CNS Injuries

Cellular Responses and Pharmacological Strategies

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CRC Press
Boca Raton New York

Contact Editor: Liz Covello
Project Editor: Maggie Mogck
Marketing Manager: Becky McEldowney
Cover design: Dawn Boyd

Library of Congress Cataloging-in-Publication Data

CNS injuries : cellular responses and pharmacological strategies / edited by Martin Berry, Ann Logan.
p. cm. -- (Pharmacology and toxicology)
Includes bibliographical references and index.
ISBN 0-8493-8309-9 (alk. paper).
1. Central nervous system--Wounds and injuries--Pathophysiology. 2. Central nervous system--Wounds and injuries--Chemotherapy. 3. Brain--Wounds and injuries--Pathophysiology.
I. Berry, M. (Martin) II. Logan, Ann. III. Series: Pharmacology & toxicology (Boca Raton, Fla.)
[DNLM: 1. Central Nervous System--injuries. 2. Central Nervous System--drug effects.
3. Nerve Regeneration--drug effects. 4. Nerve Growth Factors--therapeutic use. 5. Protease Inhibitors--therapeutic use. 6. Cytokines--therapeutic use. 7. Macrophages--physiology. 8. Microglia--physiology.
WL 300 C651 1998]
RD594.C632 1998
616.8′047—dc21
DNLM/DLC
for Library of Congress

97-52407
CIP

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International Standard Book Number 0-8493-8309-9
Library of Congress Card Number 97-52407
Printed in the United States of America 1 2 3 4 5 6 7 8 9 0
Printed on acid-free paper

Preface

The basic science of the cellular and molecular responses of the brain to injury is a rapidly expanding area of research which is providing evidence of growing opportunities for pharmacological intervention in the clinic. This book collates up-to-date reviews of most of the important areas of study and discusses possible therapeutic strategies for the manipulation of major events in the injury response, including inflammatory and immune reactions, scarring, neuron death, demyelination, remyelination, axonal regeneration, and the reestablishment of neural connectivity. All these events are controlled and modulated by complex intercellular chemical signals mediated by an ever-increasing number of cell adhesion molecules, vascular/leukocyte addressins, cytokines, and growth factors, and in which, additionally, proteases play a central role. The cellular responses to brain injury which initiate the production of the above factors and ultimately become influenced by them, partly through interaction with cell surface receptors and co-receptors, are equally multifactorial and complex. There is immediate haemorrhage into the lesion and an accumulation of haematogenous macrophages and immune-competent cells, associated with appropriate vascular reactions. Microglia and astrocyte activation quickly follows and, later, meningeal fibroblasts and new blood vessels invade the wound, leading to the deposition of a glial/collagen scar. Superimposed on this sequence of reactions there is usually a massive destruction of neurons and axons, accompanied by myelin sheath disruption and changes in the oligodendrocyte population. Subsequently, since neither neural replication nor sustained axon regrowth occur in the adult CNS, little or no recovery of neural connectivity ensues and lost functions are never restored.

As the database of knowledge on the subject expands, a growing optimism about the prospects of recovery from penetrant brain injury has developed based on experimental evidence demonstrating that most phases of the CNS injury response are therapeutically accessible. Accordingly, acute inflammation has been reduced, immune responses moderated, scar deposition lessened, neuron death and demyelination prevented, and axon regeneration promoted by pharmacological interventions which target vulnerable phases of the injury response, often with results graded in relation to the time of application. For example, cytokines mediate injury-responsive cellular reactions through a temporal cascade of factors, and thus therapy early in the cascade precipitates broad effects, like the inhibition of glial/collagen scarring with acute-phase administration of TGF-β antagonists, whilst delayed therapy does not influence inflammation but neutralises more specific downstream cellular responses such as matrix deposition mediated by CNTF, for which TGF-β is a late-phase activator. Future potential clinical applications will draw on laboratory experience in the use of neutralising antibodies, cytokine antagonist and protagonist, neurotrophins, and protease inhibitors, administered either as recombinant molecules or through gene vector delivery techniques.

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Each chapter in the monograph is self-contained and designed to benefit the casual reader, the active researcher, and the medical practitioner by providing a record of recent advances which point the way to future developments ultimately applicable in the clinic.

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Editors

Martin Berry, M.B., Ch.B., B.Sc., Ph.D., D.Sc., M.D., FRCPath, is currently investigating *in vivo* neurotrophin stimulation of axonal regeneration and inhibition of scarring in the visual system, cerebral cortex, and spinal cord, as well as growth factor control of the development of oligodendrocytes in the anterior medullary velum at Guy's Hospital in London. He accepted the post of Professor of Anatomy and Chairman of the Division of Anatomy and Cell Biology at the hospital in 1982, where he continued research into scarring and regeneration of axons in the CNS, the development of CNS glia, and myelination and remyelination.

He is a graduate of Birmingham University and obtained a Lectureship in the Department of Anatomy at the university in 1969, where he pursued a career in teaching of basic medical science and research into development of the cerebral and cerebellar cortices.

Professor Berry is a member of the Scientific Committee of the International Spinal Research Trust, Scientific Advisory Panel of the Brain Research Trust, Chairman of the Neuroscience Centre at UMDS in London, Editorial Board of the *Journal of Neurocytology*, and the Core Advisory Group for the Royal College of Surgeons.

Ann Logan, Ph.D., received her B.Sc. from the University of London in 1974 and her Ph.D. in Endocrinology from the University of Birmingham in 1978. After postdoctoral training at the University of Leeds and in the laboratory of Dr. Andrew Baird at The Whittier Institute in La Jolla, CA, Dr. Logan established her own Molecular Neuroscience Group at the University of Birmingham in 1990. She is currently Reader in Molecular Neuroscience in the Department of Medicine.

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Dr. Logan's research interests center on the role of growth factors in the scarring and regeneration responses of the mammalian CNS. She is particularly interested in the role of TGF-β in scar formation in the brain and spinal cord and is currently investigating the therapeutic potential of TGF-β antagonists as antifibrotic agents in the injured CNS. In addition she is currently investigating the potential for combined treatments of antifibrotic agents with neurotrophic factors in order to promote functional reconstruction of damaged neural pathways in the brain, visual system, and spinal cord.

