline and to reach forward into its future.

Keywords

Adaptive immunity • Alzheimer's disease • Astrogliosis • HIV-associated neurocognitive disorders • Microglia • Neuroimmune pharmacology • Neuropharmacology • Parkinson's disease

Notably, like other disciplines of science, the fields of immunology, pharmacology and neuroscience intersect. How this has come about resides in history. Simply said, it did not begin in modern times but evolved over a century ago when Dr. Elie Metchnikoff discovered that an inflammatory cell type in starfish larvae could engulf foreign materials and as a consequence protect its host, birthing the field of immunology. It is from this field that some of the most significant medical advances have been realized. Indeed, immunology has become the most active and arguably the most prolific field of medicine. At its epicenter is the mononuclear phagocyte (MP; monocyte, tissue macrophage, dendritic cell and microglia) with its nonspecific ability to protect itself against pathogenic microbial and other injurious attacks. While the 1908 Nobel Prize in Medicine and Physiology was awarded

to Dr. Metchnikoff, even he was uncertain of the exceedingly broad implications of his discovery in appreciating how these cells could affect the well-being of the human race. This realization came only decades later through a plethora of new developments in immune-based diagnostics and therapeutics. For example, MP were subsequently found to induce intracellular killing of pathogenic microbes, process antigens for presentation, secrete biologically active factors and under certain circumstances, become instigators in pathological processes. It was considerably later when scientist discovered that MP phagocytic functions were central to normal tissue homeostasis (Gardai et al. 2005; Fadok et al. 1998). So how is MP function linked to disease? The obvious answer is through inflammation. On the one hand, inflammation is essential for life as it serves as the frontline defense against infection and tumor surveillance. Inflammation, for simplicity's sake, enriches the host's abilities to fend off disease-causing bacteria, viruses and parasites. It comprises the first-line of immune defense to eliminate invaders and plays a critical role in the repair of damaged tissue. On the other hand, when these highly regulated processes go awry, inflammation evolves into pathological processes that are disadvantageous to the host as is seen in cancer, infectious, cardiovascular diseases, autoimmunity and neurodegenerative disorders.

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Neuroscience is defined broadly as the investigation of networks and models of neural communication in health and disease amongst hundreds of billions of neurons and supporting glia (microglia, oligodendrocytes and astrocytes). The intersection between the immune system and the nervous system is now considered significant. Indeed, within the central nervous system (CNS) immune activities abound. During disease, neuroinflammation perpetrated through the brain's MP has been linked to widespread neurodegeneration in adult diseases that include Parkinson's, Alzheimer's and Huntington's diseases (PD, AD, HD), HIV-1-associated neurocognitive disorders (HAND), stroke, multiple sclerosis and spongiform encephalopathies (prion-mediated neurodegeneration). We now understand that CNS injuries ranging from acute traumatic brain injury to the chronic neurodegenerative disease leads to the recruitment and activation of the adaptive immune system (Benner et al. 2008; Appel et al. 2010). The polarization of this adaptive immune response appears to have a significant impact on local CNS MP or microglial secretory functions with the capacity for both deleterious or beneficial outcomes. A range of microglial-derived products have been linked to neuronal injury including quinolinic acid, superoxide anions, matrix metalloproteinases, nitric oxide, arachidonic acid and its metabolites, chemokines, pro-inflammatory cytokines and excitotoxins. Activated microglia commonly speed disease progression while anti-inflammatory therapies appear to slow or halt it.

The ontogeny of the brain's immune system is still debated (Chan et al. 2007). However, recent lineage tracing experiments reveal that microglia begin as yolk sac-derived *Csflr*⁺ erythro-myeloid progenitor cells which enter the brain during early embryogenesis and differentiate into a mature microglial pool (Gomez Perdiguero et al. 2015). Microglia serve as the resident CNS phagocytes capable of reacting promptly to brain insults that range from pathogens to aggregated proteins. For the most part, resident microglia are equipt to detect and rapidly remove infected or damaged cells and thus are critical to brain's surveillance system. Both microglia and astrocytes were once thought of as simple support cells for neurons. However, new data suggest that these cells have a distinct and direct functions in shaping neuronal synapse formation suggesting a far more important roles in neural function (Lui et al. 2016; Singh et al. 2016). The newer discovery, in regards to microglial function show that they have evolved into cells with distinct identities as compared to peripheral myeloid cells and as such open new areas of studies of cell biology.

On balance, these glial cells serve neuroprotective functions. Astrocytes serve as a primary supporter of neuronal and microglial activities made possible by the cell's abilities to produce neurotrophins, eliminate excitotoxins, and control tissue functions. However, it is the microglia that is primarily responsible for the removal of debris and clearance of

microbes that have gained access to neural microenvironments. In particular and under a broad range of diseased states and infectious processes microglia serve with the astrocyte to promote neuronal survival. The activation of either cell type leads to profound morphological and functional changes. Activated microglia increase their numbers at the affected site and exhibit a "spider-like" ramified appearance with substantially enlarged cell bodies. Such cell signatures appear at the lesion close to injured or degenerating neurons. While such changes are clearly implicated in neurodegenerative processes the cells serve the nervous system through their abilities to affect tissue and cell function, molecular trafficking, regeneration and elimination of lipids and scavenging apoptotic cells. Such functions are coincident with innate cellular proliferation and surface expression of "activated" markers.

We can no longer propagate the perception that the CNS is an "immune privileged" site. Recent disclosures support the presence of an anatomical lymphatic system within the CNS (Louveau et al. 2015). Such a unique system serves to traffic cells and macromolecules into and out of the CNS. Brain-derived antigens drain to the deep cervical lymph nodes following injury (Benner et al. 2008) by way of this system, and CNS-derived dendritic cells function in perivascular regions that may serve to maintain immune tolerance through regulatory T cells (Treg). While naïve T cells are precluded from CNS entry, neuroinflammation aggressively recruits activated T cells to sites of active neurodegeneration (Karman et al. 2004). While the pathogenic role of T cell autoimmunity has been extensively studied, more recent studies have suggested a potential beneficial role in some settings. Nonetheless, a far more complex relationship between neurons and immunity exists than previously thought. For example, numerous interleukins and chemokines are readily expressed in neurons. Signaling between neurons, astrocytes and microglia during neural injury incite inflammatory responses and leukocyte migration. Yet another example, the major histocompatibility complex (MHC) class I and CD3 ζ molecules have a role in axonal guidance, activity-dependent remodeling, and plasticity in the developing nervous system (Huh et al. 2000). Within the neuronal synapse, MHC class I molecules may participate in synaptic connections. The cognate receptor for MHC class I peptide complexes is the $\alpha\beta$ T cell receptor (TCR) expressed on T cells. During neuroinflammatory responses MHC class I molecules are up-regulated on neurons without evidence of cytotoxic T lymphocyte (CTL) neural cell damage (Syken and Shatz 2003; Nishiyori et al. 2004). Altered peptide profiles at the neuronal synapse may affect neuronal plasticity and brain remodeling. Additionally, a myriad of animal model systems of human disease have shown that deficiencies in adaptive immunity lead to neuronal loss which are reversed following immune reconstitution. The relationship between T cell

responses following neural injury are now well known. However, the specific identity of T cell subpopulations and their divergent roles in neuroprotection and neurodegeneration are hotly debated.

How neurons are injured or die remains an important determinant in the ensuing microglial responses. This is of paramount importance as phagocytes clear cells undergoing programmed cell death and must do so in a manner that does not harm neighboring cells (Sastry and Rao 2000). The rapid clearance of such cells prevents the release of toxic or immunostimulatory molecules from dead neurons and is thought to play an important role in maintaining homeostatic functions (Magnus et al. 2001; Chan et al. 2001). Apoptosis leads to morphological and biochemical alterations at the cell surface making them suitable targets for resident phagocytes. Such changes include the reversal of membrane polarity, yielding a net anionic charge, and the surface expression of modified lipid (phosphatidylserine) and carbohydrate motifs. Apoptosis proceeds without an inflammatory response and phagocytosis of apoptotic cells inhibits innate immune activation (Savill et al. 2002). Actually this process leads to anti-inflammatory cytokine production that includes transforming growth factor beta (TGF- β) and interleukin-10 (IL-10). Alternatively, necrotic cell death alerts the immune system to danger through danger-associated molecular patterns (DAMP) and elicits significant inflammatory response (Krysko et al. 2011). Antigens from necrotic or stressed cells may trigger a class of pattern-recognition receptors (PRRs) designed to recognize evolutionarily distant microorganisms and includes Toll-like receptors (TLRs). While minimal evidence exists for any infection etiology parallel in neurodegenerative diseases, inflammation is nonetheless linked to neuronal loss. One example is the infusion of lipopolysaccharide (LPS), a pathogen-associated molecular pattern, into the brain, the eliciting of an inflammatory response and its association with neuronal loss. LPS signals through TLR4 and CD14 and results in a robust activation of microglial cells (Elward and Gasque 2003). Such immunostimulatory ligands include heat-shock proteins (HSP) (Asea et al. 2002), high mobility group 1 protein (HMG1) (Park et al. 2004), DNA (Collins et al. 2004), hyaluronan (Termeer et al. 2002), uric acid (Shi et al. 2003), and fibronectin (Okamura et al. 2001). Many of these target ligands are upregulated in stressed cells or liberated from the extracellular matrix during tissue damage. As such, they make excellent candidates for molecular triggers of immune activation following tissue injury and may therefore contribute to neurodegenerative process.

Interestingly, extracellular mitochondria, released during cell death, may also act as DAMPs to the innate immune system. Mitochondria likely evolved from a prokaryotic ancestor and have retained many phylogenetic characteristics including double membrane structures, primitive transcription and translation machinery, and stretches of unmethylated CpG

DNA motifs associated with microbial genomes. Both mitochondrial DNA (mtDNA) and DNA modified by oxidation (8-oxodG), strongly activate cells of the MP lineage (Collins et al. 2004). This has important implications for neurodegenerative disease where mitochondrial pathology and oxidative stress is associated with neuronal loss.

Aggregated proteins are also indicators of cellular stress and are potent activators of innate immunity. Most neurodegenerative diseases are associated with the accumulation of abnormal protein aggregates (proteopathies). Aggregated proteins can exert toxicity by disrupting intracellular transport, overwhelming the protein degradation machinery, or by sequestering normal proteins away from their physiological roles. In addition to direct toxicity, mounting evidence suggests that abnormal protein conformations can activate innate immune cells through scavenger receptors thus providing a mechanism for secondary tissue injury (Fischer et al. 2002; Yan et al. 1999; Bradt et al. 1998; Reynolds et al. 2008, 2009). Additionally, treatment of neurons with proteasome inhibitors and subsequent accumulation of ubiquitinated proteins elicits prostaglandin synthesis suggesting that intracellular accumulation of abnormal proteins can trigger the production of proinflammatory mediators (Rockwell et al. 2000). A major pathological hallmark of PD is the presence of eosinophilic, cytoplasmic inclusions of fibrillar, misfolded proteins called Lewy bodies (LB) suggesting derangements in proteostasis may be involved in disease pathogenesis (Dauer and Przedborski 2003). While LBs are intracellular, the fate of associated protein aggregates following cell death is unknown and can be released following cell death. Moreover, neurons go to great lengths during times of cellular stress to prevent such aggregation by upregulating ATP-dependent chaperones such as HSPs (Muchowski 2002). In addition to aggregated proteins, HSPs can be released during cell death and doing so may directly contribute to microglial activation (Kakimura et al. 2002).

Reactive oxygen species (ROS) include superoxide, hydrogen peroxide and hydroxyl free radicals as well as nitrogen intermediates (nitric oxide and peroxynitrite). These cause damage to neurons if produced in excess such as during prolonged neuroinflammatory responses. Much of the microglial-derived ROS such as superoxide cannot efficiently transverse cellular membranes, making it unlikely that these highly reactive molecules gain intracellular access to neurons and trigger intraneuronal toxic events (Ischiropoulos and Beckman 2003). However, superoxide can rapidly react with NO in the extracellular space to form a more stable oxidant, peroxynitrite (Wu et al. 2003). The stability of peroxynitrite facilitates its crossing of cell membranes where it can damage intracellular components in neighboring neurons. Its toxicity has been linked to the disruption of mitochondrial electron transport chain, lipid peroxidation, DNA damage, and the nitration of tyrosine

residues in cellular proteins. NO plays an important role in innate immunity and is associated with tumoricidal and bactericidal activities of macrophages. In addition to the physiological roles of NO, under pathological settings with excessive production it can act as a potent neurotoxin in a number of neurodegenerative models.

On a more general level, matrix metalloproteinases (MMPs) are a class of extracellular soluble or membrane bound cysteine proteases involved in remodeling of the extracellular matrix (ECM). MMPs have also been implicated in a range of neurodegenerative disease including, HAD, AD, PD and stroke. Gu et al. reported that S-nitrosylation of N-terminal cysteine residues within proMMP-9 leads to the subsequent activation of MMP-9 protease activity (Gu et al. 2002). These findings identify a potential extracellular proteolysis mechanism involved in neuronal cell death in which S-nitrosylation activates MMPs. An increase in MMP-9 expression has been determined in the MPTP model and pharmacological inhibition of MMP-9 was neuroprotective (Lorenzl et al. 2004).

The complex role of cellular and molecular inflammatory mediators, particularly within the CNS, makes it difficult to define their specific beneficial or deleterious roles in pathophysiological states (Gendelman 2002). Cytokines and chemokines are of particular importance in the initiation, maintenance, and resolution of inflammatory reactions within the CNS (Allan and Rothwell 2001). Moreover, cytokines and chemokines in particular, have important roles outside of inflammation including chemotactic axonal guidance during developmental stages of the CNS (Mennicken et al. 1999; Tran and Miller 2003a). With regards to the immune system, cytokines and chemokines act as potent survival cues and their tissue levels directly modulate the activity of inflammatory cell types. Therefore, the function of these molecules within the immune system is reminiscent of the function of neurotrophins within the nervous system (Tran and Miller 2003b). In fact, many neurotrophins are structurally related to molecules within cytokine families and these two classes of molecules appear to share functional overlap. IL-10 is a potent neuroprotective factor on purified cerebellar neurons and AD (Bachis et al. 2001; Kiyota et al. 2012) and NGF has emerged as a potent anti-inflammatory factor with beneficial effects in the mouse model of MS (Flugel et al. 2001). Indeed, neurons themselves can produce chemokines and express many of their receptors on their surface suggesting direct dialog between these two systems. Neuronal-immune communication is not restricted to soluble mediators. The CD200 receptor (CD200R) is expressed on myeloid cells including microglia and its ligand, CD200, is abundant on neuronal cell surfaces. Cognate CD200-CD200R interactions have potent inhibitory effects on cells of the myeloid lineage (Barclay et al. 2002; Hoek et al. 2000; Varnum et al. 2015). Cells of the adaptive immune system can also communicate with neurons. Activated T cells

express numerous neurotrophins in addition to the neurotrophic factor receptors, TrkB and TrkC, thus providing sufficient mechanistic means to establish direct T cell-neuron communications.

Functional cellular immune responses can provide conflicting studies. Virally or otherwise activated macrophages can have devastating consequences on neuronal function and survival. Depletion of peripheral monocyte/macrophage lineages in a spinal cord injury model can improve functional recovery (Popovich et al. 1999). Conversely, direct spinal injection of macrophages activated with damaged peripheral nerves *ex vivo* leads to enhanced functional recovery (Rapalino et al. 1998). Resident microglia, while contributing to noxious factors in CNS disease detailed above, also produce a plethora of neurotrophic factors upon activation. Local production of these factors can establish trophic gradients which stimulate and guide axonal sprouting following striatal injury (Batchelor et al. 1999, 2002). Thus, fine-tuning of cellular immune responses is critical for maximizing neuroprotection by immune cells.

The presence of T cells in CNS pathological settings has long been regarded as detrimental. One such case where T cells play a direct role in disease pathogenesis is multiple sclerosis (MS). MS is an inflammatory disease characterized by perivascular cuffs of mononuclear cells that include both lymphocyte and macrophage populations. Accumulation of these cells leads to significant damage to the myelin sheath and can involve the underlying axon (Kornek et al. 2000). However, provocative experimental data also implicate a role for adaptive immunity in otherwise non-autoimmune disorders of the CNS. Surprisingly, SCID and Rag2^{-/-} mice, both deficient in adaptive immunity, show enhanced motor neuron loss within the facial nucleus following transection of the facial nerve. Regulatory T cells such as CD4⁺CD25⁺, which comprise 10 % of the total CD4⁺ population, play a vital role in inhibiting autoreactive T cell activation and expansion and may be of paramount importance in maintaining peripheral tolerance against CNS antigens. Depletion of naturally occurring regulatory CD4⁺CD25⁺ T cells predisposes animals to development of organ-specific autoimmune diseases (Shevach 2000). Depletion of CD4⁺CD25⁺ regulatory T cells enhances T cell-dependent protective response and hence improves post-injury neuronal survival following disease (Kipnis et al. 2002). Alternatively, immunodeficient mice have improved dopaminergic neuronal survival (Benner et al. 2008) and Treg cells confers protection animal models of Parkinson's disease (Reynolds et al. 2010). Moreover, a recent study demonstrates that the influence of gut microbiomes on ischemic stroke outcomes is mediated through Treg regulation of $\gamma\delta$ T cells responses to injury (Benakis et al. 2016). In this study, Treg populations in the gut resulted in decreased $\gamma\delta$ T cell-derived IL-17 in the brain following stroke leading to improved outcomes. Thus, CD4⁺CD25⁺ regulatory T cells, at least for now are both agonistic and antagonistic and require further investigation.

How can we harness these complex immune responses within the nervous system for therapeutic gain? This has given rise to the development of neuro- and immuno-pharmacology. In the context of the immune system the drug acts on the biological system that augments cellular responses against disease or in maintaining host homeostasis. Such cellular responses can be utilized for therapeutic gain. The ensuing events that occur within the nervous system ultimately is the field of neuroimmune pharmacology. For example, an unexpected role of T cells in neuronal function is immune-mediated neuroregeneration, differentiation or protection. Surprisingly, for example, proinflammatory T_H1 cell responses generated through immunization with myelin-associated antigens can attenuate secondary neuronal loss following experimentally induced CNS injury (Weiner and Selkoe 2002; Schwartz 2001). It appears that such strategies can increase local production of proinflammatory cytokines as well as multiple neurotrophic factors within the injured tissue (Hammarberg et al. 2000; Moalem et al. 1999). These provocative observations have generated novel questions regarding neuro-immune interactions and it has been suggested that the observed neuroprotection can be directly linked to the proinflammatory nature of the infiltrating T cell population (Schwartz et al. 1999). However, detailed mechanistic data is lacking and the true mechanisms will likely prove as complicated as many other inflammatory mediators described above. Contrary to this, oral or nasal immunization that preferentially generates an anti-inflammatory T cell response (T_H2/T_H3) also has a demonstrated neuroprotective element and may prove more clinically feasible (Frenkel et al. 2003; Monson et al. 2003).