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1 Cellular Responses to Penetrating CNS Injury

Martin Berry, Arthur Butt and Ann Logan

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1.1 INTRODUCTION

Three distinct sequential cellular responses characterise the reaction of the adult spinal cord and brain to injury. An acute *haemorrhagic phase* immediately ensues after wounding, in which haematogenous cells flood the lesion site. This is followed by a *subacute period* during which macrophages clear necrotic debris, glial cell reactions are mobilised, the clot becomes organised, and scarring is initiated. Finally, the scar tissue contracts during a *consolidation phase*. Superimposed on the above primary inflammatory/scarring responses are secondary neuronal degenerative and regenerative reactions to injury, accompanied by demyelination and remyelination. The interrelations between primary and secondary responses are not understood. It was once thought that scarring arrested axon regeneration in the central nervous system (CNS), but more recent experimental data indicate a contrary proposition that regenerating axons actually prevent scarring, possibly by protease release, and thus scarring could be a consequence rather than a cause of the failure of axons to regenerate in the CNS.

Pharmacological strategies for the control of the cellular injury responses after CNS injury aim to:

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- Modulate acute inflammation to reduce oedema and necrosis in the neuropil about the wound
- Decrease the density of deposition of the glia/collagen scar to create an environment favourable for the regrowth of axons through the injury site
- Maintain the viability of neurons by controlling both excitotoxicity and the release of proteases from macrophages
- Remyelinate both demyelinated intact fibres and regenerated axons to reinstate normal conduction velocities
- Promote regeneration of the severed axons with the ultimate aim of restoring lost function

Many aspects of the injury response in the neonatal CNS are atypical and unlike those of the mature animal. Thus, although the acute haemorrhagic phase is similar, no scar tissue is deposited and axons and dendrites grow *de novo* through the wound, obliterating the site of the original lesion. In the rat, the mature injury response is attained early during the neonatal period. In the cerebrum, for example, a mature scar develops over a transition period of from 5 to 8 days postnatum (dpn). Although the factors controlling maturation are presently unknown, an ultimate pharmacological goal is to replicate a neonatal reaction to injury in the adult through an understanding of the biology of acquisition of the mature CNS injury response in the neonatal period.

1.2 INFLAMMATION/SCARRING RESPONSES TO INJURY IN THE ADULT CNS

1.2.1 Acute Haemorrhagic Phase – 0 to 3 Days Postinjury (Figure 1.1)

All penetrant wounds in the CNS impale the glia limitans externa and occasionally the cerebral ventricles are also entered through puncture of the ependyma. The blood-brain barrier is also breached through the severance of blood vessels and thus haemorrhage into the lesion, subarachnoid space, and ventricular system are a sequelae of these insults, carrying serum, platelets, neutrophils, monocytes, and macrophages into these areas. Leukocytes are also recruited into the damaged brain parenchyma, mediated by interactions with endothelial addressins expressed in the vasculature about the wound and by the release of chemokines from cells in the damaged neuropil.[1] α-Chemokines (e.g., interleukin-8 [IL-8]) and neutrophil-activation protein 2 [NAP-2]) attract neutrophils, β-chemotactins (such as monocyte chemotactic protein (MCP) and macrophage inflammatory proteins (MlP-1α and MlP-1β) chemoattract monocytes, the γ-chemokine (lymphotactin) recruits lymphocytes, and the δ-chemokine (neurotactin), a specific brain chemokine expressed by reactive microglia, appears to have a specific role in brain inflammation.[2] The adhesion of neutrophils to the perilesion vasculature leads to the loss and/or redistribution of tight junction proteins with subsequent failure of tight junction integrity, causing a breakdown of the blood-brain barrier with an exacerbation of tissue damage by oedema.[3-5] Accordingly, neutrophil depletion is likely to be beneficial in the future treatment of brain/spinal cord trauma.

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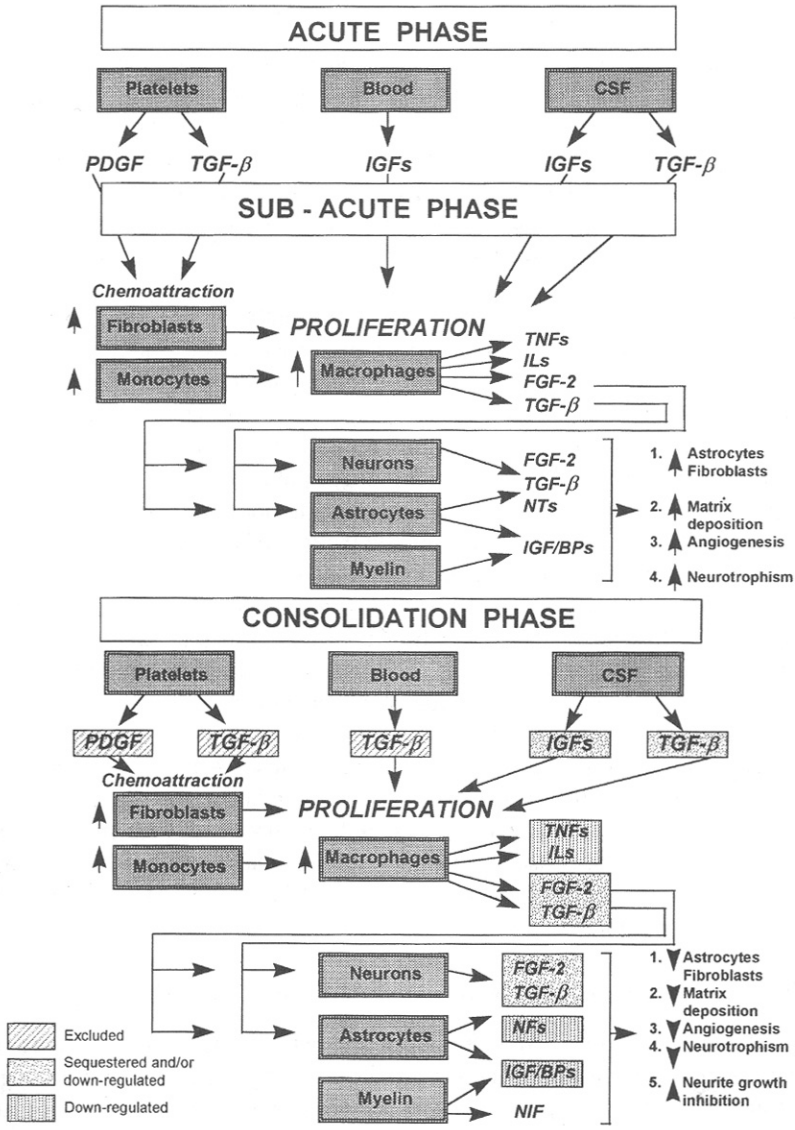

FIGURE 1.1 Up- and down-regulation of the trophic cascade initiated in the adult CNS by a penetrating lesion. In the acute and subacute phases, upregulation of numerous trophins occurs and the source, range, and interaction of the specific growth factors and cytokines released and expressed in the wound is illustrated. During the consolidation phase trophins are excluded, sequestered, or their synthesis is down-regulated as the major cellular events reach completion. PDGF — platelet-derived growth factor; TGF-β — transforming growth factor β; IGFs — insulin-like growth factors; BPs — insulin-like growth factor binding proteins; FGF-2 — fibroblast growth factor 2; TNFs — tumour necrosis factors; ILs — interleukins; NIF — neurite growth inhibitory factors; CSF — cerebrospinal fluid; NTs — neurotrophins. (From Logan, A., Oliver, J. J., and Berry, M., *Prog. Growth Factor Res.,* 5, 1, 1994. With permission.)