Neurodegenerative disorders are linked to innate immune activation comprised of an astonishing array of cellular and molecular players. There is little evidence for an infectious process in most neurodegenerative diseases however; multiple endogenous immune activators associated with cellular stress signals likely converge on resident glia leading to an inflammatory phenotype. The intended purpose of this response is beneficial to the host. However, when regulatory controls are breached and inflammation is allowed to persist, tissue damage ensues. The identification of individual activation pathways operative in disease and the subsequent response they elicit may prove beneficial in the development of novel therapeutics. In addition to innate immunity, the adaptive immune system may also play a role (beneficial or toxic) in neurodegeneration, even in the absence of overt autoimmune pathology. Moreover, the adaptive immune system may be manipulated through peripheral immunization and or immunotherapy in a way that modifies innate inflammatory mechanisms in the CNS. Strategies to maximize the benefit of protective innate and adaptive immunity against CNS disorders with emerging immunological, nanomedicinal, and molecular biological techniques is fundamental to these approaches. Careful pursuit of therapeutic strategies

with an emphasis on safety may lead to novel treatment means to combat a broad range of neuropsychiatric, developmental, infectious and degenerative disorders of the nervous system as highlighted in this 2nd edition of textbook. Here we humbly dedicate this new book for the field's future.

56.1 Review Questions

- Dr. Elie Metchnikoff's famous discovery that led to the birth of immunology was:
 - He discovered that an inflammatory cell type in starfish larvae could engulf foreign materials and as a consequence protect its host.
 - He discovered that cytotoxic T lymphocytes clear virus and cancerous cells from the body during disease.
 - He discovered that the gut is a central lymphoid organ and responsible for a plethora of immune responses in the host and during homeostasis and disease.
 - He discovered that macrophages and dendritic cells present antigens and mobilize adaptive immune responses.
 - All of the above.
- Neuroscience is defined as:
 - Cell to cell interactions and communication networks of neurons and glia.
 - The investigation of networks and models of neural communication in health and disease amongst hundreds of billions of neurons and supporting glia (microglia, oligodendrocytes and astrocytes).
 - The development of the nervous system.
 - The reaction of the brain and spinal cord to injury and infections.
 - All of the above
- The abilities to harness complex immune responses within the nervous system for therapeutic gain is defined by:
 - Neuroimmune Pharmacology
 - How a drug acts on a specific biological system and modulates cellular responses against disease
 - Finding the means to boost immune system to maintain host homeostasis.
 - Engaging cellular immune responses for therapeutic gain.
 - All of the above
- Astrocytes are NOT defined by:
 - Being the primary supporters of neuronal and microglial activities
 - The ability to produce neurotrophins, eliminate excitotoxins, and control tissue functions.
 - The removal of debris and clearance of microbes that have gained access to neural microenvironments.

- (d) The abilities to work in concert with microglia to promote neuronal survival.
 - (e) Activation leads to profound morphological and functional changes.
5. True or false.
While naïve T cells are precluded from the CNS entry, neuroinflammation recruits activated T cells to sites of active neurodegeneration.

56.2 Answers

1. A
2. E
3. E
4. C
5. True

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Glossary¹

1.1

Δ⁹THC Tetrahydrocannabinol is the major psychoactive ingredient in the Cannabis plant. **Δ⁹THC** is responsible for both the psychiatric and therapeutic effects obtained from marijuana. Its receptor, the cannabinoid receptor, is located mainly in the presynaptic gap. The areas of the brain most affected are the basal ganglia, cerebellum, cerebral cortex, and the hippocampus. The acute effects consist of degradation in short term memory, changes in sensory perception, reduced concentration, disturbances in motor abilities, hypothermia, increased blood pressure and heart rate, and reduced pain perception.

2D electrophoresis A method for separation and analysis of macromolecules and their fragments and is commonly used to analyze proteins.

6-Hydroxydopamine A neurotransmitter analogue that depletes noradrenergic stores in nerve endings and reduces dopamine levels in brain. Its mechanism of action is linked to cytolytic free-radical production.

1.2 A

ACAID Anterior chamber-associated immune deviation—a unique form of immune tolerance. ACAID is a selective, systemic immune deficiency where Antigen-specific delayed hypersensitivity and complement-fixing antibody responses are impaired by lymphocyte responses while other immune effectors are left preserved.

Ach Acetylcholine is the chemical responsible for transmitting impulses between neurons in the central and peripheral (parasympathetic and somatic) nervous systems. It is the chemical that allows nerve cells to communicate with one another.

Acetylcholinesterase The enzyme responsible for the destruction of acetylcholine at the synaptic cleft (region

between nerve cells) after its neuronal release during neurotransmission.

AIDS Acquired Immunodeficiency Syndrome (AIDS) is an acquired defect of cellular immunity associated with infection by the human immunodeficiency virus (**HIV**), a CD4- positive T-lymphocyte count under 200 cells/ μ L or less than 14 % of total lymphocytes, and increased susceptibility to opportunistic infections and malignant neoplasms. Clinical manifestations also include emaciation (wasting) and dementia. These elements reflect criteria for **AIDS** as defined by the **CDC** in 1993.

Action potential Is a spike of electrical discharge that is propagated along the cell membrane. It is essential for information transmission amongst and from nerve cells to tissues throughout the body.

Active transport Movement of materials across cell membranes and epithelial layers against an electrochemical gradient, requiring the expenditure of metabolic energy. The process involves the use of cellular energy and commonly ATP to actively pump substances into or out of the cell.

Active vaccination The process of injecting a protein (an antigen) into a host, usually together with an adjuvant (a molecule to enhance immunity) for the purpose of preventing disease. In response, the host immune system generates an immune response that includes specific cells and antibodies against the antigen. Although most commonly used to prevent microbial infections, active vaccination has been used to ameliorate disease.

Active zone Specialized region of the cortical cytoplasm of the presynaptic nerve terminal that faces the synaptic cleft. It is the place for synaptic vesicle docking and neurotransmitter release.

ADEM Acute disseminated encephalomyelitis is an immune mediated disease of brain. It is brief but significant and results in direct myelin damage. It usually occurs following a viral infection or vaccination (commonly for measles, mumps or rubella), but it may also appear spontaneously.

¹Definitions provided by contributing authors, National Library of Medicine and/or Wikipedia unless otherwise noted.

- Acute flaccid paralysis** Loss of voluntary motion, due to temporary or permanent dysfunction of lower motor neurons or associated nerve fibres, neuromuscular junction, or muscle fibres. It is the most common sign of acute polio but is also associated with West Nile Virus infection. In chronic cases of permanent flaccid paralysis muscle tone is decreased and muscle atrophy may occur.
- Adamantiades-Behçet's disease** A rare form of systemic vasculitis through immune-mediated small vessels usually accompanied by mucous membrane ulceration and ocular problems.
- Adaptive immune system** The adaptive immune system evolved in early vertebrates and allows for a specific and targeted response based upon immunological memory of antigens or related antigens previously encountered by the host innate immune systems. This requires the recognition of specific "non-self" antigens. The effectors of the adaptive immune response include T cells and B cells.
- Adenoviruses** These are a family of non-enveloped medium-sized (90–100 nm), icosahedral double-stranded DNA viruses that infect various mammalian species, including humans. These viruses commonly cause mild respiratory infections. Adenovirus-based vectors have been used for gene delivery into the nervous system, as high vector titers can be generated, and these episomally maintained vectors efficiently infect and express transgenes in a variety of cell types including the non-dividing cells.
- AAV** Adeno-associated virus is a non-pathogenic member of the parvovirus family that harbors a single-stranded DNA viral genome. Vectors derived from AAV can carry up to a 4.5-kb transgene and stably express a transgene in vivo months to several years.
- Adjunctive** Something joined or added to another thing but not essentially a part of it, used in this context to refer to additional therapies for HIV-related neurological conditions.
- Adjuvant** A formulation of substances that is used to enhance/potentiate immune response to an antigen. Complete adjuvants are water in oil emulsions composed of inactivated or killed mycobacteria-usually *Mycobacterium tuberculosis*. Whereas incomplete adjuvants are the same adjuvants, but without the mycobacterial components.
- ACTH** Adrenocorticotrophic hormone is a peptide hormone released by anterior pituitary cells in response to stressful stimuli that causes the synthesis and release of cortisol (corticosteroid) from the adrenal cortex. It is an important component of the hypothalamic-pituitary-adrenal (HPA) axis. ACTH is released from pro-opiomelanocortin and secreted from corticotropes in response to corticotropin-releasing hormone (CRH) released by the hypothalamus.
- (Adsorptive) endocytosis** Cellular uptake of extracellular materials within membrane-limited vacuoles or microvesicles. **ENDOSOMES** play a central role in endocytosis.
- (Adsorptive) transcytosis** A mechanism for transcellular transport in which a cell encloses extracellular material in an invagination of the cell membrane to form a vesicle, then moves the vesicle across the cell to eject the material through the opposite cell membrane by the reverse process.
- Adult stem cell** A cell found in adult somatic tissue that can differentiate into the cells of the particular tissue in which it resides and can self-renew while maintaining its differential capability.
- Age related macular degeneration** A gradual worsening of vision that eventually leads to no vision in the center of the visual field. It is caused by damage to the macular of the retina.
- Akt** Serine/threonine protein kinase that is a cellular homologue of the viral oncogene v-Akt. There are three genes in the Akt family: Akt1, Akt2, and Akt3. Akt1 is involved in cell survival pathways by inhibiting apoptosis. Akt2 is an important signaling molecule in the Insulin signaling pathway, and is required to induce insulin transport. The role of Akt3 is unknown, but it is predominately expressed in the brain.
- Alkylosing spondylitis** Literally "stiff vertebra" is an autoimmune inflammatory disease that primarily affects the intervertebral joints (joints of the spine) and the sacroiliac joint.
- Allele** The name given to a specific polymorphic variation.
- Allele association** Association with a specific allele but not necessarily a genotype.
- Allograft** Tissue transplanted from the same species but of a different genotype.
- Alpha adrenergic receptor** One of two general classes of G protein-coupled receptors that can be further sub-classified and responds to the neurotransmitter norepinephrine.
- α-MSH** Alpha melanocyte stimulating hormone is a tripeptide derived from proopiomelanocortin that stimulates production of melanin and acts on melanocortin-1 receptors found on immune cells.
- Alpha synuclein** An abundant presynaptic neuronal protein found in the brain of uncertain function, but thought to act as a molecular chaperone involved in vesicular transport. Mutation and/or oxidation of alpha-synuclein increase its propensity to aggregate and is a major component of Lewy bodies that plays a role in neurodegeneration and neuroprotection.
- AD** Alzheimer's disease is the most common degenerative brain disease of unknown cause resulting in progressive mental deterioration with disorientation, memory disturbance and confusion. AD leads to progressive dementia, often accompanied by dysphasia and/or dyspraxia.

The condition may also give rise ultimately to spastic weakness and paralysis of the limbs, epilepsy and other variable neurological signs. Alzheimer disease is marked histologically by the degeneration of brain neurons especially in the cerebral cortex and by the presence of neurofibrillary tangles and plaques comprised of highly insoluble β -amyloid peptides in the CNS.

Amacrine cell Lateral inhibitory interneuron in the inner retina located at the inner plexiform layer where bipolar cells and ganglion cells synapse. Amacrine cells deliver the majority of the ganglion cells input, and also regulate the output of the cone bipolar cells. These cells are thus responsible for the complex processing of the retinal image by adjusting brightness as well as detecting motion.

Amphetamine A central nervous stimulant that increases neuronal activity by releasing norepinephrine, dopamine and/or serotonin from nerve endings.

Amygdala An almond-shaped mass of gray matter, one in each hemisphere of the brain, associated with feelings of fear and aggression and important for visual learning and memory.

A β Amyloid-beta is a peptide of 39–43 amino acids that is the main constituent of amyloid plaques in the brains of Alzheimer's disease patients. Similar plaques appear in some variants of Lewy body dementia and in inclusion body myositis, a muscle disease. A β also forms aggregates coating cerebral blood vessels in cerebral amyloid angiopathy.

APP Amyloid precursor protein is a single transmembrane molecule, a causative gene of FAD, and is acted upon by proteases to form the amyloid- β peptide.

ALS **Amyotrophic lateral sclerosis** is a rare fatal progressive degenerative disease of unknown cause that affects motor neurons, usually begins in middle age, and is characterized by muscular weakness; called also Lou Gehrig's disease.

Amyloid An abnormal aggregation of proteins or protein fragments with a beta-pleated sheet secondary structure.

Animal models Normal animals modified mechanically, genetically or chemically to create laboratory paradigms with infrahuman species (usually rodents) which study behavior, neurochemistry, or physiology to demonstrate all or part of the characteristics of a disease.

Antibodies repertoire Antibody genes are pieced together from widely scattered bits of DNA. The possible combinations are nearly endless. As this gene forms, it assembles segments that will determine the variable-V, diversity-D, joining-J, and constant-C segments of this antibody molecule. For example, variable-region and joining-region rearrangements of the immunoglobulin light chain called V_LJ_L rearrangements.

Antibody A protein used by the immune system to neutralize pathogens.

Antibody titer The concentration of a specific antibody in blood.

Antidepressant drugs Pharmacological agents used for ameliorating symptoms of depressive disorders, including monoamine oxidase inhibitors, selective serotonin reuptake inhibitors, serotonin/norepinephrine reuptake inhibitors, and other agents.

Anti-ganglioside antibodies Circulating auto-antibodies directed against gangliosides. Anti-ganglioside antibodies are associated with axonal forms of GBS and can be induced experimentally in laboratory animals.

Antigen The molecule against which an immune response is raised.

Antigen presenting cells (APC) A cell that displays foreign antigens complexed with MHC on its surface. T-cells may recognize this complex using their T-cell receptor (TCR).

Anti-inflammatory cytokines A series of immunoregulatory molecules that suppress proinflammatory responses.

Antipsychotic drugs Pharmacological agents used to treat psychotic disorders, including schizophrenia, mania, and psychotic depression. These drugs are usually classified as "typical" or "atypical," depending on their affinity for the dopamine D₂ receptor, lack of extrapyramidal side effects, and action upon serotonin receptors.

Antiretroviral therapy Use of anti-HIV agents to treat disease by lowering viral titer and reducing viral replication, usually in combination of at least three drugs.

Antithymocyte globulin Purified gamma globulin from the serum of rabbits immunized with human thymocytes. It is used for induction of immunosuppression in the treatment of acute renal transplant rejection.

apoE Apolipoprotein E is a component of lipoprotein complex both in plasma and cerebrospinal fluid, responsible for cholesterol transport in the blood stream. One copy of the ApoE4 allele is an established risk factor of AD.

Apoptosis A genetically determined process resulting in cell self-destruction that is marked by the fragmentation of nuclear DNA, and is a normal physiological process eliminating DNA-damaged, superfluous, or unwanted cells (as immune cells targeted against the self in the development of self-tolerance). Apoptosis may occur prematurely in neurodegenerative conditions; also called programmed cell death.

Arbor Dendritic arbor, a single dendrite, the arbor density is called arborization.

Arboviruses (Arthropod-Borne virus) This type of virus is transmitted by arthropods.

Area postrema A structure in the brain that controls vomiting as well as other autonomic functions along with the central nervous system. It is located in the inferoposterior limit of the fourth ventricle.

Association Describes a relationship between a disease and a specific allele or haplotype of a polymorphism.

Astrocytes Major glial cells in the CNS (star-shaped cell as of the neuroglia). Astrocytes are characterized by histopathologically by glial fibrillary associated protein (GFAP). Astrocytes perform many functions including formation of the blood brain barrier, provide neurotrophic factors and metabolic support to the CNS, and have a role both in the repair and scarring process in the brain.

Atherosclerosis Characterized by the formation of plaques within the innermost layer of artery walls and narrowing of the vessel lumen. Vascular occlusion in the brain leads to stroke and in the coronary arteries leads to heart attack.

Atropine An alkaloid isolated from the belladonna plant that blocks the action of acetylcholine on muscarinic cholinergic receptors.

Autism spectrum disorders A section of neurodevelopmental disorders which include social deficits and communication problems.

Autoantibodies Immunoglobulins directed against self-antigens, representing an autoimmune process.

Autoimmune disease A condition in which a person's immune cells recognize self-produced molecules (self-antigens), leading to the inappropriate destruction of host tissue, e.g. rheumatoid arthritis, multiple sclerosis, and possibly Addison's disease.

Autoimmune T cells T cells that recognize self-antigens and are responsible for cell-mediated destruction of host tissue in an autoimmune disease.

Autoimmunity A condition in which an individual's immune system reacts against his or her own tissues, causing diseases such as lupus erythrocytes.

Autonomic nervous system The portion of the peripheral nervous system which acts autonomously through either of two divisions (parasympathetic and sympathetic) and regulates the action of smooth muscle, exocrine glands and the heart.

Axon The long extension of a neuron that carries nerve impulses away from the cell body.

Axonal damage Via inflammation not only the myelin sheath, but also the axon itself is destroyed.

Axon terminals The hair-like ends of the axon.

Axonal transport Bidirectional transport process, consisting of anterograde (transport of presynaptic molecules) and retrograde transport systems (neurotrophic signaling).

1.3 B

Bad A pro-apoptotic member of the Bcl-2 gene family involved in initiating apoptosis. BAD- Bcl-2-associated death promoter protein.

β -amyloid peptide (A β) A principal component of senile plaque, a pathological hallmark of AD, which is generated by β and γ -processing of APP.

Barbiturates A group of sedative-hypnotic agents that also have anti-anxiety effects. Barbiturates act on GABA-A receptors.

Basal ganglia Many subcortical nuclei at the base of the forebrain. They are involved in the control of motor movements, learning and routine behaviors.