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Other events probably contributing to the development of acute oedema include the delivery into the wound of platelet-derived growth factor (PDGF) and transforming growth factors β(TGF-βs) by platelet lysis. The latter cytokine has been implicated as a prime organiser of a cascade of events which control many of the subsequent cellular responses[6] (Figure 1.1). Monocytes and macrophages also appear in large numbers at the wound margins, probably homing into the lesion in response to both platelet-derived factors from the clot and also through the expression of vascular addressins by the endothelium of the perilesion vasculature and the counterreceptors on leukocyte membranes.[7] Most monocytes entering the wound ultimately transform into macrophages.[8,9]

Perivascular brain macrophages,[10] which normally occupy space between the basal lamina and the endothelium of the cerebral vasculature, and are also found in the pia mater, probably become displaced into the parenchyma after penetrant brain injury. At first, macrophages remove erythrocytes from the haemorrhagic core of the wound. The volume of the core is thereby reduced and becomes filled with masses of macrophages and monocytes and a few neutrophils, all of which release a range of trophic cytokines into the wound including tumour necrosis factors (TNFs), interleukins (ILs), TGF-βs, fibroblast growth factors (FGFs), and insulin-like growth factors (IGFs) which also induce the release of endogenous trophic factors from target glia, and probably neurons as well[6,11,12] (Figure 1.1). Also, within the first 24 h microglia are activated.[13-15] They withdraw their processes and express major histocompatibility antigens (MHC I and II) and leukocyte common antigen (LCA), and also have elevated levels of nucleoside diphosphatase (NDPase) and complement type 3 receptor (CR3) recognised by the 0X-42 antibody. They migrate and accumulate about neuronal debris, which they phagocytose. Astrocytes in the neuropil surrounding the lesion also become reactive, upregulating the expression of glial fibrillary acidic protein (GFAP).[16,17] Although mature astrocytes may proliferate about the lesion,[18-20] the consensus favours the view that reactive astrocytes appear about the wound as a result of the upregulation of GFAP in existing astrocytes rather than by migration and/or mitosis.[21]

1.2.2 Subacute Phase – 3 to 8 Days Postinjury (Figure 1.1)

During the subacute period, the number of haematogenous cells in the core of the lesion is reduced and the endogenous glial reaction by astrocytes and microglia is augmented. Necrotic neuropil is removed and the wound margins become organised by astrocyte processes to form the glial component of the scar about the central mesenchymal core, into which meningeal fibroblasts have migrated. The latter cells deposit matrix material into the core of the wound including collagens, fibronectins, laminin, tenascin, and sulphated chondroitin and keratin proteoglycans. A basal lamina is deposited at the interface between core and astrocyte processes. The scar thereby reconstitutes a glia limitans (sometimes called the accessory glia limitans) over the exposed parenchymatous surfaces of the original penetrant cavity — the astrocytic, basal lamina, and mesenchymal parts of which become contiguous with the complementary laminae of the glia limitans externa.[17,22]

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1.2.2.1 Reaction of Astrocytes to Injury

The intercellular matrix molecules chondroitin and keratin sulphated proteoglycans and tenascin, produced by reactive astrocytes at the lesion site,[23-29] are all implicated in inhibiting the growth of fibres regenerating after injury (see later). The upregulation of GFAP after wounding is not confined to cells in the region of direct injury, but also extends into the undamaged neuropil. In the cerebrum, for example, most astrocytes in the lesioned hemisphere become intensely GFAP positive during the first week after wounding.[16] Astrocyte processes accumulating at the interface between the viable neuropil and the mesodermal core produce a glia limitans rich in collagen types IV and V[30] and laminin.[17,22] The formation of the accessory glia limitans begins at the pial surface as an extension of the glia limitans externa and progresses over the exposed surfaces of the neuropil into the depths of the wound, completely investing the penetrant cavity by the end of the subacute period. The cavity itself becomes filled with macrophages and also fibroblasts migrating in from the pia, and is later permeated by blood vessels formed by neovascularisation. All these elements eventually replace the blood clot.

The factors mediating astrocyte reactivity, as measured by the upregulation of GFAP, are manifold and have been summarised by Eng[31] (Figure 1.2). After a penetrant brain injury, it has long been thought that serum flooding into the neuropil contacts astrocytes and triggers their activation.[32] GFAP is upregulated and proliferation is induced in cultures of astrocytes by the application of a number of growth factors and hormones present in the blood[33-35] and, both *in vivo* and *in vitro*, by other serum constituents including albumin,[36] thrombin,[37-39] angiotensin II,[40] cAMP,[41-43] and inflammatory cytokines.[44-47] Degenerating neuronal somata and their processes might also release synaptic mediators which could activate the GFAP gene.[41,48,49] Astrocyte processes are linked by gap junctions[50,51] and may form a functional network in the brain by signalling to one another through intracellular Ca^{2+} wave propagation,[36,52,53] providing a mechanism for spreading GFAP reactivity within the vicinity of the wound. Eddleston and Mucke[54] reviewed the protective role of the astrocyte reaction to injury which, aside from repair of the blood-brain barrier, includes (1) remodelling of the extracellular matrix of the scar and the clearance of debris by protease secretion; (2) release of cytokines, including TGF-βs and ILs, which mediate the inflammatory reaction; (3) secretion of neurotrophins (e.g., FGFs and IGFs) which enhance neuron survival; (4) production of transporter molecules and enzymes for the metabolism of excitotoxic amino acids; and (5) reactive astrocytes which may also transform monocytes into microglia to establish the primary population of microglia in the CNS during development.[55,56]

Two subtypes of astrocyte have been recognised *in vitro*, type 1 and type 2.[57,58] Type 1 cells are analogous to GFAP-positive protoplasmic and fibrous astrocytes, but type 2 cells are thought to be a specialised glial astrocyte derived from a bipotential progenitor cell which also produces oligodendrocytes. The type 2 astrocyte was claimed to exist *in vivo*, confined to myelinated tracts, with processes which ramified about the nodes of Ranvier, subserving a specialised but as yet undefined perinodal function.[59-60] After injury it was thought that type 2 astrocytes largely died,

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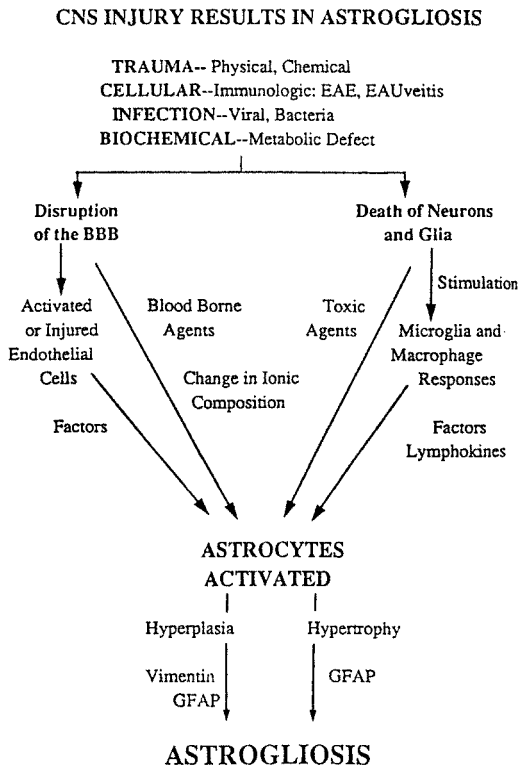

FIGURE 1.2 Flow chart of the possible sequence of events leading to activation of astrocytes and astrogliosis. (From Eng, L. F., *The Biochemical Pathology of Astrocytes*, Alan R. Liss, New York, 1988. With permission.)

suggesting that reactive gliosis was an exclusive property of the type 1 subpopulation.[61] The results of studies in the rat optic nerve combining the techniques of intracellular dye injection of single astrocytes with electron microscopy have challenged the existence of these two astrocyte subpopulations, since the processes of all cells have both nodal extensions and end-feet abutting the basal lamina of the vasculature and the glia limitans externa, at least in the optic nerve.[62,63] Moreover, after enucleation, reactive astrocytes in optic nerves undergoing Wallerian degeneration are all of the same morphological phenotype with end-feet contributing to both the pial and vascular glia limitans,[64,65] exhibiting less complex branching patterns, and becoming predominantly longitudinally orientated. Some cells, however, do transform into a unique GFAP+/vimentin-hypertrophic form.

A small, irregularly shaped stellate type of glial cell which constitutively expresses a chondroitin sulphate proteoglycan recognised by the NG2 antibody is found in the mature CNS.[66] The cell has thin, highly branched processes which are orientated randomly within grey matter, but run parallel to axons in tracts. Despite being neither GFAP+, S-100+, nor vimentin+, they have been classed as protoplasmic astrocytes on the basis of their fine structural characteristics. In the immature

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brain, NG2+ cells express PDGF-α receptor, and are considered to be oligodendrocyte progenitor cells.[67-71] In the adult brain, most NG2+ cells are also PDGF-α receptor+,[69,71] suggesting an origin from the O-2A progenitor lineage representing either adult progenitor cells,[72-74] or perhaps type 2 astrocytes, although the absence of GFAP would contraindicate this latter proposition. NG2+ cells in the adult CNS become reactive in experimental autoimmune encephalitis (EAE),[75] and after brain injury,[76] increasing in both cell number and staining intensity and also shortening and thickening their processes.

1.2.2.2 Reaction of Oligodendrocytes to Injury

Within the acute period, axons severed by a penetrant injury of the CNS start to degenerate and their myelin sheaths undergo secondary degeneration; primary demyelination may also be initiated as a consequence of the acute inflammation.[77] In the subacute period, demyelination and the associated cellular reactions become florid. Oligodendrocytes lose their characteristic morphology when dissociated from myelin sheaths[64,78-81] and elaborate fine attenuated processes which ramify within the demyelinating/degenerating axon bundles. It is generally accepted that mature oligodendrocytes are not dependent on axons for their continued survival. In the absence of axons, oligodendrocytes continue to express carbonic anhydrase II (CA II) and the myelin-associated proteins such as myelin basic protein (MBP), myelin oligodendrocyte protein (MOG),[65] myelin oligodendrocyte-specific protein (MOSP), and 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP).[82] Moreover, many oligodendrocytes continue to form myelin,[83] and appear to maintain cytoplasmic continuity with aberrant loops and whorls of myelin.[64,83] An intriguing possibility is that the myelin debris which persists within CNS lesions may be supported by surviving oligodendrocytes, thus explaining why myelin bodies continue to express both CA II and myelin proteins months or years after axon degeneration — long after the half life of these myelin-associated molecules has expired.

The question of whether the original population of mature oligodendrocytes reacts to injury by proliferation is conjectural. There is certainly evidence of increased numbers of oligodendrocytes after wounding,[84,85] but it is unclear if these cells arise from mitosis of dedifferentiated mature cells or from an independent adult progenitor pool.[72-74,86] Despite the survival of mature oligodendrocytes and the formation of new cells, there is only limited remyelination of the demyelinated axons and of regenerating fibres in and about the lesion.[83] The ensuing conduction block has grave consequences for the restoration of functional recovery although the potassium blocker, 4-aminopyridine, offers the potential of restoring normal propagation, thereby improving neurological function in chronic spinal injury both in animal models and human subjects.[87,88]

1.2.2.3 Reaction of Microglia to Injury

The numbers of resident microglia in the normal brain are stable, but after trauma there is hyperplasia, particularly about the wound.[81] New microglia probably derive from the endogenous resting population rather than from transformed monocytes

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invading the lesion from the blood.[89,90] Reactive microglia withdraw their processes, increase the expression of CD4, ED1, OX42, MHC class I and II antigens, secrete cytokines (e.g., TGF-βs, IL-1, and IL-6), and may become phagocytic, actively stripping synapses from postsynaptic sites,[91,92] and removing neuronal and glial debris.[81] Microglia release cytotoxins such as proteases, free oxygen intermediates, nitric oxide, arachidonic acid, quinolinic acid, and TNF-α, and also neurotrophins with the potential for promoting neuron survival and axonal regeneration.[77] These apparently paradoxical activities suggested to Banati and Graeber[93] that the cells have overall surveillance and protective functions after injury subserving both scavenger and neuroprotective/regenerative roles. Microglia may remain active indefinitely, providing a record of the site of past brain trauma. The immune functions of microglia are discussed in depth in Chapter 4 and other aspects of the wounding responses of microglia are covered in Chapters 5 and 7.