Basiliximab Humanized monoclonal antibody directed against the IL-2 receptor on T cells. It is used for the prophylaxis of acute organ rejection in adult patients.

Bax A protein of the Bcl-2 gene family that promotes apoptosis by competing with Bcl-2.

B cell B lymphocytes; a white blood cell that specialize in the secretion of antibodies and cytokines.

Bcl2 The prototype for a family of mammalian proteins located in the membranes of the endoplasmic reticulum (ER), nuclear envelope, and in the outer membranes of the mitochondria. They can be either pro-apoptotic (Bax, BAD, Bak and Bok among others) or anti-apoptotic.

BCR B cell receptor is an antigen-specific receptor is a sample of the antibody it is prepared to manufacture; it recognizes antigen in its natural state.

Bcl11a C2H2 type zinc-finger protein. The corresponding mouse gene is a common site of retroviral integration in myeloid leukemia, and may function as a leukemia disease gene, in part, through its interaction with BCL6. During hematopoietic cell differentiation, this gene is down-regulated. It is possibly involved in lymphoma pathogenesis since translocations associated with B-cell malignancies also deregulate its expression.

BDNF Brain derived neurotrophic factor is a member of the nerve growth factor family of trophic factors. In the brain, BDNF has a trophic action on retinal, cholinergic, and dopaminergic neurons, and in the peripheral nervous system it acts on both motor and sensory neurons (From Kendrew, The Encyclopedia of Molecular Biology, 1994).

Belladonna alkaloid Any of several compounds that block the effect of acetylcholine on muscarinic cholinergic receptors.

Benzodiazepines A group of anti-anxiety agents that can also produce sedation, hypnosis and that have anti-convulsant effects. In the CNS, benzodiazepines act on GABA-A receptors. They also act on peripheral benzodiazepine receptors in the immune system.

Beta adrenergic receptor One of two general classes of G protein-coupled receptors that can be further sub-classified and responds to the neurotransmitter norepinephrine.

Beta-amyloid precursor protein converting enzyme (BACE) A single transmembrane aspartic proteinase, which processes APP at β -site; the β -secretase.

Beta-pleated sheet A molecular structure where proteins spontaneously fold into a layered shape, analogous to the accordion folds of a fan.

Bethanechol A derivative of acetylcholine with stimulant effects primarily on the smooth muscle of the gastrointestinal tract and the urinary bladder.

- Biogenic amine hypothesis** The hypothesis that major depressive disorder and bipolar disorder are functionally related to abnormal levels of catecholamines and/or serotonin.
- Bioinformatics** Field of science in which biology and computer science merge into a single discipline to create, manage and interpret massive sets of complex biological data.
- Biomarker** Distinctive biological indicator of biological state, process or condition.
- Bipolar cell** Retinal interneuron that transfers visual information from photoreceptors to ganglion cells.
- Bipolar disorder** A mental disorder characterized by cyclic episodes of mania or hypomania, usually accompanied by depressive episodes.
- BLIMP1** A protein that acts as a repressor of beta-interferon gene expression. The protein binds specifically to the PRDI (positive regulatory domain I element) of the β -IFN gene promoter. Transcription of this gene increases upon virus induction.
- Block copolymer** A polymer in which all of one type of monomer are grouped together and all of the other type are grouped together. A block copolymer can be thought of as two homopolymers joined together at the ends.
- Block ionomer complex** A complex between two oppositely charged block copolymers.
- Blood-brain barrier** Specialized non-fenestrated tightly-joined ENDOTHELIAL CELLS with TIGHT JUNCTIONS that form a transport barrier for certain substances between the cerebral capillaries and the BRAIN tissue.
- Botulinum toxin** A group of zinc proteases that can degrade one or more of the key proteins required for the process of exocytosis thus preventing the neuronal release of acetylcholine.
- Brain** The part of the central nervous system contained within the cranium, comprising the prosencephalon, mesencephalon, and rhombencephalon. It is derived from the anterior part of the embryonic neural tube.
- Brain endothelial cell** Highly specialized cells that form the blood-brain barrier.
- Butoxamine** An antagonist of norepinephrine action selective for the subtype two beta adrenergic receptor.
- to the cytoplasm in response to external stimuli. Elevation of intracellular Ca^{2+} activates a series of enzymes.
- CA1** Carbonic anhydrase 1; a gene containing an N-terminus active site, zinc binding site, and substrate-binding site.
- CAG repeats** Primary determinant of the onset of HD and other neurodegenerative disorders, including SBMA, DRPLA, SCA 1–3, 6, 7 and 17. CAG codes for the amino acid Glutamine, and extra repeats are thought to lead to a “toxic gain of function,” and increased propensity of the translated protein to form aggregates. Genetic testing of the HD CAG repeat is a useful tool for clinical diagnosis.
- Calcium excitability** Refers to the dynamics of calcium homeostasis.
- cAMP signaling** Signal transduction cascade initiated by the action of external stimuli on binding to a G-protein couple receptor (Gs) resulting in activation of adenylyl cyclase, production of cyclic AMP and activation of cyclic AMP-dependent protein kinase.
- Campylobacter jejuni* Gram-negative bacterium, which is one of the most frequent causes of bacterial gastroenteritis world-wide. *Campylobacter jejuni* gastroenteritis is the most frequently recognized preceding infection in GBS.
- Cannabinoids** Substances that bind to cannabinoid receptors.
- Case-control** Study describes an association study that compares allele frequencies using unrelated affected cases and matched controls.
- Caspase** Known as proteases, which play essential roles in apoptosis (cell death) and inflammation.
- CD1d** The sole group-2 member of the CD1 family of major histocompatibility (MHC)-like glycoproteins.
- CD3** Complex of delta, epsilon, gamma, zeta and eta chains of integral membrane glycoproteins that associates with T cell antigen receptor (TCR), and is required for TCR cell surface expression and signal transduction. CD3 is a universally expressed by T cells. Member of immunoglobulin superfamily.
- CD4** Nonpolymorphous glycoproteins belonging to immunoglobulin superfamily. Expressed on surface of T helper cells, accessory cells, macrophages and serves as co-receptor in MHC class II-restricted antigen induced T cell activation. Major receptor for HIV-1.
- CD5** Belongs to ancient scavenger receptor superfamily. CD5+ B cells, which may arise from B-1 cells (subset of B cells) produce “generalist antibodies”—polyreactive low affinity “natural” antibodies to exogenous antigens (tetanus toxoid, lipopolysaccharide) as well as autoreactive antibodies. CD5 may serve as a dual receptor, giving either stimulatory or inhibitory signals depending both on the cell type and the development stage. Key regulator of immune tolerance; abnormalities may produce autoimmunity.

1.4 C

- C5a** The 74-residue anaphylatoxin derived from C5 during complement activation that possesses inflammatory and immune stimulatory properties causing the accumulation of white blood cells at the site of complement activation.
- C5a agonist** A peptide mimetic of natural C5a that exhibits certain C5a-like activities.
- Ca^{2+} signaling** Signal transduction mechanism in which Ca^{2+} is recruited from extracellular or intracellular stores

- CD8** Cell surface glycoprotein, member of immunoglobulin superfamily. Heterodimer of an alpha and a beta chain linked by two disulfide bonds; heterodimer on thymocytes and homodimer on peripheral blood T cells MHC class I restricted receptor; binds to nonpolymorphic region of class I molecules; may increase avidity of interactions between cytotoxic T cell and target cell during antigen-specific activation. Can kill target cells by recognizing peptide-MHC complexes on them or by secreting cytokines capable of signaling through death receptors on target cell surface. CD8 alpha cells promote survival and differentiation of activated T cells into memory CD8+ T cells, which may become clonal (but not malignant) in the elderly.
- CD11b** Member of integrin receptor family; also called integrin alpha M, Mac-1. Mediates adhesion to substrates by opsonization with iC3b and subsequent phagocytosis, neutrophil aggregation, and chemotaxis. Ligands include fibrinogen, Factor X, ICAM1, iC3b, some bacteria. CD11b is a marker for both macrophages and microglia, and expression increases with cell-activation.
- CD11c** Member of b2 of integrin receptor family; also called integrin alpha X, CR4, LeuM5. Clears opsonized particles and immune complexes; also binds to fibrinogen and is involved in adhesion of monocytes and neutrophils to endothelium. Myeloid cell marker.
- CD19** Response regulator that plays a dominant role in establishing signaling thresholds for antigen receptors and other surface receptors on B cells; also regulates B cell development, activation and differentiation. Assembles with the antigen receptor of B lymphocytes in order to decrease the threshold for antigen receptor-dependent stimulation. Co-receptor with CD21. Earliest B cell antigen in fetal tissue. B cell marker.
- CD21** Complement component receptor-2 (CR2) that binds to C3d.
- CD25** IL-2 receptor alpha chain; exists in at least three forms. Limited expression may safeguard against catastrophic T-cell proliferation by immunogenic stimulus. Expressed, typically at high levels, on PHA-stimulated T cells, and is commonly used as a marker for activated T cells. Expressed on B cells stimulated with anti-IgM antibody. Expressed on monocytes/macrophages stimulated with LPS. Full signaling of IL-2 requires trimer with CD122 (IL-2R beta) and CD132 (IL-2R gamma) chains.
- CD27** Marker of T cell activation. CD27/CD70 interactions also regulate B cell proliferation and T cell differentiation. The protein is a member of the TNF-receptor superfamily. This receptor is required for generation and long-term maintenance of T cell immunity. It binds to ligand CD70, and plays a key role in regulating B-cell activation and immunoglobulin synthesis. This receptor transduces signals that lead to the activation of NF-kappaB and MAPK8/JNK. Adaptor proteins TRAF2 and TRAF5 have been shown to mediate the signaling process of this receptor. CD27-binding protein (SIVA), a pro-apoptotic protein, can bind to this receptor and is thought to play an important role in the apoptosis induced by this receptor.
- CD28** Cell adhesion molecule; co-receptor for B cell-T cell cooperation. Promotes T cell activation. Receptor for CD80 (B7.1) and CD86 (B7.2), found on activated B cells. Constitutive, high abundance, low affinity receptor; opposite signals are mediated by CTLA4 (CD152). Result of T cell antigen stimulation depends on sum of effects of T cell receptor, CD28 and its ligand, CTLA4 and its ligand. CD8+, CD28+ T cells mediate antigen specific cytotoxic T cells (class I restricted) (90% of CD8+ T cells). CD8+, CD28- T cells mediate suppressor T cells (10% of CD8+ T cells). CD28 costimulation is essential for CD4-positive T-cell proliferation, survival, interleukin-2 production, and T-helper type-2 (Th2) development.
- CD30** Tumor necrosis factor receptor superfamily, member 8, is a member of the TNF-receptor superfamily. This receptor is expressed by activated, but not by resting, T and B cells. TRAF2 and TRAF5 can interact with this receptor, and mediate the signal transduction that leads to the activation of NF-kappaB. This receptor is a positive regulator of apoptosis, and also has been shown to limit the proliferative potential of autoreactive CD8 effector T cells and protect the body against autoimmunity. Two alternatively spliced transcript variants of this gene encoding distinct isoforms have been reported.
- CD34** Cell-cell adhesion molecule and cell surface glycoprotein. May mediate attachment of stem cells to bone marrow extracellular matrix or directly to stromal cells. Constitutively expressed on endothelial cells.
- CD38** A multifunctional ectoenzyme widely expressed in cells and tissues especially in leukocytes and endothelial cells. CD38 also functions in cell adhesion, signal transduction and calcium signaling. Positive and negative regulator of cell activation and proliferation, depending on the cellular environment.
- CD40** Member of the TNF-receptor superfamily. This receptor has been found to be essential in mediating a broad variety of immune and inflammatory responses including T cell-dependent immunoglobulin class switching, memory B cell development, and germinal center formation. AT-hook transcription factor AKNA is reported to coordinately regulate the expression of this receptor and its ligand, which may be important for homotypic cell interactions. Adaptor protein TNFR2 interacts with this receptor and serves as a mediator of the signal transduction. The interaction of this receptor and its ligand is found to be necessary for amyloid-beta-induced microglial activation, and thus is thought to be an early event in Alzheimer disease pathogenesis. Two alternatively

spliced transcript variants of this gene encoding distinct isoforms have been reported.

CD44 Family of cell surface glycoproteins with isoforms generated by alternate splicing of mRNA. CD44 is important in epithelial cell adhesion (cell-cell and cell-matrix) to hyaluronate in basement membranes and in maintaining polar orientation of cells; also binds laminin, collagen, osteopontin, matrix metalloproteinases (MMPs) and fibronectin. Involved in leukocyte attachment and rolling on endothelial cells, homing to peripheral lymphoid organs and sites of inflammation and leukocyte aggregation, hematopoiesis, and tumor metastasis.

CD45 Leukocyte common antigen (LCA); a protein tyrosine phosphatase. Critical requirement for T and B cell antigen receptor-mediated activation. Expressed on all hematopoietic cells except erythrocytes and platelets at different levels. Some isoforms of CD45 are CD45RO, CD45RA, and CD45RB. Each CD45 isoform is distinguished from one another depending on the type of exon the CD45 has or the exons the CD45 does not have. The CD45RA isoform contains the A exon only and the CD45RB has the B exon only whereas the CD45RO has none of the exons: A, B, or C.

CD45RA CD45RA—expressed by naive/resting T cells, medullary thymocytes

CD45RO CD45RO—expressed by memory/activated T cells, cortical thymocytes

CD56 N-CAM (neuronal adhesion molecule). Regulates homophilic (like-like) interactions between neurons and between neurons and muscle. Also associates with fibroblast growth factor receptor and stimulates tyrosine kinase activity of receptor to induce neurite outgrowth. When neural crest cells stop making N-CAM and N-cadherin and start displaying integrin receptors, cells separate and migrate. Contributes to cell-cell or cell-matrix adhesion during development. Lymphocyte activated killer phenomenon mediated by IL-2 activated CD56+, CD3-, NK cells. Prototypic marker of NK cells, also present on subsets of CD4+ and CD8+ T cells.

CD62L L selectin; LECAM-1. Mediates lymphocyte homing to high endothelial venules of peripheral lymphoid tissue, leukocyte rolling on activated endothelium at inflammatory sites.

CD64 High affinity receptor binds to Fc region of IgG. CD64 is important in phagocytosis via receptor-mediated endocytosis of IgG-antigen complexes. Mediates antigen capture for presentation to T cells, antibody-dependent cellular cytotoxicity, release of cytokines and reactive oxygen intermediates.

CD68 A 110-kDa transmembrane glycoprotein that is highly expressed by human monocytes and tissue macrophages. It is a type I integral membrane protein with a heavily glycosylated extracellular domain. May have

a role in macrophage phagocytic activities. Specific to lysosomes, not cell lineage. Expressed by macrophage/monocytes, basophils, dendritic cells, mast cells, myeloid cells, CD34+ progenitor cells, neutrophils, osteoclasts, activated platelets, B and T cells.

CD117 (c-Kit) The tyrosine kinase type receptor. The c-Kit gene encodes a 145-kDa transmembrane protein that is a member of the receptor tyrosine kinase subclass III family that includes platelet-derived growth factor receptor (PDGF-R), macrophage colony-stimulating factor receptor (M-CSF-R or c-fms), and fms-like tyrosine kinase-3/fetal liver kinase-2 (Flt-3/Flt-2). The ligand for c-Kit, Steel factor (SF) or kit ligand, stem cell factor, and mast cell growth factor, exists both as a secreted and membrane-bound form where the latter appears to be most important for biologic activity in vivo. Important for development and survival of mast cells, hemopoietic stem cells, melanocytes, germ cells, and interstitial cells of Cajal.

CD122 IL-2 receptor beta chain.

CD123 Interleukin 3 receptor, alpha (low affinity). The receptor is comprised of a ligand specific alpha subunit and a signal transducing beta subunit shared by the receptors for interleukin 3 (IL-3), colony stimulating factor 2 (CSF2/GM-CSF), and interleukin 5 (IL-5). The binding of this protein to IL-3 depends on the beta subunit. The beta subunit is activated by the ligand binding, and is required for the biological activities of IL-3.

CD134 (OX40) Tumor necrosis factor receptor superfamily, member 4. This receptor has been shown to activate NF-kappaB through its interaction with adaptor proteins TRAF2 and TRAF5. Knockout studies in mice suggest that this receptor promotes the expression of apoptosis inhibitors BCL2 and BCL2L1/BCL2-XL, and thus suppresses apoptosis. The knockout studies also suggest the roles of this receptor in CD4+ T cell response, as well as in T cell-dependent B cell proliferation and differentiation.

CD154 CD40 ligand (TNF superfamily, member 5, hyper-IgM syndrome) is expressed on the surface of T cells. It regulates B cell function by engaging CD40 on the B cell surface. A defect in this gene results in an inability to undergo immunoglobulin class switch and is associated with hyper-IgM syndrome.

CD278 Inducible T-cell co-stimulator, belongs to the CD28 and CTLA-4 cell-surface receptor family. It forms homodimers and plays an important role in cell-cell signaling, immune responses, and regulation of cell proliferation.

Cell body The body of the neuron containing the nucleus (also called the soma or perikaryon).

Cell-mediated delivery Delivery of therapeutic agents to the site of action inside carrier cells.

Central memory t cells Memory T cells that express L-selectin and CC-chemokine receptor 7 (CCR7) and have the capacity to circulate from the blood to the

- secondary lymphoid organs. They have a non-polarized differentiation state in that they secrete IL-2 but not IFN- γ or IL-4; however, on restimulation, they rapidly differentiate into cytokine-producing effector cells.
- Central nervous system (CNS)** The main information-processing organs of the nervous system, consisting of the brain, spinal cord, and meninges that controls and coordinates most function of the body and mind.
- Centroblast** A proliferating germinal-centre B cell, which undergoes somatic hypermutation and immunoglobulin class switching.
- Centrocyte** The non-dividing progeny of a centroblast. These cells need to be selected on the basis of affinity for antigen, following interaction with immune complexes that are associated with follicular dendritic cells, and ability to elicit help from follicular B helper T (T_{FH}) cells.
- Ceramide** A sphingosine-based lipid molecule that regulates a wide spectrum of biological processes such as cellular differentiation, proliferation, apoptosis and senescence.
- Cerebellum** A part of the brain that specializes in motor control as well as attention, language, fear, and pleasure regulation.
- Cerebral amyloid angiopathy** The deposition of β -amyloid in brain blood vessels.
- CSF** Cerebrospinal fluids are fluid in the ventricles of the brain, between the arachnoid and pia mater, and surrounding the spinal cord that absorbs shocks and maintains uniform pressure.
- Chemoattractant-receptor homologous molecule expressed by t_H2 cells (crth2)** A cell-surface marker for human T helper 2 cells.
- Chemokines** A group of secreted proteins within the family of cytokines that by definition relate to the induction of migration. These “chemotactic cytokines” are produced by and target a wide variety of cells, but primarily address leukocyte chemoattraction and trafficking of immune cells to locations throughout the body via a concentration-dependent gradient. Chemokines are categorized on the basis of the protein structure according to the types of cysteine motifs (e.g., C-C, C-X-C)
- Chemotaxis** Attraction and trafficking of cells to locations via a gradient.
- Choline acetyltransferase** The enzyme present in nerve endings that is responsible for the esterification of choline yielding the neurotransmitter acetylcholine.
- Cholinergic receptors** One of two general classes of receptors (either G protein-coupled or ligand-gated) that can be further sub-classified and responds to the neurotransmitter acetylcholine.
- Chorea** An abnormal voluntary movement disorder, and subcategory of dyskinesias, which are caused by overactivity of the neurotransmitter dopamine in the areas of the brain that control movement. Chorea is a primary feature of HD and important diagnostic symptom.
- Chromogranin A** A type of chromogranin which was first isolated from chromaffin cells of the adrenal medulla but is also found in other tissues and in many species including human, bovine, rat, mouse, and others. It is an acidic protein with 431–445 amino acid residues. It contains fragments that inhibit vasoconstriction or release of hormones and neurotransmitter, while other fragments exert antimicrobial actions.
- Chronic inflammatory neuropathy** A chronic autoimmune neuritis, which involves demyelination of peripheral nerves and spinal roots. This disease is thought to be mediated by autoantibody production to peripheral myelin. The effector cell types include macrophages, B cells, and CD4+ T cells.
- Choroid** A layer of the eye adjacent to the RPE containing a dense bed of fenestrated capillaries (also called the choriocapillaris).
- Circumventricular organ** Brain structures that lack a normal blood brain barrier, which allows for a link between the central nervous system and peripheral blood flow of peptides and hormones. These include the area postrema, subfornical organ, vascular organ of lamina terminalis, subcommissural organ, posterior pituitary, pineal gland and median eminence.
- Clade** A group of biological taxa (as species) that includes all descendants of one common ancestor.
- Class-switch recombination** The process by which proliferating B cells rearrange their DNA to switch from expressing IgM (or another class of immunoglobulin) to expressing a different immunoglobulin heavy-chain constant region, thereby producing antibody with different effector functions.
- Clinical trials** An experiment to test quality, value, or usefulness of a therapeutic agent in human subjects.
- CNPase** Refers to 2',3'-cyclic nucleotide 3'-phosphodiesterase that is present in myelin.
- CNS homeostasis** Level of maintenance needed for normal central nervous system health and function.
- Cocaine** A compound present in the leaves of coca trees that is a central nervous stimulant, which increases neuronal activity by lengthening the duration of action of norepinephrine, dopamine and/or serotonin in the neuronal synapse.
- Combination antiretroviral therapy** Combination of several classes of antiretroviral drugs for the treatment of HIV infections. The major classes of combination antiretroviral therapy include nucleotide reverse transcriptase inhibitors, non-nucleotide reverse transcriptase inhibitors, protease inhibitors and integrase inhibitors.
- Combination therapy** The use of two or more therapies, especially drugs, to treat a disease or condition.
- Complement system** A host defense mechanism made up of a group of serum proteins involved in the control of inflammation, the activation of humoral and acquired immune responses, and the lytic attack on cell membranes.

Complex disease Is a disease due to the interaction of multiple susceptibility genes and/or environmental risk factors. Most common medical problems fall within this category

Cone Photo-sensitive retinal cell mediating color vision and responsive to higher light levels than rods.

Connexins (Cx) Connexins, or gap junction proteins, are a family of structurally-related transmembrane proteins that assemble to form vertebrate gap junctions.

Cornea A transparent part of the eye that covers the iris, pupil, and anterior chamber from the front. It refracts light and provides most of the eye's focusing power.

Corticosteroids A group of natural and synthetic hormones that are immunosuppressive agents and are used for treatment of transplant rejection and autoimmune disorders. Natural corticosteroids are synthesized from cholesterol in the adrenal cortex and are involved in the stress response and regulation of inflammation, carbohydrate metabolism, protein catabolism, blood electrolyte levels, and behavior. Corticosteroids are further classified as glucocorticoids and mineralocorticoids.

CRH Corticotropin releasing hormone is a peptide hormone produced by cells in the hypothalamus in response to stressful stimuli that causes the synthesis and release of ACTH from anterior pituitary cells.

Creutzfeldt-Jakob disease (CJD) The most common prion disease of humans and can have a sporadic, familial or acquired etiology.

Crohn's disease Is a chronic autoimmune disease affecting the gastrointestinal tract, and can affect any region from mouth to anus.

CXCR4 receptor The receptor for the chemokine stromal cell-derived factor (SDF-1).

Cyclic nucleotide 3' phosphohydrolase (CNPase) A component of myelin that demonstrates increased activity in active stages of MS:

Cyclooxygenase-2 (COX-2) An inducibly-expressed subtype of prostaglandin-endoperoxide synthase. It plays an important role in many cellular processes and inflammation. It is the target of cox2 inhibitors.

Cyclophosphamide Nitrogen mustard alkylating agent that is used as an anticancer agent and immunosuppressive agent mainly in the treatment of autoimmune diseases.

Cyclosporine A cyclic polypeptide that acts as a calcineurin inhibitor. It is an immunosuppressive agent that is used in organ transplantation and autoimmune diseases.

Cytokines Regulatory soluble glycoproteins (8–30 kDa), such as interleukins and lymphokines, secreted by inflammatory leukocytes and some non-leukocytic cells that act as intercellular mediators. They differ from classical hormones in that they are produced by a number of tissue or cell types rather than by specialized glands. They generally act locally in a paracrine or autocrine rather than

endocrine manner, and facilitate communication among immune system cells and between immune system cells and the rest of the body.

Cytotoxic T lymphocytes (CTL) T cells that can kill other cells. Most cytotoxic T cells are MHC class I-restricted CD8+ T cells. Cytotoxic T cells are important in host defense against cytosolic pathogens.

1.5 D

Daclizumab Humanized monoclonal antibody directed against the IL-2 receptor on T cells. It is used for the prophylaxis of acute organ rejection in adult patients.

Delusions Fixed false beliefs

Dementia A term describing memory loss and other cognitive impairments that result from a number of causes, including Alzheimer's disease; marked by the development of multiple cognitive deficits (including memory impairment, aphasia, and inability to plan and initiate complex behavior). Deteriorated mentality is often accompanied by emotional apathy.

Demyelination The act of demyelinating, or the loss of the myelin sheath insulating the nerves, and is the hallmark of some neurodegenerative autoimmune diseases, including: multiple sclerosis, transverse myelitis, chronic inflammatory demyelinating polyneuropathy, Guillain-Barre Syndrome, and adrenoleukodystrophy. When myelin degrades, conduction of signals along the nerve can be impaired or lost, and the nerve eventually withers.

Dendrites The branching structures projecting from the cell body of a neuron that receive messages from other neural cells and work to transmit these messages via electrical impulses to the cell body from which they project.

Dendritic cells Antigen-presenting cells that process material to the T cells of the immune system. They connect the innate and adaptive immune systems.

Dendritic spine A small protrusion from the dendrite comprised of a head that forms one half of a synapse and a thinner neck that connects the head to the dendrite. Responsible for the controlled diffusion of molecules from the synapse to the dendrite. They contain the highest concentrations of actin, which retains dynamic activity and can drive rapid changes in its shape and therefore its function.

Dentate granule cell layer Tiny neurons (10 μm in diameter) located in the dentate gyrus of the hippocampus, which contain glutamatergic projection axons, and are one of only two major types of adult neuronal populations to undergo neurogenesis.

Dentate gyrus A subfield in hippocampus.

Depression Mood disorder (clinically, major depressive disorder or a milder form referred to as dysthymia)

characterized by persistent sadness, hopelessness, cognitive impairment and other symptoms that interfere with work, sleep, etc. and in severe cases can lead to suicide.

Dexamethasone suppression test Measures cortisol levels in response to dexamethasone (synthetic cortisol) administration. Specifically, it tests the response of adrenal glands to ACTH (adrenocorticotrophic hormone which is secreted from the anterior pituitary gland of the brain). Cortisol levels should decrease following dexamethasone administration. Abnormally high cortisol levels are seen following dexamethasone administration in up to 50 % of patients with a major depressive disorder.

DICER (Endoribonuclease Dicer); An enzyme in humans that cleaves double-stranded RNA and pre-microRNA into small interfering RNA and microRNA.

Differentiation The ability of a pluripotent cell to become a specialized cell type by expressing distinct lineage specific genes and undergoing morphological changes.

Dobutamine A norepinephrine derivative that mimics norepinephrine action primarily at the subtype one beta adrenergic receptor.

Dopamine One of the catecholamine neurotransmitters in the brain. It is derived from tyrosine and is the precursor to norepinephrine and epinephrine. Dopamine is a major transmitter in the extrapyramidal system of the brain, and important in regulating movement. A family of receptors (receptors, dopamine) mediates its action.

Dopamine hypothesis The hypothesis that schizophrenia is functionally related to excessive dopamine activity. Evidence for this is the observation that many antipsychotics have DA-antagonistic effects.

Dopaminergic neurons Neurons that utilize dopamine as a neurotransmitter.

Doublecortin Binds to the microtubule cytoskeleton, stabilizing them and causing bundling of microtubules.

Drug delivery The transport of therapeutic agents to the site of action.

d-tubocurarine An alkaloid that blocks the action of acetylcholine on skeletal muscles causing paralysis

Dynorphins Endogenous opioid peptides, 17 amino acids in length, derived from prodynorphin.

Dysthymia Minor depression, with fewer and less intense symptoms as major depressive disorder.

Dystonia A neurological movement disorder consisting of repetitive muscle contracts and abnormal fixed postures. Over time, the symptoms may spread to nearby muscles.

EBF Early B-cell factor.

Effector memory t cells Cells that have an L-selectin⁻CCR7 phenotype. They have immediate effector functions, including rapid production of cytokines (IFN- γ or IL-4), and they migrate to sites of inflammation, such as the skin and the gut. They recognize foreign invaders with a faster and stronger immune response than the initial immune response.

Embryonic germ cell (EG) A totipotent cell that can differentiate into all the cells of the organism, self-renew while maintaining its differential capability, the precursor for adult germ cells, and can be isolated from primordial germ cells of 5–9 week human fetuses.

Embryonic stem cell (ES) A multipotent stem cell that can differentiate into all the cells of the organism except the trophoblast, self-renew while maintaining its differential capability, and is isolated from the inner cell mass of the blastocyst stage embryo.

Encephalopathy A disease of the brain; especially one involving alterations of brain function and/or structure.

Endocytosis Uptake of molecules that cannot readily pass through the cell membrane through engulfment by the membrane resulting in the formation of a vesicle. This process is employed for a variety of functions within the cell such as reloading of synaptic vesicles with vesicular components at the active site through receptor-mediated endocytosis.

Endorphins Endogenous opioid peptides, 31 amino acids in length, derived from proopiomelanocortin.

Endothelins 21-Amino-acid peptides produced by vascular endothelial cells and functioning as potent vasoconstrictors. The endothelin family consists of three members, ENDOTHELIN-1; ENDOTHELIN-2; and ENDOTHELIN-3. All three peptides contain 21 amino acids, but vary in amino acid composition. The three peptides produce vasoconstrictor and pressor responses in various parts of the body. However, the quantitative profiles of the pharmacological activities are considerably different among the three isopeptides.

Enkephalins Short endogenous opioid peptides derived from proenkephalin and proopiomelanocortin.

Epidemiology Science of the incidence, distribution, and control of disease in a population.

Epidermal growth factor (EGF) A 6-kDa polypeptide growth factor initially discovered in mouse submaxillary glands. Human epidermal growth factor was originally isolated from urine based on its ability to inhibit gastric secretion and called urogastrone. EGF exerts a wide variety of biological effects including the promotion of proliferation and differentiation of mesenchymal and epithelial cells.

Epinephrine An analog of norepinephrine that is formed in the adrenal medulla and acts primarily as a hormone on

1.6 E

E2A Immunoglobulin enhancer binding factors, also E12/E47. Binds the immunoglobulin k chain enhancer and is involved in promoter regulation.

either alpha or beta adrenergic receptors. Responsible for the “fight or flight” response to danger.

EPSC Excitatory postsynaptic currents are the flow of ions resulting in EPSP

EPSP Excitatory postsynaptic potentials are temporary increases in postsynaptic membrane potentials caused by the flow of positively charged ions into the postsynaptic cell. The influx of positively charged ions leads to an excitatory response therefore simplifying the neuron’s generation of an action potential.

Epstein Barr virus (EBV) A gamma human herpesvirus.

Exocytosis Vesicles carrying molecules to be secreted from the cell merge with the cell membrane resulting in the vesicular contents being released to the exterior of the cell. This is the method by which neurotransmitters are released at the active site.

EAE Experimental allergic/autoimmune encephalitis is an animal model for demyelinating diseases, such as multiple sclerosis. It is induced by injection of myelin basic protein or whole CNS tissue together with adjuvants.

Exosome A cell-derived vesicle present in many biological fluids.

Experimental allergic encephalomyelitis (EAE) An animal model of brain inflammation, used mostly with rodents, and serves as a model of the human disease multiple sclerosis.

Experimental allergic neuritis (EAN) Animal model of acute inflammatory demyelinating polyradiculoneuropathy that shares many features with human GBS. EAN can be induced in rodents, rabbits, and monkeys by immunization with peripheral nerve homogenate, myelin, and myelin proteins such as PO.

Each ferritin molecule consists of ferric iron in a hollow protein shell (apoferritins) made of 24 subunits of various sequences depending on the species and tissue types.

Fetal liver kinase (Flk)-2 A member of the tyrosine kinase receptor family, this 135–150 kDa molecule is expressed by multipotential progenitor cells including primitive B cell and myelomonocytic progenitors in fetal liver and adult bone marrow. Also called as Fms-like tyrosine kinase 3 (Flt3) and CD135 in human.

Fibroblast growth factor (FGF)-2 A single-chain polypeptide growth factor that plays a significant role in the process of wound healing and is a potent inducer of physiologic angiogenesis. Several different forms of the human protein exist ranging from 18 to 24 kDa in size due to the use of alternative start sites within the fgf-2 gene. It has a 55 % amino acid residue identity to fibroblast growth factor 1 and has potent heparin-binding activity. The growth factor is an extremely potent inducer of DNA synthesis in a variety of cell types from mesoderm and neuroectoderm lineages. It was originally named basic fibroblast growth factor based upon its chemical properties and to distinguish it from acidic fibroblast growth factor (Fibroblast Growth Factor 1).

Follicular dendritic cells (FDCs) Cells found in lymph follicles that present immune complexes to B cells, aiding in B cell maturation.

Fovea The high acuity region of the retina upon which the center of the visual image is focused. The fovea contains only cones.

Friedreich ataxia An autosomal recessive inherited disease, which causes damage to the nervous system and potentially scoliosis, heart disease, and diabetes.

1.7 F

Facilitated diffusion A process of passive transport where molecules move across membranes with the assistance of transport proteins

Family-based association study Is an association study that uses families as the source, where the parents are the controls and affected are their offspring.

Fas Fas/APO-1/CD95 (36 kDa) is a member of the tumor necrosis factor (TNF) receptor superfamily. Fas has been shown to be an important mediator of apoptotic cell death, as well as being involved in inflammation.

Fatal insomnia Rare prion disease of humans that is initially characterized by sleeplessness, later developing into hallucinations, followed by extreme weight loss, dementia and finally death

Ferritin Iron-containing proteins that are widely distributed in animals, plants, and microorganisms. Their major function is to store iron in a nontoxic bioavailable form.

1.8 G

Gadolinium A radio-opaque dye injected in conjunction with magnetic resonance imaging (MRI) to evaluate the brain parenchyma for breaches in the cerebrovascular system.

Ganglia A collection of cell bodies of the postganglionic motor neurons which join synapse with nerves from the central nervous system; they act as relay stations for CNS impulses to peripheral effector cells and as control loops for reflex actions.

Gangliosides Sialic acid-containing glycosphingolipids which are widely distributed in mammalian tissues but highly enriched in the nervous system, due to their incorporation into the myelin sheath. Gangliosides consist of a tetraose core to which variable numbers of sialic acids are attached; the ceramide or lipid portion of the molecule is attached to the internal glucose. GM1, GD1b, GD1a, and GT1b are the most abundant complex gangliosides in the nervous system.