1.2.3 Consolidation Phase – 8 to 20 Days Postinjury (Figure 1.1)

The duration of this phase is variable and is marked by a volume reduction in the core of the lesion, compaction of subbasal lamina astrocyte processes, and a downregulation of GFAP about the wound. ED1+ microglia remain in the perilesion neuropil, but in the core of the wound most of the fibroblasts and macrophages disappear, although a few of each persist indefinitely.[22] The greatly contracted core remains rich in fibronectin and collagen.[30] During the subacute stage, astrocyte processes form an intensely GFAP+ multilayered palisade about the margins of the wound, but over the compaction period they either lose or contain less GFAP+ intermediate filaments. Processes become attenuated and thinned, bound to each other by multiple tight junctional complexes with minimal extracellular material between them. The laminin/collagen IV+ basal lamina of the accessory glia limitans coating the opposed faces of the lesion may thus become separated by a thin sheet of acellular connective tissue matrix contiguous with that of the pia mater. No axons traverse the lesion and, interestingly, no axons accumulate along the wound margins. Thus, in the absence of neuromatous formations about the scar it is difficult to defend the hypothesis that the cicatrix acts as an impenetrable barrier to the growth of axons.

1.3 INFLAMMATION/SCARRING RESPONSES TO INJURY IN THE FOETAL/NEONATAL CNS

The marked differences between scarring reactions in the skin of adult as compared with foetal/neonatal animals have long been recognised. The documentation of similar ontogenetic differences in the scarring reactions of the brain have come to light relatively recently.[23,94] Thus, although the acute haemorrhagic phase appears similar to that of the adult — with the invasion of haematogenous cells into the wound, the removal of necrotic tissue, and GFAP upregulation in astrocytes about the lesion — no scar is formed over the subacute period in the rat cerebrum lesioned before 8 dpn. The growth of glial and neuronal elements across the wound ultimately obliterates all signs of the original lesion site. Normal mature scarring is acquired slowly over the

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period of 8 to 12 dpn. Scarring first develops subpially as fibroblasts and macrophages invade from the meninges and over the 8- to 12-dpn transitional period these cells penetrate more deeply to ultimately fill the wound, apparently organising astrocytes to form a basal lamina where core cells become opposed to the latter.

The absence of an astrogliosis in the neonatal brain after injury could be related to the low titres of inflammatory cytokines[95] released by reactive microglia and macrophages, since the delivery of cytokines into neonatal brain wounds promotes scarring.[96,97] A capacity for basal lamina production by reactive astrocytes perinatally is also demonstrated by the observation that a breached glia limitans externa is invariably healed after penetrant lesions of the immature cerebral hemisphere.[94] Several recent findings suggest that it is the presence of growing axons in brain wounds which actively inhibits scarring. For example, axons and dendrites grow out of foetal brain grafts implanted into adult CNS and integrate well with host neuropil, with little or no scar tissue formed by the adult host about such grafts.[98,99] At the site of grafting a peripheral nerve into adult CNS, no scar tissue forms unless regeneration of CNS axons into the graft fails across the anastomosis.[100,101] When regeneration is promoted in the adult optic system by grafting Schwann cells into the vitreous body of the eye, the presence of masses of regenerating axons traversing optic nerve transection sites is invariably correlated with a failure to develop the basal lamina and mesodermal core components of the scar.[102,103] Moreover, delaying the time of grafting beyond that of maturation of the scar in optic nerve lesions (e.g., at 12 dpn) does not deter the regenerative response of the quiescent fibres arrested at the proximal edge of the scar. Delayed stimulation promotes florid regrowth, and the new axons penetrate the cicatrix in numbers comparable with those seen after Schwann cell implantation at the time of optic nerve lesioning, and extend into the distal optic nerve segment at least as far as the chiasm.[104] In the neonatal cerebrum, scarring develops between 8 to 12 dpn, when the period of establishment of the major tracts is coming to an end. After 12 dpn a mature scar is established in the wound and no axons accumulate in its walls or penetrate the structure.

Growing axons may inhibit scar production by releasing factors from growth cones which inhibit fibroblast migration into the wound and/or block the secretion of matrix components. Growth cones may also be capable of digesting a path through connective tissue extracellular matrix. All these properties might be attributable to metalloproteases and plasminogen activators, known to be released from growth cones during development.[105-110] Like axon growth and regeneration, protease gene expression is growth factor regulated.[111]

1.4 RESPONSES OF NEURONS TO INJURY

The somata of neurons respond to axotomy by chromatolysis in the adult;[112] those of neonates are more sensitive and degenerate.[113] The release of neurotoxins from reactive glia in damaged neuropil (see above) also causes neuronal cell death. Within wounds there are elevated titres of the excitotoxic amino acids, glutamate and aspartate,[114] released from damaged neurons and glia,[115] which activate *N*-methyl-D-aspartate (NMDA) receptors on neurons. The resulting raised intracellular levels

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of Ca^{2+} lead to protein breakdown, lipid peroxidation, and free-radical production. Excitotoxic injury can be blocked by a glutamate receptor antagonist.[116,117]

The distal segments of all transected axons degenerate together within the myelin sheaths although, as mentioned above, those myelin segments not dissociated from the oligodendrocyte process may remain viable. There is dieback of a variable segment of the proximal axonal stumps accompanied by Wallerian degeneration. The debris is cleared by both haematogenous macrophages and activated microglia, although degenerating myelin is slow to clear and may persist for months. There is also bystander degeneration of oligodendrocytes through cytotoxic activity, leading to secondary demyelination of uninjured axons. The capacity for remyelination of the latter axons and those which have regenerated is limited,[83] leading to a permanent conduction block and a poor prognosis for functional recovery.