- Gap junctions** Gap junction or nexus is a junction between certain animal cell-types that allows cell-to-cell passage of ions, hormones, and neurotransmitters. One gap junction is composed of two connexons (or hemichannels), which connect across the intercellular space, therefore connecting the cytoplasm of the cells. They are analogous to the plasmodesmata that join plant cells.
- GATA3** Binding protein 3. GATA-3 expression inhibits the differentiation of Th1 cells *in vivo*, induces Th2 cell differentiation, and increases functional capacity to secrete Th2 cytokines and enhance surface expression of markers for antigen-experienced Th2-committed cells. GATA-3 expression must be sustained to maintain the Th2 phenotype.
- Gene arrays** High throughput technique used to analyze different expression profiles using a collection of gene-specific nucleic acids.
- Gene modifiers** Are genes that affect the expression of the phenotype of a trait.
- General anesthetics** A group of diverse agents used in surgery to produce loss of pain perception and loss of consciousness. They depress most CNS modalities, including respiration.
- Genetic heterogeneity** Describes when a disorder has more than one genetic cause that causes the same clinical presentation.
- Genome** One haploid set of chromosomes with the genes they contain; *broadly*: the genetic material of an organism.
- Genomic convergence** A process in which multiple independent genomic research techniques are used to identify possible causal genes, with the overlap between the results in each technique converged to produce the genes with the highest likelihood of being involved in the disorder.
- Genomics** Field of science that combines genetics and molecular biology techniques to create genetic maps and DNA sequences; analyze and organize genetic information into databases.
- Genotype association** Association with a specific genotype but not necessarily an allele.
- Germinal centre** A lymphoid structure that arises within follicles after immunization with, or exposure to, a T-cell-dependent antigen. It is specialized for facilitating the development of high-affinity, long-lived plasma cells and memory B cells.
- Gerstmann-Straussler-Scheinker (GSS)** Syndrome is a prion disease linked to germline mutations or insertions in the human prion protein gene resulting in a neurodegenerative brain disorder.
- Glatiramer acetate** An FDA approved therapy for relapsing remitting multiple sclerosis that consists of acetate salts of polypeptides that are found in myelin and contains four naturally occurring amino acids randomly synthesized: L-glutamic acid, L-alanine, L-tyrosine, and L-lysine; available as Copaxone®, 20 mg of glatiramer acetate and 40 mg of mannitol for subcutaneous injection daily.
- Glaucoma** A group of eye diseases that include damage to the optic nerve and vision loss. Other symptoms can include severe eye pain, blurred vision, and nausea.
- Glia** Non-neural cellular element that has diverse functions in the nervous system including participation in innate immunity, myelin formation, signal transmission, and homeostasis maintenance.
- Glial activation** Refers to activation of glial cells (astrocytes and microglia) in response to proinflammatory stimuli triggered by injury or disease. This can usually be detected by the presence of glial fibrillary acidic protein (GFAP) in astrocytes.
- GDNF** Glial cell line-derived neurotrophic factor is the founding member of the glial cell line-derived neurotrophic factor family. It was originally characterized as a nerve growth factor promoting the survival of midbrain dopaminergic neurons, and it has been studied as a potential treatment for Parkinson's disease.
- GFAP** Glial fibrillary acidic proteins form the main intermediate filament in astrocytes and are therefore used as the marker protein of astrocytes.
- Glial scar** Scar formed due to insults to the CNS.
- Glucocorticoids** Steroid hormones (esp. cortisol in humans) produced by the adrenal cortex in response to stressful stimuli.
- Glutamate** The most important excitatory neurotransmitter in the CNS. Glutamate binds to either metabotropic, or ionotropic receptors. Ligation of glutamate at the NMDA receptor—one of the ionotropic receptors—induces calcium influx into the neuron. Astrocytes are crucially involved in glutamate metabolism, storage and re-uptake.
- Glutamine** An amino acid that is formed from glutamate. Glutamine in excess is toxic for the brain.
- Growth factor** Growth factors stimulate cell proliferation and/or differentiation during embryonic development, tissue growth and wound healing. The term “growth factors”, used in this chapter, also refers to a broad range of structurally diverse molecular families and individual proteins.
- Guanine nucleotide binding proteins (G-proteins)** Membrane-associated, heterotrimeric proteins composed of three subunits: alpha, beta and gamma subunits. G proteins and their receptors (GPCRs) form one of the most prevalent signaling systems in mammalian cells, regulating systems as diverse as sensory perception, cell growth and hormonal regulation. Binding of ligands such as hormones and neurotransmitters to a G-protein coupled receptors causes a conformational change, which in turn activates the bound G protein on the intracellular-side of the membrane. The activated receptor promotes the exchange of bound GDP for GTP on the G protein alpha subunit.

Guillain Barré syndrome An acute monophasic neuropathy characterized by an autoimmune response to peripheral myelin, and is the most common acquired demyelinating peripheral neuropathy. Pathogenesis involves mononuclear cell infiltration in the peripheral nerves, along with both B cells and CD4+ helper T cells. Symptoms begin as tingling sensations and weakness in the legs and in extreme cases can lead to the development of total paralysis. Current treatment includes high-dose intravenous Ig and plasma exchange.

1.9 H

Hallucinations Sensory misperceptions in which a person will experience an auditory, visual, or other sensory experience in the absence of observable stimuli

Haplogroup A haplotype of the mitochondrial genome.

Haplotype With two polymorphisms in linkage disequilibrium the alleles of those two polymorphisms travel together as a single combined allele or haplotype.

Hemorrhagic stroke Accounts for 15–20 % of all strokes. Rupture of a blood vessel into the brain parenchyma results in an intracerebral hemorrhage (ICH); this is commonly caused by hypertension-induced vascular disease of the small perforating intracranial arteries. Rupture of a vessel into the subarachnoid space results in a subarachnoid hemorrhage; this most commonly results from rupture of a brain aneurysm or an arteriovenous malformation.

Herpes simplex virus (HSV) A naturally neurotropic virus capable of establishing latent infection within neurons, but also possesses the ability to infect a wide range of cellular targets. Recombinant HSV vectors comprise a wild-type HSV genome rendered replication defective via disruption/deletion of an indispensable viral gene(s). Typically, the immediate-early gene loci, which encode for potent transactivation proteins that initiate the viral lytic cycle, are targeted for insertion of therapeutic transcription units via homologous recombination. The HSV-1 amplicon, another type of HSV vector, is a uniquely designed eukaryotic expression plasmid that harbors two non-protein encoding virus-derived elements: an HSV origin of DNA replication (OriS) and the cleavage/packaging sequence ("a" sequence). Both *cis* sequences are specifically recognized by HSV proteins to promote the replication and incorporation of the vector genome into viable viral particles, respectively. This highly versatile plasmid can be readily manipulated to contain desired promoters, enhancers, and transgenes of substantial size (~130 kb).

High endothelial venules (hevs) Specialized venules found in lymphoid tissues, which are the site of entry for lymphocytes from the bloodstream into lymph nodes and Peyer's patches.

Hippocampus Part of the brain located inside the temporal lobe. It forms a part of the limbic system and plays a part in memory and spatial navigation.

Histone acetyl transferase Enzymes that acetylate conserved lysine residues on histone proteins by transferring an acetyl group from acetyl CoA to form -N-acetyl lysine.

Histone deacetylase A class of enzymes that remove acetyl groups from an -N-acetyl lysine on histones.

HIV-1 associated dementia HIV-1 associated dementia is a cognitive disorder specific to HIV, HAD is the clinical consequence of HIV-1 infection in the CNS and chronic inflammatory response that ultimately causes neuronal damage.

Horizontal cell Lateral inhibitory interneuron in the outer retina.

HIV-associated neurocognitive disorders (HAND) These disorders can include AIDS dementia complex, HIV associated dementia, HIV encephalopathy, and Mild Neurocognitive Disorder. They share the features of cognitive impairment and motor dysfunction.

Homeostasis The property of a system to actively regulate a variable and maintain a constantly consistent level.

HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) A chronic progressive inflammatory disorder of the CNS caused by infection with HTLV-. This disorder affects 0.25–3 % of HTLV-I-infected individuals.

Human herpes virus 6 (HHV-6) A beta human herpesvirus, which is a common opportunistic viral infection for the immunosuppressed.

Human leukocyte antigen (HLA) The genetic designation for the human major histocompatibility complex (MHC). This group of genes resides on chromosome 6, and encodes cell-surface antigen-presenting proteins and many other genes. Individual loci are designated by upper-case letters, as in HAL-A, and alleles are designated by numbers, as in HLA-A0201. HLA-B27 is a genetic marker present in patients' ankylosing spondylitis and Reiter's syndrome

Human T cell lymphotropic virus type I (HTLV-I) An exogenous human retrovirus that infects 10–20 million people worldwide. Endemic areas of HTLV-I infection include southern Japan, certain regions of Africa and South American, and the Caribbean. HTLV-I is the etiologic agent of both an aggressive T cell leukemia and an inflammatory disorder of the CNS.

Huntingtin (htt) A causative gene of Huntington's Disease (HD), with its N-terminal CAG trinucleotide repeat expansion correlated with the onset of the disease. Predicted to function as a scaffolding molecule for multiple transporting/transcriptional molecules in cytoplasm and nucleus. Inclusion of htt and/or its N-terminal polyglutamine tract have been proposed as a potential mechanism of neurodegeneration in HD.

Huntington Disease A progressive neurodegenerative disorder that is inherited as an autosomal dominant trait, that usually begins in middle age, that is characterized by choreiform movements (involuntary, rapid, jerky movements) and mental deterioration leading to dementia. Histologically, Huntington disease shows atrophy of the caudate nucleus and the loss of brain cells with a decrease in the level of several neurotransmitters—called also Huntington's—abbreviation HD:

Hydrophilicity A physico-chemical characteristic of a compound attributed to its ability to dissolve in water solutions.

Hyphenated techniques Combination of individual techniques, such as liquid chromatography (LC) and mass spectrometry (MS), to create one encompassing method (LC-MS) capable of new analytical possibilities.

Hypomania A less severe and less persistent form of mania. Seen in such disorders as bipolar II and cyclothemia.

Hypothalamic-pituitary-adrenal (HPA) axis Neuroendocrine pathway that mediates the stress response by regulating systemic cortisol levels and brain CRH levels.

Hypothalamo-pituitary-adrenocortical (HPA) axis The major endocrine route in stress response. Activation of the HPA axis results in the secretion of corticotropin-releasing factor (CRF) from the hypothalamic paraventricular nucleus (PVN), adrenocorticotropin (ACTH) from the pituitary, and glucocorticosteroids (cortisol) from the adrenal cortex. Cytokines such as IL-1, IL-6, TNF- α are known to activate the HPA axis.

1.10 I

ID2 A helix-loop-helix protein that can inhibit transcription factors with a basic helix-loop-helix motif. Besides its role in lymphoid organogenesis, ID2 is required for the generation of natural killer cells.

IFN β -1a An FDA approved therapy for relapsing remitting multiple sclerosis. IFN β -a is available as Avonex[®] (Biogen-Idec) 33 μ g for intramuscular injection (IM) once a week and Rebif[®] (Serono) with doses of 22 μ g and 44 μ g for subcutaneous (SQ) injection 3X/week.

IFN β -1b An FDA approved therapy for relapsing remitting multiple sclerosis, available as Betaseron[®] for subcutaneous injection 22 μ g (6MIU) per dose for 66 μ g/week and 44 μ g (12 MIU) per dose for 132 μ g per week.

IKAROS This gene encodes a family of zinc-finger transcription factors that regulate transcription required for the development of all lymphoid lineages, as well as lymph nodes and Peyer's patches.

IL-7R A receptor for interleukine 7 (IL-7). The function of this receptor requires the interleukin 2 receptor, gamma

chain (IL2R γ), which is a common gamma chain shared by the receptors of various cytokines, including interleukines 2, 4, 7, 9, 15, 21. This protein has been shown to play a critical role in the V(D)J recombination during lymphocyte development. This protein is also found to control the accessibility of the TCR gamma locus by STAT5 and histone acetylation. Knockout studies in mice suggested that blocking apoptosis is an essential function of this protein during differentiation and activation of T lymphocytes. The functional defects in this protein may be associated with the pathogenesis of the severe combined immunodeficiency (SCID).

Imipramine An agent effective in the treatment of depression that acts to increase neuronal activity by lengthening the duration of action of norepinephrine and/or serotonin in the neuronal synapse.

Immune deviation Modification of cells having or producing antibodies or lymphocytes capable of an immune response to an antigen after prior exposure to that antigen.

Immune neuropathies Heterogeneous group of neuropathies with an assumed autoimmune pathogenesis. This includes acute forms such as Guillain-Barré Syndrome (GBS) and chronic conditions such as chronic inflammatory demyelinating polyneuropathy (CIDP) as well as polyradiculoneuropathies associated with monoclonal paraprotein.

Immune neuropathy Disease of the peripheral nerves that is mediated by autoimmune processes, either cellular or humoral autoimmunity

Immune privileged Organs or tissues where entry of effector lymphocytes is blocked by anatomic barriers and is not recognized by immune cells due to absence or low levels of expression of MHC class I and II antigens.

Immunoglobulins Natural immunomodulatory compounds that provide passive immunization and are used in a variety of diseases.

Immunomodulator A type of adjuvant that directly activates cells of the immune system, usually via cytokine release

Immunosuppression A decrease in any functional assay of immune competence from its baseline level in normal animals.

Immunotherapy The treatment of a disease by vaccination or by administering antibodies.

Induced pluripotent stem cells Stem cells which can be directly generated from adult cells.

Infliximab Chimeric anti-TNF- α monoclonal antibody. It is used in the treatment of rheumatoid arthritis and other autoimmune diseases.

Innate immune system The dominant system of host defense in most organisms. Innate immune defenses are non-specific, and do not confer long-lasting immunity against a pathogen.

Inflammation A local response to cellular injury that is marked by capillary dilatation, leukocytic infiltration, and often loss of function. Inflammation serves as a mechanism initiating the elimination of noxious agents and of damaged tissue:

Insulin degrading enzyme (IDE) Responsible for the degradation of the β -chain of insulin, expressed abundantly in microglia and also other neuronal cells in brain.

Insulin like growth factor Resembles insulin but is contained in part of a complex used to communicate with their physiologic environment, contributing to cell such processes as proliferation and inhibition of cell death. The complex consists of two cell-surface receptors, two ligands, and six high affinity binding proteins.

Intercellular adhesion molecule 1 (ICAM-1) Molecules that promote adhesion between cells.

Interferon-gamma The major interferon produced by mitogenically or antigenically stimulated lymphocytes. It is structurally different from type I interferon and its major activity is immunoregulation. It has been implicated in the expression of class II histocompatibility antigens in cells that do not normally produce them, leading to autoimmune disease.

Interferons (IFNs) Natural and recombinant compounds with important immunomodulatory activities, such as inflammation, antigen presentation, and antiviral activity. IFN type I binds to IFN- α receptor complex, and IFN type II binds to IFN- γ receptor complex.

Interleukin A group of cytokines (secreted signaling molecules) that mediate autocrine, paracrine and endocrine communication between cells of the immune system and other systems of the body. They enhance cell proliferation and differentiation, proteins **DNA** produced by the cells of the immune system of most vertebrates in response to challenges by foreign agents such as viruses, bacteria, parasites and tumor cells. They were first seen to be expressed by white blood cells (leukocytes, hence the -leukin) as a means of communication (inter-). The name is something of a relic though; it has since been found that interleukins are produced by a wide variety of bodily cells. The function of the immune system depends in a large part on interleukins. They are designated by IL and a number. IL-1 causes fever. IL-12 biases towards a Th1 response. IL-10 is anti-inflammatory. IL-8 is chemotactic. The interleukins are comprised of some of the cytokines and some of the chemokines.

Interleukin-2-recombinant form of IL-2 An immunostimulant used to treat adults with metastatic renal carcinoma and melanoma.

Intraocular pressure A measurement of the fluid pressure inside the eye. This fluid, or aqueous humor, nourishes the cornea, iris, and lens, and it helps the eye maintain its globular shape. Normal eye pressure, as measured by

an eye doctor, usually ranges between 10 and 21 mm of mercury, with an average of 16.

Ion channel Protein that mediates the transport of ions across membranes through the formation of a pore in the cell membrane, therefore allowing for the establishment of a voltage gradient across the cell membrane.

Ipratropium A derivative of atropine that blocks the action of acetylcholine on muscarinic cholinergic receptors.

IRF4 and IRF8 IFN regulatory factors (IRFs) are a family of transcription factors involved in the regulation of both innate and adaptive immunity. By gene knockout studies, IRF1, IRF4, and IRF8 have been found to be essential for the proper differentiation and functions of immune cells. Lymphocyte development requires IRF1 and IRF4, whereas IRF7 and IRF8 are needed for monocyte-to-macrophage differentiation, and together with IRF1, IRF8 orchestrates the development of Th1 immune responses by regulating the gene expression of IL-12, the major Th1 immune response-inducing cytokine. Recently, IRF4 and IRF8 have also been shown to participate in DC differentiation and functions. An interesting feature of IRF4 and IRF8 is their ability to function as transcriptional activators and repressors, depending on the promoter context.

Ischemic stroke Account for around 80–85% of all strokes and are nearly always caused by arterial vascular occlusions; rarely occlusion in the cerebral venous system may result in ischemic and/or hemorrhagic stroke.

Isoproterenol An analog of norepinephrine that mimics norepinephrine action primarily at the beta adrenergic receptors. It is used to treat asthma, chronic bronchitis, and emphysema; by relaxing the airways to allow for increased airflow.

Isotypes Classification of various immunoglobulins by the type of chain they are composed of. This classification varies between species.

1.11 J

JAK-STAT pathway A signaling pathway utilized by cells to respond to cytokines and growth factor stimuli. This pathway transduces the signals carried by cytokines to the cell nucleus, where activated STAT proteins modify gene expression. JAKs—Janus Kinases; STATs—Signal Transducers and Activators of Transcription.

1.12 K

Keratinocytes Epithelial cells dividing in the stratum basale of the epidermis, they accumulate keratin, undergo apoptosis and are sluffed off at the skin surface. In psoriasis, the cycle is accelerated from 30 days to 7–10 days.

KIRs Killer immunoglobulin like receptors are MHC-binding receptors on NK cells that triggers NK cell-mediated responses. KIRs diversify human natural killer cell populations and T cell subpopulations. Ly49, the analogous system in rodents.

Kinase A kinase is a type of phosphotransferase that catalyzes the transfer of a phosphate from ATP to specific substrates.

Kuru A prion disease of the Fore speaking tribes in the New Guinea highlands in which disease was transmitted via endocannibalism.

Kynurenic acid The only known endogenous NMDA receptor antagonist. Kynurenic acid is the end product of a side arm of the kynurenine pathway. Besides its neuroprotective NMDA binding properties, kynurenic acid also blocks the $\alpha 7$ nicotinic acetylcholine receptor.

Kynurenine pathway More than 95 % of tryptophan in the organism are metabolised through the kynurenine pathway, which is entitled by the first stable intermediate, kynurenine. The activity of one of the key enzymes, indoleamine 2,3-dioxygenase, is regulated by cytokines: IFN- γ and TNF- α are inducers, while IL-4 and IL-10 are inhibitors of IDO. Neuroactive kynurenine pathway intermediates include the free-radical generators 3-hydroxykynurenine, 3-hydroxyanthranilic acid, and quinolinic acid. The latter is also an excitotoxic NMDA receptor agonist. The major end product of the kynurenine pathway is nicotinamide adenine dinucleotide phosphate (NADP⁺/NADPH), a major source of electrons e.g. for reductive biosynthesis.

1.13 L

Learned helplessness A condition induced by inescapable, unavoidable, unpleasant stress, in which impaired coping and maladaptive behavior results.