Spontaneous axonal regeneration after CNS injury in adults has been observed only in poorly myelinated monoaminergic and cholinergic fibres,[118-119] neurosecretory axons,[120] fibres of the olfactory nerve within the olfactory bulb,[121] axons from foetal brain grafts implanted into the adult brain,[122] and fibres of the trochlear nerve within its CNS course through the anterior medullary velum.[123-125] All other axons in the mature CNS are incapable of regrowth after transection and currently acceptable hypotheses propose that (1) growth inhibition, (2) lack of trophic factors, or (3) a combination of (1) and (2) are explanations for growth failure.

Axon growth arrest after injury may be mediated by interaction between a growth-inhibitory ligand in the damaged CNS neuropil and receptors on growth cones.[126-128] Growth-inhibitory ligands have anti-adhesive and growth-cone-collapsing properties which either temporarily or irreversibly arrest axon extension.[129-132] Although a growth-inhibitory receptor has not been isolated, several candidate ligands with axon growth-blocking potency have been identified. The most important of these include myelin/oligodendrocyte-derived molecules,[133-135] and extracellular matrix molecules like chondroitin-6-sulphate proteoglycan,[24,136-141] and tenascin,[25,26,142-144] secreted by reactive astrocytes.

Recent data favours a lack of neurotrophic factors as a major cause of abortive CNS regeneration, since adult optic nerve fibres will regenerate across a transection site, invade the distal segment in large numbers,[102,104] and traverse the optic chiasm into the optic tracts[103] after the implantation of a Schwann cell graft into the vitreous body. The latter presumably provides a trophic stimulus to retinal ganglion cells which respond by regenerating their severed axons. Regrowth of the optic projection system is achieved without concomitant neutralisation of putative growth-inhibitory molecules in the optic nerve, thought to be concentrated in myelin membranes and on the plasmalemma of oligodendrocytes (see above), and which saturate the distal trajectory path throughout the nerve, chiasm, and tract for a protracted period after injury. Moreover, the scar does not constitute a barrier to regenerating axons, since growth cones both inhibit the *de novo* formation of a cicatrix and also digest a path through an established scar.[104] Accordingly, in addition to mobilising the axon growth machinery within an injured neuron, neurotrophins may downregulate genes for receptors engaging axon growth-inhibitory ligands and also activate those for the production and secretion of proteases.

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2 Cellular Responses to Ischaemic CNS Injury

William L. Maxwell

CONTENTS

2.1 INTRODUCTION

The brain has the richest blood supply of any organ in the body, the highest energy demand, and receives the largest proportion of the cardiac output. Perhaps the commonest cause of ischaemic injury to the brain in human beings is cardiac arrest

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where there is diffuse ischaemic damage over a very wide area of the brain,[1] but cerebrovascular accidents to vessels supplying the brain, reduction in cerebral perfusion due to periods of elevated intracranial pressure (ICP), and responses to trauma are also major sources of compromised blood flow. In these situations morphological evidence for ischaemic damage is obtained only in those parts of the brain where transient reductions in the cerebral blood flow (CBF) fall below certain critical values.

2.1.1 Reductions in Cerebral Blood Flow

Experimental work has demonstrated that there is not a single value of CBF below which level ischaemic damage is obtained. Rather, it is now acknowledged that there are two critical levels of reduced CBF. First, a reduction in CBF to values between 15 to 22 ml/100 g/min results in an immediate loss of neuronal function with abolition of electrocortigram and evoked potentials (EPs),[2-4] but once normalisation of blood flow occurs, even up to 1 h after cessation of that flow,[5,6] spontaneous cellular activity and EPs may be restored. Second, the development of irreversible, morphological damage is dependent upon two factors: the period of time that brain tissue is ischaemic and whether there is any residual flow at levels at or below 12 ml/100 g/min for periods of 2 to 3 h. But even in this condition it is clear that there is considerable variation in the susceptibility of neurons in different parts of the brain to ischaemic insult.[7] As a result of a fall in CBF below 18 ml/100 g/min, the threshhold for infarction,[8-10] the brain is exposed to hypoxia/anoxia which results in rapid loss of ionic homeostasis in both neurons and glial cells as a result of the energy failure giving rise to major changes in neuronal electrical activity, since the shortage of ATP disturbs ionic pump activity and there is an accumulation of Na^+ in neurons.[11,12] Long-term damage, on the other hand, has been suggested to be due to overstimulation of a combination of glutamate receptors[13] after abnormal release of excitatory neurotransmitters, disruption of Ca^{2+} homeostasis, generation of free radicals, activation of second messenger systems, and changes in gene expression.[12]

However, it is clear that ischaemic injury will affect the activity of all types of cells within the affected region of the brain. The purpose of this chapter is to provide an overview of cellular responses by all of the cell types found within the brain. These will be treated in alphabetical order rather than to give greater emphasis to changes in one cell type.

2.2 ASTROCYTES

2.2.1 Early Responses

A widespread early reponse by astrocytes is that they demonstrate swelling and cytoplasmic lucency within minutes of reduction in CBF.[14-16] However, there is increasing evidence that the microglial response (see below) precedes or at least parallels that of astrocytes. The most notable response by astrocytes occurs in perivascular foot processes (Figure 2.1), possibly related to the high concentration of transport systems in the membranes of these processes. Swollen astrocyte foot processes demonstrate a lucent cytoplasm lacking any content of cytoplasmic

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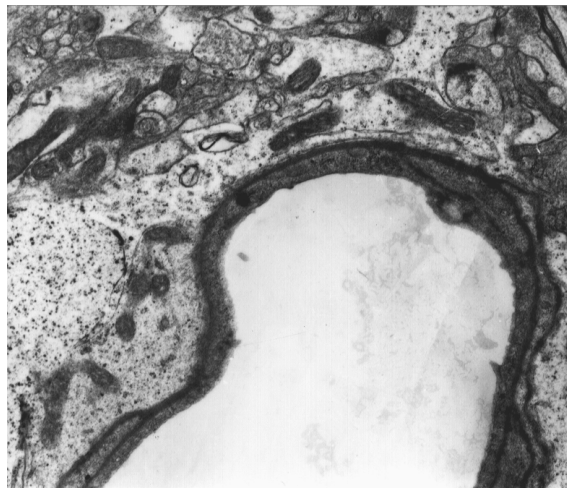

FIGURE 2.1 A transmission electron micrograph of part of the wall of an intraparenchymal blood vessel in the ischaemic region from a rat brain after endothelin-1 constriction of the right middle cerebral artery. Perivascular astrocyte foot processes are enlarged but contain mitochondria with a normal structure. (Original magnification × 13,600.)

organelles; however, mitochondria possess either a normal morphology or become contracted. This latter finding is perhaps indicative that astrocyte swelling is not a direct response to ischaemia/anoxia. Astrocytes *in vitro* do not swell during anoxic injury.[17] It has been suggested that astrocytic swelling is an exaggerated pathological extension of the normal astrocyte functions of regulation of extracellular ion levels and brain pH[18] such that factors released by injured neurons, for example, potassium, glutamate and lactate, among others, are ultimately responsible for astrocyte swelling.[18,19] The conclusion must be drawn that probably a number of different mechanisms lead to astrocytic swelling and that the precise interaction of these mechanisms may differ with the insult eliciting that swelling.