Lectin Carbohydrate-binding proteins, macromolecules that are highly specific for sugar moieties.

LEF1 Lymphoid enhancer-binding factor 1 is a 48-kDa nuclear protein expressed in pre-B and T cells. LEF1 binds to a functionally important site in the T-cell receptor- α enhancer and confers maximal enhancer activity. LEF1 belongs to a family of regulatory proteins that share homology with high mobility group protein-1.

Lentivirus A member of the retrovirus family of enveloped RNA viruses associated with causing serious immunosuppressive diseases (i.e., AIDS). Vectors derived from lentiviruses can harbor a transgene up to 9 kb in size and express the transgene in vivo months to years.

Leucine-rich repeat kinase-2 (LRRK2) A complex protein with multiple domains, including a leucine-rich repeat (LRR), a ROC-COR GTPase, a mitogen-activated protein kinase kinase Kinase, and WD40 domains. Mutations have

been found in all domains in LRRK2 and have been identified as the cause of the late-onset, autosomal-dominant Park8 type of Parkinson's disease.

Lewy body Intracytoplasmic neuronal inclusions which are intensely eosinophilic under routine haematoxylin-eosin stain. Immunocytochemically, Lewy bodies share epitopes with phosphorylated and non-phosphorylated α -synuclein, neurofilament subunits, tubulin, microtubule-associated protein 1 and 2, and positively immunostain with ubiquitin. Lewy bodies are classically associated with Parkinson's disease, but also are present in a distinct form of dementia termed diffuse Lewy body disease, described recently in the elderly.

Limbic system An interconnected system of brain nuclei associated with basic needs and emotion, for example hunger, pain, pleasure, satisfaction and instinctive motivation.

Lin Lineage marker negative cells are those lacking distinctive antigen associated with lymphoid, myeloid, erythroid or natural killer cells.

Linkage Describes the relationship between two measurable traits that travel together through a pedigree more than expected by chance, suggesting the two lie physically near each other.

Linkage disequilibrium Two genetic variations that lie physically close to each other (linkage) and travel together through multiple generations in a population unchanged. This means the allele of one variation will be on the same piece of DNA as a specific allele of another variation, and thus they are correlated. This correlation can be measured and is referred to as linkage disequilibrium. The actual term comes from the fact that these variations do not follow the expectations of Hardy-Weinberg equilibrium and thus are in disequilibrium do to linkage.

Lipophilicity A physico-chemical characteristic of a non-polar compound attributed to its ability to dissolve in organic solvents.

Lipopolysaccharide (LPS) Large molecule consisting of a lipid and a polysaccharide (carbohydrate) joined by a covalent bond. LPS is a major component of the outer membrane of Gram-negative bacteria, contributing greatly to the structural integrity of the bacteria, and protecting the membrane from certain kinds of chemical attack. LPS is an endotoxin, and induces a strong response from normal animal immune systems. LPS acts as the prototypical endotoxin, because it binds the CD14/TLR4/MD2 receptor complex, which promotes the secretion of pro-inflammatory cytokines in many cell types, particularly in macrophages.

Liposome A spherical vesicle with a membrane composed of a phospholipid and cholesterol bilayer.

Liquid chromatography A method to separate mixtures using a liquid medium and high pressures.

Long terminal repeat A region of the retroviral genome principally involved in mediating insertion of the viral genome into the host chromosome.

Long-term potentiation A long-lasting enhancement of synaptic efficacy. Regarded as a primary mechanism in memory formation.

Ly49 Killer cell lectin-like receptor subfamily A, member 1. Analogous for human KIRs. A natural killer cell receptor.

Lymphocyte A subsection of white blood cells, which include natural killer cells, T cells, and B cells that are most commonly found in lymph.

Lymphoid chemokines (CCL-13, -21) These chemotactic cytokines are constitutively expressed in lymphoid tissues and mediate the formation and maintenance of micro-domains in lymphoid organs (See chemokines).

Lymphotoxin (It) Protein belongs to the tumour-necrosis factor family and can be produced as a secreted homotrimer, LT β , or as a membrane-bound heterotrimer, LT $\alpha_1\beta_2$. The heterotrimer LT $\alpha_1\beta_2$ binds to the lymphotoxin-receptor (LTR). LT β is an inducer of the inflammatory response system and involved in normal development of lymphoid tissue.

1.14 M

Macrophage A phagocytic cell of the reticuloendothelial system or mononuclear phagocyte system that may be fixed or freely motile. Myeloid-derived cell of the innate immune system matures from a monocyte, and functions in the protection of the body against infection and noxious substances.

Magnetic resonance imaging (MRI) The use of a nuclear magnetic resonance spectrometer to non-invasively produce electronic images of specific atoms and molecular structures in solids, especially human cells, tissues, and organs.

Magnetic resonance spectroscopy A method to determine physical and chemical properties of atoms and molecules through their magnetism.

Magnocellular ganglion cells Large retinal ganglion cells with low resolution but high sensitivity to intensity changes and motion.

Major depressive disorder A mental illness characterized by a persistent low mood and decreased interest and pleasure, as well as symptoms such as loss of energy and sleep and appetite disturbances

Major histocompatibility complex (MHC) A large genomic region with the primary immunological function to bind and "present" antigenic peptides on the surfaces of cells for recognition (binding) by the antigen-specific T cell receptors (TCRs) of lymphocytes. Differential structural properties of MHC class I and class II molecules account for their respective roles in activating different

populations of T lymphocytes. Class I molecules play a central role in the immune system by presenting peptides derived from the endoplasmic reticulum lumen. They are expressed in nearly all cells. The class II molecule plays a central role in the immune system by presenting peptides derived from extracellular proteins. Class II molecules are expressed in antigen presenting cells (APC: B lymphocytes, dendritic cells, macrophages).

Mania A phase of bipolar disorder characterized by elevated or irritable mood, as well as symptoms such as increased high risk behavior and decreased need for sleep.

Mantle zone The area of a secondary follicle that surrounds the germinal centre and contains IgD⁺ naive, resting B cells.

MAP kinase pathways Signaling pathway utilized by cells to respond to extracellular stimuli. Activation of this pathway regulates varied cellular activities including gene expression, mitosis, differentiation, and cell survival/apoptosis.

Mass spectrometry Technique used to measure the mass-to-charge ratio of ions to identify the components of a sample.

Mast cell A type of white blood cell derived from the myeloid stem cell and contains many granules with histamine and heparin.

MDR inhibitor A compound terminating multidrug resistance in cells.

Mecamylamine A synthetic compound which blocks the effect of acetylcholine at autonomic ganglia.

Medium spiny neurons (MSNs) The most numerous and principal projection neurons from the striatum. The death of GABAergic MSNs in the striatum is associated with Huntington's Disease.

Melanocortin-1 receptors G-protein-coupled receptors that are stimulated by α -MSH (melanocyte stimulation hormone). These receptors are found in pigmented cells as well as immune cells.

Melanopsin The photopigment found in intrinsically light-sensitive ganglion cells involved in setting circadian rhythms.

Mendelian disease A disease caused by a single gene mutation such as sickle-cell anemia.

Meningitis Inflammation of the meninges and especially of the pia mater and the arachnoid.

Metallothionein (MT)-I/II A family of Cys-rich, low molecular weight (MW ranging from 3500 to 14,000 Da) proteins. MTs have the capacity to bind both physiological (such as Zn, Cu, Se) and xenobiotic (such as Cd, Hg, Ag) heavy metals through the thiol group of its cysteine residues, which represents nearly the 30 % of its amino acid residues. Important to uptake, transport and retention of metals.

mGluR6 Type III metabotropic glutamate receptor that mediates responses of ON type bipolar cells in the vertebrate retina. mGluR6 couples to the G protein, G_o,

- stimulating a signaling cascade that causes the closing of cation channels.
- Micelle** An aggregate of surfactant molecules dispersed in a liquid colloid. A typical micelle in aqueous solution forms an aggregate with the hydrophilic “head” regions in contact with surrounding solvent, sequestering the hydrophobic tail regions in the micelle center.
- Microglia** Cells derived from myeloid progenitor cells (as are macrophages and dendritic cells) which come from the bone marrow and are key cellular mediators of neuroinflammatory processes in the CNS.
- microRNA** A small non-coding RNA molecule that functions in RNA silencing and post-transcriptional regulation of gene expression.
- Mitogen** A substance that induces cell division in lymphocytes in an antigen nonspecific way. Concanavalin A (Con A) is extracted from the Jack Bean plant and triggers T cell proliferation. Lipopolysaccharide (LPS) is part of the outer membrane of Gram-negative bacteria and triggers B cell proliferation.
- Mitoxantrone** FDA approval for use in progressive MS; available as (Novantron®) with recommended dosage of 5–12 mg/mL IV every 3 months. Because of cumulative cardiotoxicity, the drug can be used for only 2–3 years for a cumulative dose of 120–140 mg per m².
- Molecular adjuvant** An adjuvant comprised of a single molecular entity that targets antigens to and/or activates specific pathways of antigen processing and presentation.
- Molecular mimicry** Immunological mechanism, whereby epitopes incidentally shared by microbial antigens and target tissues elicit an autoreactive T- or B-cell response in the wake of an infective illness. This autoimmune response then injures the ‘self’ target tissues.
- Monoclonal antibody** A laboratory-produced antibody clone of one isotype that reacts to a single epitope.
- Mononuclear phagocytes (MP)** Phagocytic cells that typically initiate the host immune response. Mononuclear phagocytes include monocytes, mature macrophage, microglia and dendritic cells. These cells are widely distributed throughout the body in blood, tissue, and immune tissue identifying pathogens and steering the immune response, as well as clearing debris.
- Mood disorders** Mental disorders characterized by inappropriate or abnormal emotional states, such as depression and bipolar disorder.
- Mood stabilizers** Pharmacological agents used to treat bipolar disorder, including lithium, valproate, carbamazepine, and lamotrigine.
- Morphine** An alkaloid present in opium, an extract of the poppy plant, which binds principally to the mu opioid receptor and relieves pain.
- MPP+** A product of MPTP metabolism, capable of inducing a Parkinson like state through inhibition of complex I of the electron transport chain. (see MPTP)
- MPTP** 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, a lipophilic pro-neurotoxin that is metabolized by monoamine oxidase B to the active dopaminergic toxin, 1-methyl-4-phenylpyridinium (MPP+).
- Muller cell** A radial glial cell that is the predominant glial cell type in retina.
- Multidrug resistance** The ability of pathologic cells to withstand chemicals that are designed to aid in the eradication of such cells.
- Multiple Sclerosis** (Abbreviated MS, also known as disseminated sclerosis or encephalomyelitis disseminata) a chronic, inflammatory, demyelinating disease that affects the central nervous system (CNS). MS can cause a variety of symptoms, including changes in sensation, visual problems, muscle weakness, depression, difficulties with coordination and speech, severe fatigue, cognitive impairment, problems with balance, overheating, and pain.
- Muromonab-CD3** A monoclonal antibody directed at the epsilon chain of CD3 adjacent to the T cell receptor on human T lymphocytes, preventing T-cell activation. It is indicated for the treatment of acute organ transplant rejection.
- Muscarine** An alkaloid which blocks the effects of acetylcholine at G protein-coupled cholinergic receptors in the peripheral (parasympathetic) and central nervous systems.
- Mutation** Is a genetic change so severe that the disruption in the processing or gene function causes a disease by itself.
- Mycophenolate mofetil** A prodrug that is hydrolyzed to the active agent mycophenolate. Mycophenolate inhibits *de novo* purine synthesis and is indicated for prophylaxis of transplant rejection.
- Myelin** Ensheathes the axons of the nervous system
- Myelin associated glycoprotein (MAG)** A myelin protein that is a candidate autoantigen in multiple sclerosis.
- Myelin basic protein (MBP)** An abundant component of myelin that is a candidate autoantigen in MS.
- Myelin inhibitors** Adult mammalian central nervous system (CNS) lacks the ability to regenerate after injury. This is due in part to inhibitors within myelin. These inhibitors are myelin-Nogo, myelin-associated glycoprotein (Mag) and oligodendrocyte myelin glycoprotein (Omgp).
- MOG** Myelin oligodendrocyte glycoprotein is a glycoprotein believed to be important in the process of myelination of nerves in the central nervous system. The gene for MOG, found on chromosome 6, was first sequenced in 1995. It is a transmembrane protein expressed on the surface of oligodendrocyte cell and on the outermost surface of myelin sheaths. Interest in MOG has centered on its role in demyelinating diseases, particularly MS. Several studies have shown a role for antibodies against MOG in the pathogenesis of MS.

Myelin sheath The fatty substance that surrounds and protects some nerve fibers.

Myelination The formation of the myelin sheath around a nerve fiber.

1.15 N

nanoART Nanoformulated AntiRetroviral Therapy is a method of drug delivery using the patient's immunocytes as the transportation toward virus-targeted tissues.

Nanofiber A microscopic fiber whose diameter is measured in nanometres.

Nanogel A nanoscale size polymer network of cross-linked chains.

Nanoparticle A microscopic particle whose size is measured in nanometres.

Nanosphere A microscopic hollow particle whose size is measured in nanometres.

Nanosuspension A colloidal dispersion (mixture) in which a finely-divided species of a nanoscale size does not rapidly settle out.

Nanotube A microscopic tube whose diameter is measured in nanometres.

Natalizumab Tysabri® is a recombinant humanized monoclonal antibody that binds to the $\alpha 4$ subunits of $\alpha 4\beta 1$ and $\alpha 4\beta 7$ integrins that are expressed on the surface of all leukocytes except neutrophils and inhibit the $\alpha 4$ mediated adhesion to their receptors. Effective in phase I/II FDA trials for MS it was withdrawn due to deaths from progressive multifocal Leukoencephalopathy.

Natural killer cell A lymphocyte (white blood cell) that can kill target cells that express nonself proteins, such as viral antigens or tumor antigens.

Neostigmine An inhibitor of acetylcholinesterase which prolongs the actions of acetylcholine.

Neprilysin A neutral zinc metallopeptidases and important $A\beta$ degrading enzyme, which is involved in the clearance of $A\beta$ in the brain.

Nerve conduction Salutory propagation of nerve impulses along the axon

Nestin A large (200 kDa) intermediate filament protein found in developing rat brain. Often used as a specific marker of neuronal progenitor cells.

Neural progenitor cells (NPC) The term progenitor cell is used to refer to immature or undifferentiated cells, typically found in post-natal animals. While progenitor cells share many common features with stem cells, the term is far less restrictive.

Neuritis Inflammation of the peripheral nerve

Neurodegeneration A progressive loss of brain tissue structure or function due to a disease process; in the case of Alzheimer's and Parkinson's disease, due to accumulation of aggregated cellular products.

Neurodegenerative diseases The subset of neurological disorders that include neuron pathologies, but exclude diseases of the nervous system due to cancer, edema, hemorrhage, trauma, poisoning, hypoxia, etc.

Neurofibrillary tangle (NFT) Intraneuronal protein aggregates and a pathological hallmark of AD, composed of filamentous hyperphosphorylated tau (PHF-tau) and other ubiquitinated proteins.

Neurogenesis The formation of nervous tissue through a process that involves proliferation, migration, differentiation, and survival of neural stem cells.

Neuroimmune process The biochemical and electrophysiological interactions between the NERVOUS SYSTEM and IMMUNE SYSTEM that result in regulation of the immune system by the nervous system.

Neuroinflammation Chronic, CNS-specific, inflammation that is mediated predominantly by microglia that may engender neurodegenerative events.

Neuroinvasion The process by which prions enter the nervous system.

Neuromelanin A modified form of melanin pigment normally found in certain neurons of the nervous system, especially in the substantia nigra and locus ceruleus.

Neuromyelitis optica Also known as Devic's disease, is a severe variation of a demyelinating disease affecting the optic nerve and spinal cord.

Neuron Nerve cell that transmits nerve signals to and from the brain, consisting of a cell body, axon(s) and dendrite(s).

Neuronal apoptosis Death of neurons via programmed cell death.

Neuronal plasticity Refers to the changes that occur in the organization and/or function of the brain as a result of experience.

Neuropeptides Endogenous compounds made of amino acids that act upon receptors within the nervous system.

Neuroprotection Mechanisms that protect the brain from apoptosis (programmed cell death) or degeneration, following a brain injury or in the course of a neurodegenerative disorder.

Neuroproteomics Analysis of proteins and protein complexes of the nervous system (see Proteomics).

Neuropsychology The science of integrating psychological observations on behavior and the mind with neurological observations of the brain and nervous system.

Neuroregeneration The regrowth or repair of the nervous system or its components: tissues, cells or cell products.

Neurosphere A spherical group of neural progenitor cells growing in a suspension culture.

Neurotoxin A toxic complex typically of protein that acts preferentially on the nervous system.

Neurotransmitter Chemical that mediates information transfer between neurons.