2.2.2 Differential Astrocyte Responses

There is a differential astrocyte response depending upon whether the ischaemic insult is long or severe enough to result in irreversible or reversible neuronal injury. In the former case there is somal swelling of astrocytes to a doubling of cell size from a control value of 59.2 ± 21.2 μm^2 to 122.7 ± 31.6 μm^2 within 3 h after 30 min of 4-vessel occlusion.[16] The cells become electron lucent with a reduced content of normal cytoplasmic organelles — for example, small stacks of rough endoplasmic reticulum cisternae, scattered microtubules, but no intermediate filaments in the cell soma. There is nuclear enlargement with a finely dispersed chromatin and an incidence of pleiomorphic and contracted mitochondria. On the contrary, however, in reversible ischaemic injury there is not a statistically significant increase in cell size at 2 h and the cell soma contains bundles of intermediate filaments. In this latter

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case astrocyte morphology is indistinguishable from control animals 24 h after ischaemia.[16]

In both types of ischaemic injury, swollen astrocytic processes extend through both the ischaemic core and for a considerable distance into the otherwise morphologically intact neuropil surrounding the ischaemic lesion.[20] Such astrocytic swelling probably results in a decrease in the extracellular space which has been documented in both ischaemia and spreading depression[21] and contusion injury to the human cerebral cortex.[22] But whether astrocytic swelling is the major or only mechanism leading to raised ICP has not yet been demonstrated experimentally.

2.2.3 Reactive Astrocytosis and GFAP Upregulation

Reactive astrocytes[19] are distinguished from swollen astrocytes by the occurrence of bundles of intermediate filaments, consisting of glial fibrillary acidic protein (GFAP) and vimentin, within the astrocyte cytoplasm. However, there is also an increase in the numbers of mitochondria, Golgi complexes, endoplasmic reticulum, lysosomes, microtubules, dense bodies, and lipofuscin pigment. There are differences between species as to the time at which these cells occur after a lesion. The response is maximal between 3 and 4 days in rats but not until 2 to 3 weeks in humans.[23,24] A number of proteins/chemicals are upregulated in astrocytes after ischaemic insult (reviewed by Norenberg).[19] GFAP is the most widely used marker for reactive astrocytes. Vimentin and S-100 protein occur in cells found at the site of a lesion.[19] Basic fibroblast growth factor (bFGF) and β-amyloid precursor protein (β-APP) may be synthesised by reactive astrocytes. However, a detailed consideration is beyond the scope of this chapter. The interested reader is referred to several review articles.[19,25]

The intimate role of perivascular astrocytes in the maintenance of the blood-brain barrier (BBB) is well established; but in models of brain ischaemia it has become established[18] that astrocytic swelling precedes the later breakdown of the BBB such that, although perivascular astrocytic swelling occurs within minutes of induction of ischaemia, extensive breakdown of the BBB starts at 4 to 6 h and becomes maximal only 2 to 4 days after induction of ischaemia.[26]

2.3 ENDOTHELIAL AND MICROVASCULAR CHANGES

2.3.1 Breakdown of the Blood-Brain Barrier

It is clear that the initiation of the breakdown of the BBB occurs within minutes of insult as demonstrated by the use of either [^{3}H] sucrose,[27] infusion with hyperosmolar L(+)arabinose[28] or horseradish peroxidase (HRP) tracer studies.[29] However, the opening of the BBB continues over several hours after an ischaemic insult and at least two additional phases of BBB opening may occur.[27] There is also evidence for a differential localisation of such openings between different parts of the brain. Thus, after bilateral carotid artery occlusion for 10 to 25 min, followed by recirculation, there is acute opening of the BBB in neocortical regions, possibly due to reactive hyperaemia,[30] with recovery suggested to occur by 24 h.[27] In the striatum and

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hippocampus regions, where neuronal death occurs one to several days after ischaemia, there is marked deterioration of integrity of the BBB at 24 h. This has been attributed to the release of excessive vasoactive neurotransmitter substances, for example, glutamate and eicosanoids.[31] A third opening of the BBB coincident with the development of oedema occurs in the cerebral cortex between 6 and 24 h of ischaemia and has been attributed to release of leukotrienes and arachidonic acid, lipid peroxidation, and platelet/leucocyte accumulation in injured tissue.[32]

2.3.2 Smooth Muscle and the Tunica Media

There is good evidence that both smooth muscle of the tunica media and the endothelium of the brain microvasculature respond rapidy to an ischaemic or other type of brain insult. Within 10 min of cardiac arrest in rats, followed by several hours of recirculation, transverse circumferential ridging of large arteries occurs that is suggestive of arterial vasospasm.[4] Analysis of thin sections of large arteries provides evidence for shortening of smooth muscle fibres in the tunica media (Figure 2.2),[33] and thus the arterial ridges (Figure 2.3) may be explained by localised contraction of muscle fibres in the arterial wall. When the period of ischaemia is increased to 2 h, ultrastructural changes occur more rapidly in smooth muscle than in endothelium.[34] Smooth muscle shows fragmentation of myofilaments, decreased density of the cytosol, swelling of mitochondria containing disorganised cristae, and oedematous swelling (Figure 2.2). Muscle cells show marked degeneration with condensation of nuclear chromatin and further oedematous swelling of the cytosol

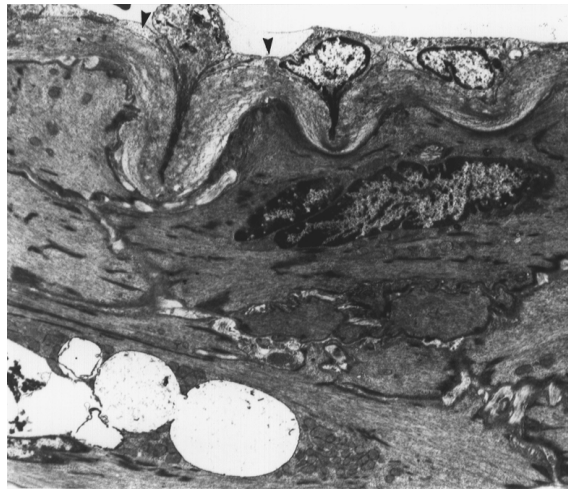

FIGURE 2.2 A transmission electron micrograph of the luminal aspect of the wall of a large cortical arterial branch of the middle cerebral artery of the rat after application of endothelin-1 to the latter. Endothelial cells are lucent with a vacuolated cytoplasm (top), there is denudation of the basal lamina (arrowhead), and there is structural damage to smooth muscle cells in the tunica media (lower half of figure). (Original magnification × 3,600.)