- Neurotrophin** A small secretory molecule needed for growth, maintenance, or repair in the nervous system
- Neurovascular unit** A composition of endothelial cells, astrocytes, neurons, and a contractile apparatus of either smooth muscle cells or pericytes that form a functionally integrated network.
- NF- κ B** Transcription factor found in all cell types and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, and bacterial or viral antigens.
- NG2** Expressed by a variety of immature glia in the CNS including oligodendrocyte progenitor cells, paranodal astrocytes and perisynaptic glia. The protein has a large extracellular domain with two LNS/Lam G domains at the N-terminus and a short intracellular tail with a PDZ-recognition domain at the C-terminus.
- NADPH oxidase** Nicotinamide adenine dinucleotide phosphate oxidase, a primary producer of reactive oxygen species.
- Nicotine** An alkaloid that blocks the effects of acetylcholine at ligand-gated ion channels in the peripheral (parasympathetic and somatic) and central nervous systems.
- Nigrostriatal pathway** The neural pathway connecting the substantia nigra to the striatum.
- Nitric oxide** A free radical gas produced endogenously by a variety of mammalian cells, synthesized from arginine by nitric oxide synthase. Nitric oxide is one of the endothelium-dependent relaxing factors released by the vascular endothelium and mediates vasodilation. It also inhibits platelet aggregation, induces disaggregation of aggregated platelets, and inhibits platelet adhesion to the vascular endothelium. Nitric oxide activates cytosolic guanylate cyclase and thus elevates intracellular levels of cyclic gmp.
- Nitric oxide signaling** Nitric oxide is a gaseous molecule released from the vascular endothelium in response to a variety of chemical and physical stimuli. It triggers smooth muscle cells in the vessel wall to relax by activating soluble guanylate cyclases (sGC), increasing the cyclic guanosine monophosphate (cGMP) concentration and activating the protein kinase G.
- NMDA hypothesis** Based on the observation that NMDA receptor antagonists like phencyclidine frequently induce schizophrenia-like psychotic symptoms, the NMDA hypothesis of schizophrenia proposed an endogenous NMDA receptor antagonist that may be expressed in higher amounts in individuals suffering from schizophrenia. Because of the close functional relationship between glutamatergic neurotransmission via NMDA receptors and dopaminergic neurotransmission, the NMDA hypothesis includes the generally accepted disturbance of dopamine signaling in schizophrenia.
- NMDA receptor** *N*-methyl-D-aspartate receptor, named for the chemical agonist NMDA. The receptor is a heterodimer, forming an ionic channel that opens upon the binding of agonists, glutamate and glycine. NMDA receptors are permeable to a number of ions, but primarily sodium and calcium. NMDA function is fundamental to proper CNS function, but overstimulation is harmful resulting in excitotoxicity.
- Nociception** Pain perception.
- Nociception/orphanin FQ** An endogenous opioid peptide that acts on the nociceptin/Orphanin FQ receptor.
- Node of Ranvier** One of the many gaps in the myelin sheath—this is where the action potential occurs during saltatory conduction along the axon.
- Non-coding RNA** An RNA molecule that is not translated into a protein.
- Norepinephrine** The chemical responsible for transmitting impulses between neurons in the central and peripheral (sympathetic) nervous systems and acts on either alpha or beta adrenergic receptors.
- Notch** Family of evolutionarily conserved proteins that regulates a broad spectrum of cell-fate decisions and differentiation processes during fetal and post-natal development. The best characterized role of Notch signaling during mammalian hematopoiesis and lymphopoiesis is the essential function of the Notch1 receptor in T-cell lineage commitment. More recent studies have addressed the roles of other Notch receptors and ligands, as well as their downstream targets, revealing additional novel functions of Notch signaling in intra-thymic T-cell development, B-cell development and peripheral T-cell function.
- Nuclear hormone receptor** Receptor in which hormone binding is required to control the activity of the nuclear receptor.
- Nucleotide** Organic molecules that serve as the monomers, or subunits, of nucleic acids, composed of a nitrogenous base, a five-carbon sugar, and at least one phosphate group.
- Nucleus** The organelle in the cell body of the neuron that contains the genetic material of the cell.

1.16 O

- Obsessive compulsive disorder** (OCD) A mental disorder where people feel the need to check things, perform rituals, or think thoughts repeatedly.
- Ocular immunology** A branch of immunology studying immune processes in the eye
- Ocular inflammation** An inflammatory process affecting the eye:
- Olfactory bulb** Ovoid body resting on the cribriform plate of the ethmoid bone where the olfactory nerve terminates. The olfactory bulb contains several types of nerve cells

including the mitral cells, on whose dendrites the olfactory nerve synapses, forming the olfactory glomeruli. The accessory olfactory bulb, which receives the projection from the vomeronasal organ via the vomeronasal nerve, is also included here.

Oligoclonal bands (OCB) Bands of immunoglobulins that are seen when a patient's blood plasma or cerebrospinal fluid (CSF) is analyzed by protein electrophoresis:

Oligodendrocytes Are myelin-synthesizing cells of the CNS.

Olivopontocerebellar atrophy The degeneration of neurons in the cerebellum, pons, and inferior olives of the brain.

Opiate peptides Endogenous peptides with opiate-like activity. The three major classes currently recognized are the ENKEPHALINS, the DYNORPHINS, and the ENDORPHINS. Each of these families derives from different precursors, proenkephalin, prodynorphin, and PRO-OPIOMELANOCORTIN, respectively. There are also at least three classes of OPIOID RECEPTORS, but the peptide families do not map to the receptors in a simple way.

Opioids Substances typically used to relieve pain through the binding of opioid receptors.

Opioid receptors G-protein-coupled receptors that are the targets of the endogenous opioid peptides and of the opioid drugs, such as morphine.

Opiopeptides A general name for the whole class of endogenous peptides acting upon opioid receptors.

Opsin G-protein coupled receptor activated by light in photoreceptor outer segments. Cones have cone opsins and rods possess rhodopsin.

Optic nerve A paired nerve that sends visual information from the retina to the brain using ganglion cell axons and glial cells.

ORL-1 receptor Also known as the nociceptin/orphanin FQ receptor—a G-protein-coupled receptor for which the endogenous ligand is nociceptin.

Oxidative damage The oxidation of proteins, lipids, and DNA, which results in neuronal dysfunction. An important component of neuronal damage and aging, where neuroinflammation is significantly involved.

Oxidative stress An imbalance between formation and neutralization of reactive oxygen species.

Outer segment Photoreceptor cell structure that contains visual pigments and the enzymatic machinery for phototransduction.

p75^{NTR} signaling A member of the tumor necrosis factor receptor superfamily that facilitates apoptosis during development and after injury to the CNS. It activates Jun kinase (JNK), caspases 9, 6, and 3 and accumulates cytochrome c within the cytosol.

PASAT Paced auditory sequential addition test is a segment of the multiple sclerosis functional composite that tests one component of cognitive function.

Paraquat Trade name for the herbicide *N,N'*-dimethyl-4,4'-bipyridinium dichloride. Dangerously poisonous to humans as the compound is easily reduced resulting in superoxide generation.

Parasympathetic The division of the autonomic nervous system that regulates various body functions and is active primarily during periods of rest.

Parkin An E3 ligase in the ubiquitin-proteasome system. Parkin mutations have been associated with familial Parkinson's disease.

Parkinson's Disease (PD) A chronic progressive neurodegenerative disease chiefly of later life that is linked to decreased dopamine production due to loss of dopaminergic neurons in the substantia nigra. The clinical features are tremor of resting muscles, rigidity, slowness of movement, impaired balance, and a shuffling gait—called also paralysis agitans. Current therapies include L-DOPA and deep brain stimulation.

Parvocellular ganglion cells Small retinal ganglion cells that possess the high resolution and color sensitivity required for fine feature analysis and color vision.

Pax-5 Paired box gene 5 (B-cell lineage specific activator). The PAX proteins are important regulators in early development, and alterations in the expression of their genes are thought to contribute to neoplastic transformation. The PAX5 gene encodes the B-cell lineage specific activator protein (BSAP) that is expressed at early, but not late stages of B-cell differentiation. Its expression has also been detected in developing CNS and testis. Therefore, PAX5 gene product may not only play an important role in B-cell differentiation, but also in neural development and spermatogenesis.

Passive immunization Administering antibodies for temporary immune protection against an antigen. Immunity exists as long as the antibodies remain in the body.

Pathogen-associated molecular patterns (PAMPs) Small molecular motifs consistently found on pathogens; recognized by toll-like receptors and other pattern recognition receptors (PRRs) in plants and animals.

Pathogenesis The biological mechanism that leads to the diseased state.

Peripheral benzodiazepine receptor A G-protein-coupled receptor for benzodiazepines found primarily in the periphery, particularly in the immune system, such as microglia in brain.

1.17 P

p53 protein 53 (TP53) is a transcription factor critical factor to the prevention of mutation that regulates the cell cycle and hence functions as a tumor suppressor.

- Peripheral blood mononuclear cells (PBMC)** Lymphocytes and monocytes isolated from the peripheral blood, usually by Ficoll Hypaque density centrifugation.
- Peripheral nervous system** Part of the nervous system consisting of the nerves and neurons outside the central nervous system.
- Phagocytosis** The process in which a cell engulfs a solid particle to form an internal vesicle known as a phagosome.
- Pharmacogenomics** The study of the role of genetics in drug response.
- Phenotype** A notation of an organism's observable characteristics such as phenology, behavior, and products of behavior. It forms from the expression of genes as well as environmental stimuli.
- Phenylephrine** A norepinephrine analog that mimics norepinephrine action primarily at the alpha adrenergic receptors.
- Photoreceptor** Principle photo-sensitive cell in the retina
- Physostigmine** An inhibitor of acetylcholinesterase which acts to prolong the actions of acetylcholine.
- PI-3 kinase Phosphoinositide 3-kinases** A family of enzymes that are capable of phosphorylating the three position hydroxyl group of the inositol ring of phosphatidylinositol.
- PK11195** 1-(2-chlorophenyl-*N*-methylpropyl)-3-isoquinolinecarboxamide, a ligand for peripheral benzodiazepine receptors.
- PLP** Refers to proteolipid protein that is present in myelin.
- Polymorphism** A genetic variation in a base pair, repeat, deletion or duplication that is neutral or acts as a susceptibility allele
- Positron emission tomography (PET)** is a nuclear medicine, functional imaging technique used to observe metabolic processes within the body.
- Postsynaptic density** An electrondense region located opposite the active zone on the postsynaptic plasma membrane.
- Pralidoxime** A reactivator of acetylcholinesterase that has been inhibited by organophosphorus compounds.
- Prazosin** Synthetic compound that blocks the action of norepinephrine on alpha adrenergic receptors.
- Preproenkephalin** A precursor peptide for the enkephalins. It is processed into proenkephalin before being converted to multiple enkephalin molecules.
- Presenilin-1 (PS1)** A principal component of γ -secretase complex, processes APP at the γ -site, and a causative gene of the early onset of FAD. The γ -secretase complex is also critically involved in Notch processing and its signaling.
- Prion** Infectious protein agent that induces a fatal neurodegenerative disease in animals and humans that is characterized by the accumulation of the abnormal isoform of the prion protein.
- Prion disease** (Transmissible spongiform encephalopathies) are a group of progressive conditions that affect the brain and nervous system. The linking feature is a weakening of mental and physical capabilities as well as tiny holes appearing in the cortex.
- Prodrug** A medication that is metabolized within the body into a pharmacologically active drug.
- Progenitor/precursor cell** A cell that can differentiate into multiple cell types and has limited self-renewal capacities, so that the cell can proliferate while maintaining its differential capability for a short period of time but not for the entire life of the organism.
- Proinflammatory cytokines** Cytokines that induce an inflammatory response such as IFN- γ , IL-1(β), IL-18, and TNF- α . some authors also include IL-6, though this cytokine has an extremely broad range of actions.
- Proliferation** The ability to multiply cell population numbers by undergoing self-renewal or division.
- Proopiomelanocortin** A large precursor peptide that is the source of many active peptides, including some endorphins, enkephalins, alpha-MSH and others.
- Propranolol** A synthetic compound that blocks the action of norepinephrine on beta adrenergic receptors.
- Protein fingerprinting** Method of classifying new protein families based on conserved regions within alignments of related proteins.
- Protein microarrays** High throughput technology to detect differentially expressed proteins using protein-protein interactions.
- Protein-only hypothesis** Proposed by Dr. Stanley Prusiner to describe the novel properties of prions; prions are proteinaceous infectious particles that lack an agent-specific nucleic acid genome.
- Protein profiling** Identification of proteins in a sample.
- Proteolipid protein (PLP)** The most abundant myelin protein; a candidate autoantigen in MS.
- Proteomics** Field of science using high throughput techniques to analyze proteins expressed under a certain set of conditions within an individual cell or organism.
- PU.1** Spleen focus forming virus (SFFV) proviral integration oncogene *spi1*. The PU.1 plays indispensable and distinct roles in hematopoietic development through supporting HSC self-renewal as well as commitment and maturation of myeloid and lymphoid lineages.
- Pyroptosis** An inflammatory form of programmed cell death, occurs most frequently with infection of intracellular pathogens.

1.18 R

Radial glia Neuronal progenitor cells that arise from neuroepithelial cells after the onset of neurogenesis with more restricted differentiation abilities. In the developing nervous system, radial glia function both as neuronal progenitors and as a scaffold upon which newborn neurons

migrate. In the mature brain, the cerebellum and retina retain characteristic radial glial cells. In the cerebellum, these are Bergmann glia, which regulate synaptic plasticity. In the retina, the radial Müller cell is the principal glial cell, and participates in a bidirectional communication with neurons.

Randomized controlled trials An experiment in which investigators randomly allocate eligible people into (e.g. treatment and control) groups to receive or not to receive one or more interventions that are being compared. The results are assessed by comparing outcomes in the treatment and control groups. This form of trial is often used in performing scientific research due to its reliability in avoiding false causality.

Reactive oxygen species (ROS) Generally very small molecules that are highly reactive due to the presence of unpaired valence shell electrons. They include oxygen ions, free radicals and peroxides both inorganic and organic. ROS are formed as a natural byproduct of the normal metabolism of oxygen and have important beneficial roles in cell signaling and innate immunity. However, during times of environmental stress ROS levels can increase dramatically, potentially resulting in significant damage to cell structures. This culminates into what is known as oxidative stress and can lead to such results apoptosis or necrosis.

Redox signaling pathways Signal transduction pathways based on electron-transfer processes that play important messaging roles in biological systems. These pathways are activated by free radicals, reactive oxygen species (ROS), and other electronically-activated species and are turned off by reducing species.

Regulatory T cells Also called suppressor T cells, regulatory T cells are a small population of CD4⁺ T cells that have regulatory (that is, suppressor) activities towards other cells of the immune system and serve to limit immune responses, particularly those which may be against host tissue. These cells express the transcription factor forkhead box P3 (FOXP3) which is used as a specific marker for these cells. An absence of T_{Reg} cells or their dysfunction is associated with severe autoimmunity.

Relative risk A statistical tool used to measure the ratio of two conditional probabilities. For example, in genetic disease inheritance, relative risk can measure the degree of genetic contribution to a trait, defined as the risk of the disease in first degree offspring (sibs, parents, offspring) of an affected individual relative to the risk of the disease in the general population.

Reserpine An alkaloid that prevents the accumulation of catecholamines such as norepinephrine and dopamine in the synapses. Reserpine performs this action by irreversibly binding to these neurotransmitters and inhibiting their uptake into synaptic vesicles. The compound has been used as an antihypertensive as well as a sedative but

is rarely used any longer due to its risk of depression and the availability of better drug therapies.

Resting membrane potential Membrane potential prevailing in a resting unstimulated cell. The value is often negative, indicating an excess of negative potential inside the cell in comparison with the external environment. The difference in potential is based on the ion concentrations within and surrounding the cell membrane, as well as the ion transport proteins embedded in the cell membrane.

Retina A thin sheet of neural cells at the back of the eye that is continuous with the optic nerve. The retina is responsible for phototransduction and the initial stages of vision. The retina's photoreceptor cells, called rods and cones, produce neural signals in response to light that then undergo complex processing by ganglion cells of the retina. The resulting information is sent to the brain via the optic nerve to be interpreted into a visual image.

Retinal ganglion cell The output cell of the retina whose axons form the optic nerve and project to higher visual center. These cells process the signals produced by the photoreceptor cells of the retina and then send the resulting information to the brain via the optic nerve for interpretation.

Retinal pigment epithelium The pigmented epithelial cells forming the outermost layer of the retina. This layer of cells is attached to the choroid vascular tissue and provides nourishment to the cells of the retina.

Retinoid-related orphan receptor (ROR) An orphan nuclear hormone receptor that regulates the survival of CD4⁺CD8⁺ doublepositive (DP) thymocytes and is essential for the development of the lymph nodes and Peyer's patches.

Retrovirus Any of a family (Retroviridae) of enveloped RNA viruses (such as HIV and numerous tumorigenic viruses) that replicate in a host using a DNA intermediate produced using their RNA template and reverse transcriptase. Through the process, their viral genome is incorporated into the genome of infected cells. There are three common genes found in most retroviruses, the gag, pol, and env genes, which encode for the viral proteins, enzymes, and envelope of the viruses respectively.

Ribbon Structure in synaptic terminals of photoreceptors, retinal bipolar cells, and hair cells that is specialized for sustained neurotransmitter release.

RISC (RNA-induced silencing complex) is a multiprotein complex which contains one strand of a single-stranded RNA fragment.

Rivastigmine An inhibitor of acetylcholinesterase which prolongs the actions of acetylcholine with prominent effects in the central nervous system. The compound has been shown effective in treating the symptoms of dementia associated with Alzheimer's disease.

RNA binding proteins (RBP) bind to the double or single stranded RNA in cells and forms RNA complexes.

RNA interference A posttranscriptional genetic mechanism, which suppresses gene expression. In this mechanism, double-stranded RNA cleaved into small fragments initiates the degradation of a complementary messenger RNA. This pathway is thought to have developed as part of the innate immune system to protect against viruses, as in controlling development and genome preservation. This mechanism is used as a technique (as the introduction of double-stranded RNA into an organism) that artificially induces mRNA inhibition and is used for studying or regulating gene expression.

Rod A type of Photo-sensitive retinal cell mediating scotopic vision near visual threshold. These cylindrical-shaped photoreceptor cells are located primarily on the outer edges of the retina and are therefore used also for most peripheral vision.

Rostral migratory stream The route for neuronal precursors (periglomerular and granule cells) to migrate to the olfactory bulb where they differentiate into interneurons throughout the life of rodents. Overlying the rostral migratory stream is the anterior or olfactory limb of the anterior commissure (or commissura anterior pars bulbaris), which carries centrifugal fibers, including some from the medial forebrain bundle, to the region.

Rotenone A botanical insecticide that is an inhibitor of mitochondrial electron transport. Inhibition is achieved through the hindrance of the electron transfer chain preventing NADH from converting to ATP thus limiting usable cellular energy. The compound is considered moderately hazardous and mildly toxic to mammals, including humans.

1.19 S

Sca-1 A mouse member of the interferon-inducible Ly-6 family of genes that can conveniently be used as a marker for stem/early progenitor cells. Sca-1 is used to isolate many stem cells in the mouse. The density of this antigen declines with differentiation. Sca-1 belongs to a family of proteins bearing a UPAR (urokinase plasminogen activator receptor) domain. The UPAR protein plays a role in cellular adhesion and migration by modulating integrin function and degradation of the extracellular matrix.

Schaffer collaterals Axon collaterals given off by CA3 pyramidal cells in the hippocampus. They are part of memory formation and the emotional network of the Papez circuit.

Schizophrenia A mental illness characterized by cognitive dysfunction, hallucinations, delusions, thought disorder and poor social and vocational functioning. The term can also be used to describe a group of mental disorders characterized by the same or similar indicators.

Schwann's cells Cells that produce myelin in the peripheral nervous system. These cells are located between the axon and axon terminal of neurons and create the myelin sheath which aides in insulating axons and in increasing impulse speeds as they are propagated through the neuron (allowing for salutatory conduction).

Sclerosis A hardening of tissue as a result of scarring or plaques such as in the central nervous system when formed by inflammatory perivascular lesions.

SELDI-TOF Surface-enhanced laser desorption/ionization—time of flight is a soft ionization method used in mass spectrometry to analyze protein mixtures within tissue or clinical samples.

Senility A term once used to describe dementia.

Self-renewal The ability of a cell to divide into two daughter cells with identical properties and retaining equal differential capabilities as the parent cell.

Scavenger receptors Receptors that identify modified low-density lipoprotein by means of oxidation or acetylation.

SCID Severe combined immunodeficiency, a genetic disorder in which both “arms” (B cells and T cells) of the adaptive immune system are crippled. This disease is fatal if untreated due to extreme susceptibility to infectious diseases.

Sickness behaviour One of the immunological models of major depression. The cytokines IL-1, IL-6, and TNF- α have been identified to induce depression-like mood disturbance upon administration or during disease states that are accompanied by increased or over-production of these cytokines.

Signal serotonin Serotonin (5-hydroxytryptamine, 5-HT) is a biogenic indoleamine derived from the dietary amino acid tryptophan. Serotonergic neurons are located almost exclusively in the raphe nuclei of the brainstem, but their projections innervate virtually all other parts of the central nervous system. A reduced serotonergic neurotransmission is supposed to play a crucial role in the pathophysiology of major depression among other disorders.

Signal transduction Mechanism by which external stimuli are interpreted by cells. This transformation is often done through biochemical reactions involving secondary and cascade pathways within and between cells.

Simian virus 5 (SV-5) A paramyxovirus belonging to the family Paramyxoviridae. The genomes of these viruses are composed of negative-sense single-stranded RNA and this family of viruses is responsible for a number of human and animal diseases.

Single-chain antibodies Engineered antibodies composed of the minimal antibody-binding site formed by non-covalent association of the V_H and V_L variable domains joined by a flexible polypeptide linker. Sequences encoding eukaryotic secretion signals can be appended to the scFv

genes and subsequently be cloned into gene transfer vectors for prolonged *in vivo* expression. These antibodies are significantly smaller than natural antibodies but retain the same affinity as natural antibodies and can be produced in large quantities at relatively low costs using biotechnology.

Single nucleotide polymorphism The most common polymorphism in the human genome, a nucleotide polymorphism at a single base. The result of these differences creates alleles.

Single photon emission computed tomography By specifically using gamma rays, this imaging technique is able to provide true 3D information.

Sirolimus A macrolide antibiotic that inhibits T lymphocyte activation and proliferation. It is an immunosuppressive agent that is used mainly to prevent rejection during organ transplantation. The compound works to inhibit a response to interleukin-2 which is necessary to activate T cells.

Sjogren's syndrome An autoimmune disorder affecting the salivary and lacrimal glands. In this disorder, immune cells attack host exocrine glands that are responsible for the production of tears and saliva.

Smac/Diablo A mitochondrial protein that promotes some forms of apoptosis by neutralizing one or more members of the IAP family of apoptosis inhibitory proteins.

Substantia nigra pars compacta (SNpc) The portion of the substantia nigra within the midbrain that encompasses the dopaminergic neurons which innervate the caudate nucleus and putamen of the basal ganglia. This area of the brain plays an important role in the pleasure/reward system and in addiction. Additionally, degeneration of the neurons in this area of the brain is an indicator of Parkinson's disease.

Superoxide dismutase-1, copper-zinc superoxide dismutase (SOD1) A cytoplasmic enzyme that works as an antioxidant converting superoxide to oxygen and hydrogen peroxide. Mutations in the enzyme have been shown to be a factor in the motor neuron disease amyotrophic lateral sclerosis (ALS).

Suppressor of cytokine signaling (SOCS) proteins A family of proteins that inhibit the JAK-STAT signaling pathway by competitively binding to phosphotyrosine binding sites on cytokine receptors.

Soman An extremely toxic long acting organophosphorus compound with prominent inhibitory effects on acetylcholinesterase causing interference with nerve functioning

Somatic hypermutation Point mutations that occur in cycling centroblasts and are targeted to the immunoglobulin variable-region gene segments. Some mutations might generate a binding site with increased affinity for the specific antigen, but others can lead to loss of antigen recognition by the B-cell receptor and generation of

a self-reactive B-cell receptor. Each mutation occurs only within individual cells and the mutations are not passed on to offspring.

Sox4 A member of the SOX (SRY-related HMG-box) family of transcription factors involved in the regulation of embryonic development and in the determination of the cell fate. The encoded protein may act as a transcriptional regulator after forming a protein complex with other proteins, such as syndecan binding protein (syntenin). The protein may also function in the apoptosis pathway leading to cell death as well as to tumorigenesis and may mediate downstream effects of parathyroid hormone (PTH) and PTH-related protein (PTHrP) in bone development.

Spatial memory A type of memory responsible for recording information about one's environment and its spatial orientation. For example, a person's spatial memory is required in order to navigate around a familiar city, just as a rat's spatial memory is needed to learn the location of food at the end of a maze.

STAT4 A member of the STAT family of transcription factors. In response to cytokines and growth factors, STAT family members are phosphorylated by the receptor associated kinases, and then form homo- or heterodimers that translocate to the cell nucleus where they act as transcription activators. This protein is essential for mediating responses to IL-12 in lymphocytes, and regulating the differentiation of T helper cells.

STAT6 Protein that plays a central role in exerting IL-4 mediated biological responses. It is found to induce the expression of BCL2L1/BCL-X(L), which is responsible for the anti-apoptotic activity of IL-4. Knockout studies in mice suggested the roles of this gene in the differentiation of T helper 2 (Th2) cells, expression of cell surface markers, and class switching of immunoglobulins.

Stem cell A single cell that can differentiate along multiple lineages and can self-renew throughout the life of an organism while maintaining its differential capability. The three primary categories of stem cells are embryonic stem cells (originating from blastocysts), adult stem cells (derived from adult tissues), and cord blood stem cells (found in the umbilical cord).

Stem cell therapy Treatments and related research involving the use of undifferentiated cells which have self-renewal capacity and the unique potential to produce any kind of cell in the body.

Stress Any physical or psychological stimulus that disrupts homeostasis.

Stress granules Aggregations in the cytosol made of proteins and RNA which appear when the cell is under stress.

Striatum A continuous part of the basal ganglia comprised of the caudate nucleus and putamen. This portion of the

brain plays roles in modulating movement pathways as well as taking part in a variety of cognitive functions.

Stroke Brain injury caused by occlusion or rupture of blood vessels into or around the brain causing a subsequent loss of oxygen and glucose to the affected brain area. Stroke is commonly termed a “cerebrovascular accident” or a “brain attack” and can cause severe neurological damage or death.

Stromal cell-derived factor 1 (SDF-1) The ligand for CXCR4 receptor, this CXC chemokine (also designated CXCL12) is involved in chemotaxis, brain development, and immune cell recruitment.

Subgranular zone A brain region deep within the hippocampal parenchyma, at the interface between the granule cell layer and the hilus of the dentate gyrus, where adult neurogenesis occurs. It is one of the two major known adult neurogenesis sites of the brain, along with the subventricular zone.

Subluxation Partial dislocation of a joint or organ.

Substantia nigra A layer of large pigmented nerve cells in the midbrain that produce dopamine and whose destruction is associated with Parkinson’s disease.

Subventricular zone A paired brain structure situated throughout the lateral walls of the lateral ventricles. Along with the subgranular zone of dentate gyrus, this zone serves as a source of neural stem cells in the process of adult neurogenesis. It harbors the largest population of proliferating cells in the adult brain of rodents, monkeys and humans. Neurons generated in this zone travel to the olfactory bulb via the rostral migratory stream.

Susceptibility gene A gene with multiple polymorphic forms, of which one or more forms causes differences in the processing or function of the gene that makes an individual more susceptible to a specific disease, but only in the presence of other interacting or contributing susceptibility genes or environmental factors.

Sympathetic The division of the autonomic nervous system that regulates various body functions and is always active on a basal level, but becomes more active during periods of stress and/or exertion. It causes the “fight-or-flight” response during times of stress by promoting the release of adrenaline and noradrenaline which bind to adrenergic receptors in peripheral tissues and cause such reactions and increased heart rate and blood pressure and pupil dilation.

Symptomatic therapy Treatment or remedy for physical disturbance observed by the patient due to an underlying disease. Also called palliative care, this form of treatment does not address the basic cause or provide a cure for the underlying disease but rather focuses on preventing or easing suffering associated with the disease.

Synapse From the Greek meaning “to grasp”, a junction between two neural cells where the cell body (or dendrite) of one nerve is positioned across a small gap from the

axon of another nerve. Signal transmission occurs across these gaps via neurotransmitters, allowing the neurons to communicate with each other through the conversion of electrical impulses into chemical signals.

Systems biology A field of science that studies the interactions between the components of biological systems and how these interactions give rise to the function and behavior of that system.

1.20 T

Tacrolimus A macrolide antibiotic that acts as a calcineurin inhibitor to hinder T cell signal transduction and IL-2 transcription. It is an immunosuppressive agent that is used mainly to prevent rejection during allogeneic organ transplantation.

Tauopathies Neurodegenerative diseases featuring aggregation of tau proteins in neurofibrillary or gliofibrillary tangles.

T-bet T-box genes encode transcription factors involved in the regulation of developmental processes. This gene is the human ortholog of mouse Tbx21/Tbet gene. Studies in mouse show that Tbx21 protein is a Th1 cell-specific transcription factor that controls the expression of the hallmark Th1 cytokine, IFN γ . Expression of the human ortholog also correlates with IFN γ expression in Th1 and natural killer cells, suggesting a role for this gene in initiating Th1 lineage development from naive Th precursor cells.

T-cell receptor (TCR) A heterodimeric molecule found on the surface of T lymphocytes (T-cells) that is responsible for recognizing antigens bound to major histocompatibility complex (MHC) molecules. Upon antigen-receptor binding with MHC, a series of biochemical reactions occur which activate the associated T-cell.

T-cells T (thymus derived) cell. These white blood cells, known as T lymphocytes, mature in the thymus and have regulatory immune functions. As part of the adaptive (cell-mediated) immune system, these cells can recognize antigens processed by antigen-presenting cells in the context of specific receptors. The cell surface protein CD3 is a marker for these cells.

Temporal lobe Either of two lobes of the brain, located on either side of the cerebral hemisphere and part of the cerebrum. This portion of the brain is involved in auditory processing, semantics, and also contains the hippocampus which is responsible for learning and memory.

Terbutaline A norepinephrine derivative that mimics norepinephrine action primarily at the subtype two beta adrenergic receptor. This compound is used as a bronchodilator and tocolytic.

Th1 cells Also called inflammatory CD4 T cells, a subset of T cells that secrete the cytokines IL-2, IFN γ , and lym-

phototoxin and are most useful against intracellular pathogens that are best attacked by a cell-mediated response involving macrophage and granulocyte activation.

Th2 cells Also called helper CD4 T cells, a subset of T cells that secrete the cytokines IL-4, IL-5, and IL-10, and induce antibody and allergic responses that are useful for combating infections.

Th1/Th2 Subclasses of T helper cells that are distinguished by their cytokine profiles. Th1 cells are characterized by production of IFN- γ . Th1 cells are characterized by production of IL-4. Th2 cells polarize the immune response towards cellular immunity and Th2 cells polarize towards antibody formation.

Th1/Th2 balance According to the Th1/Th2 paradigm, naïve CD4⁺ T helper cells can be activated to differentiate into two types of effector cells: either Th1 or Th2 cells. In general, macrophages and dendritic cells tend to stimulate the Th1 differentiation, whereas extracellular antigens tend to stimulate Th2 cell differentiation. Th1 cells in turn stimulate macrophages and induce IgG production by B cells, while Th2 cells stimulate the humoral immune response including IgA, IgE, and certain IgG subtypes. An imbalance between Th1 and Th2 cells is observed in several immunological disease conditions; typical examples are Th1 predominance in rheumatoid arthritis and Th2 predominance in IgE-mediated allergy.

Thalidomide A drug originally used to treat morning sickness during pregnancy that caused severe limb deformities. This compound downregulates TNF α and is under investigation for treatment of severe Crohn's syndrome and other autoimmune disorders.

Therapeutics A branch of medical science dealing with the application of remedies to diseases.

Thymocyte Hematopoietic progenitor cells present in the thymus. They mainly generate T cells, but also respond to foreign pathogens.

Tissue profiling A tool for rapid detection of molecules, usually proteins and peptides, obtained directly from intact tissues to identify pathological changes.

T lymphocyte or T cell Please see "T cell".

Tolerance Refractoriness to physiological effects of a stimulus which develops with prolonged exposure, such as with drug tolerance. Alternatively, the failure of the immune system to react to an antigen, such as in the case of tolerance to self antigens

Toll-like receptors (TLR) A class of single membrane-spanning non-catalytic receptors that recognize structurally conserved molecules derived from microbes and activate immune cell responses. TLRs are believed to play a key role in the innate immune system. They are a type of pattern recognition receptors (PRRs) and recognize molecules that are broadly shared by pathogens but distinguishable from host molecules, collectively referred to as pathogen-associated molecular patterns (PAMPs). These

receptors are present in both vertebrates and invertebrates and have similarities with receptors found in bacteria and plants. Due to this, these receptors are thought to be one of the oldest and most conserved components of the immune system.

Tourette's syndrome Neuropsychiatric disorder consisting of motor and vocal tics.

Transcription nuclear factor-k-B A protein complex found in all cell types that is involved in cellular responses to stimuli such as stress, cytokines, and foreign antigens. This transcription factor plays a regulatory role in immune response to infection and has also been suggested to be involved in processes of synaptic plasticity and memory. When improperly regulated, this transcription factor has also been implicated in causing cancer, autoimmune diseases, and other immune system malfunctions.

Transducin G protein expressed in retinal photoreceptor cells that is activated upon absorption of a photon by opsin. Activation of this protein stimulates cGMP-specific phosphodiesterase which leads to the vertebrate phototransduction cascade.

Transgene A segment of DNA (generally coding DNA) which is taken from one cell or organism and is introduced into different cells or organisms to modify a phenotype.

Transmembrane protein transport (diffusion) The process of moving proteins from one cellular compartment (including extracellular) to another by various sorting and transport mechanisms such as gated transport, protein translocation, and vesicular transport. The tendency of a gas, solute or protein is to pass from a point of higher pressure or concentration to a point of lower pressure or concentration and to distribute itself throughout the available space:

Transport Movement or transference of biochemical substances that occurs in biological systems.

Traumatic brain injury A general term for an external force injuring the brain with subdivisions based on severity, mechanism, injury location, etc.

Trimethaphan A synthetic compound which blocks the effect of acetylcholine at autonomic ganglia thereby blocking the sympathetic and parasympathetic nervous system. This compound has been used to reduce bleeding during neurosurgery, as well as to treat such emergencies as pulmonary edema and hypertensive crisis.

Tropicamide An atropine derivative which blocks the action of acetylcholine on muscarinic cholinergic receptors. This compound is often used during eye examinations to dilate the pupil and may also be used before or after eye surgery.

Trk receptor Cell surface proteins that are involved in transducing the actions of neurotrophins to promote neuronal survival, proliferation, migration, axonal and dendritic outgrowth and patterning, synapse strength and plasticity,

injury protection, as well as controlling the activity of ion channels and neurotransmitter receptors.

Tryptophan L-tryptophan (TRP) is an essential amino acid, present in nearly all natural proteins. It is the precursor of the neurotransmitters serotonin and melatonin and of kynurenine, which is quantitatively the most important TRP metabolite.

Tumor necrosis factor Serum glycoprotein produced by activated macrophages and other mammalian mononuclear leukocytes that acts as a cytokine to help stimulate inflammation and the acute phase reaction of the immune system. It has necrotizing activity against tumor cell lines and increases ability to reject tumor transplants. Also known as TNF-alpha, it is only 30 % homologous to TNF-beta (lymphotoxin), but they share TNF receptors. Disregulation of this cytokine has been implicated in causing cancer, among other human diseases.

Tyramine The decarboxylated form of the amino acid tyrosine which is present in food (e.g., cheese, beer, wine) and promotes the release of norepinephrine from sympathetic nerve endings. Significant displacement of norepinephrine by this compound when ingested in large quantities or while taking monoamine oxidase inhibitor medication is thought to cause vasoconstriction and increased heart and blood pressure.

1.21 U

Urocortins Most recently discovered peptides belonging to the corticotropin-releasing factor family whose functions are generally unknown, but may play roles in immune function in addition to having anorexigenic and hypotensive effects, among other functions.

1.22 V

Vaccination The introduction of antigenic stimulants to the immune systems of humans or animals for the purpose of inducing the artificial development or regulation of immunity. Multiple kinds of vaccines exist to serve this

purpose including inactivated, live attenuated, subunit, and DNA vaccines.

Varicella zoster virus (VZV) An alpha human herpesvirus that causes the diseases chickenpox and, if later reactivated, shingles. A vaccine is currently available against the virus.

Vector A "vehicle", such as a modified virus or DNA molecule, used to deliver genetic material into the body for gene therapy. Alternatively, an organism that does not cause disease itself but can transmit infection through transferring pathogens among different hosts (such as a mosquito).

Vehicle A type of adjuvant that possesses no medicinal action of its own, but delivers or targets an antigen to cells of the immune system.

Vimentin A member of the intermediate filament family of proteins that is an important component of eukaryotic cell cytoskeletons. These proteins are responsible for cell flexibility and resilience under mechanical stress as well as the transport of low density lipoprotein intended to undergo esterification.

Viral vector Tools commonly used to deliver genetic material into cells.

Virus A small infectious agent that replicates only inside the living cells of another organism.

visual-evoked potential-(VEP) viruses Minute infectious agents whose genomes are composed of DNA or RNA, but not both. They are characterized by a lack of independent metabolism and the inability to replicate outside living host cells.

Vogt-Koyanagi-Harada disease A multisystem disease of presumed autoimmune cause, which affects pigmented tissues.

1.23 W

Withdrawal Signs and symptoms that occur as a result of a discontinuation of certain activities or drugs (such as opioids) and includes perturbations in various physiological systems that are indications of physical dependence.

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