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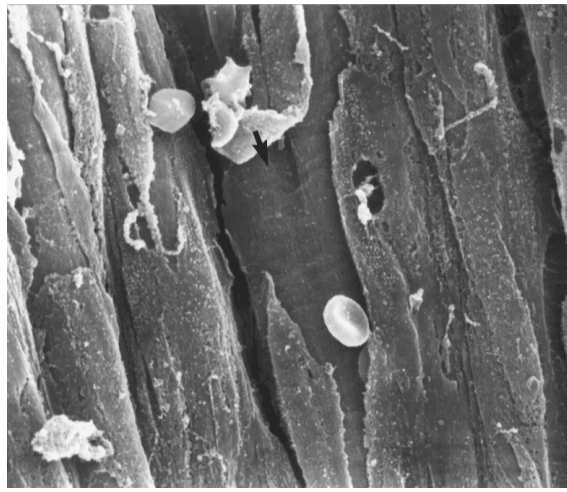

FIGURE 2.3 A scanning electron micrograph of the luminal aspect of the wall of a large cortical branch of the right middle cerebral artery of the rat after application of endothelin-1 to the latter artery. There is marked ridging of the wall, the occurrence of numerous holes in the endothelium, and a zone of endothelial denudation (arrow). (Original magnification × 2,000.)

after an 8-h occlusion of the middle cerebral artery.[34] With recirculation, necrotic and lytic smooth muscle cells allow penetration by erthrocytes and platelets. In addition, some arteries are occluded by thrombi.[34] It may be worth noting[34] that comparable changes occur much less frequently on the venous side of the circulation.

2.3.3 Endothelial Responses

Changes in the morphology of the endothelium of the brain vasculature after ischaemia have been demonstrated by means of both transmission and scanning electron microscopy. The latter, in particular, has supplied quantitative data for such changes. But this sort of information may only be obtained from studies that present a clear record of the site within the brain providing the data. A large proportion of studies do not provide such detailed data. Thus, it is difficult to compare different experiments because the precise area sampled is unknown. It is therefore suggested that a more rigorous experimental procedure would considerably enhance the majority of experiments and allow a more realistic comparison between them.

Nonetheless, with respect to the endothelium, morphological evidence has been obtained for a thickening of junctional leaflets between endothelial cells, for the occurrence of large numbers of endothelial microvilli (Figures 2.3 and 2.4), for endothelial pits either located randomly on the endothelial luminal surface or in close relation to the limiting tight junctions of these cells (Figure 2.4), for an increased number of pinocytotic openings on the luminal aspect of the endothelium, and for endothelial denudation exposing the underlying basal lamina (Figure 2.3). Recent evidence has demonstrated that damage to endothelial cells in sites of opening

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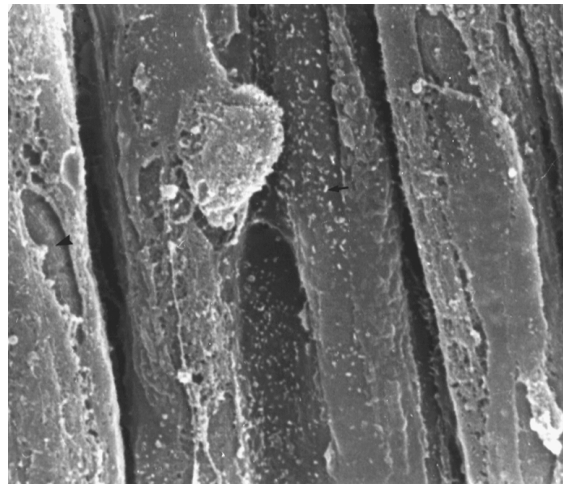

FIGURE 2.4 A scanning electron micrograph of the luminal aspect of the wall of a large cortical branch of the right middle cerebral artery in a rat after application of endothelin-1 to the latter artery. The endothelium demonstrates numbers of endothelial pits (arrowhead) and numerous microvilli (arrow). Note the variation in the number of microvilli between adjacent endothelial cells. (Original magnification × 3,000.)

of the BBB, as reflected either by immunocytochemical labelling for protein A[28] or use of HRP tracer studies,[29] results in both patency of interendothelial junctional complexes and passage of tracer to the endothelial basal lamina through the cytoplasm of injured endothelial cells after hyperosmotic injury. It is also clear that these changes occur with differing degrees of severity in spatially closely related endothelial cells.[29] Thus, some endothelial cells demonstrate shrinkage or swelling (Figures 2.3 and 2.4) while other spatially closely related cells are morphologically unaltered.[29] Additionally, there is the postischaemic formation of numerous microvilli on the luminal aspect of endothelial cells, but imprecise definition of the sites of sampling in these experiments makes it difficult to compare them with others.

In an attempt to overcome the aforementioned experimental deficiencies, endothelin-1-induced constriction of the middle cerebral artery in the rat was used to provided ultrastructural evidence that endothelial responses differ between vessels of different calibres[33] and between different parts of the ischaemic brain. Thus, it has been shown that small arterioles and venules with calibres between 50 and 100 µm within the ischaemic brain demonstrate the greatest rise (by 169%) in number of endothelial microvilli. However, it must not be forgotten that such endothelial changes have also been documented in a wide variety of brain insults ranging from photochemically induced infarction[35] to models of head acceleration.[36] The quantitative data derived from the endothelin-1 model of ischaemic injury clearly demonstrate that endothelial microvilli occur in elevated numbers in both the ischaemic (+169%) and contralateral nonischaemic brain (+130%).[33] Thus, it may be suggested that endothelial and vascular responses probably should be regarded as generalised responses to any type of brain insult. It must also be acknowledged that the endothelial

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changes noted are transient. Thus, in the rat the occurrence of endothelial border thickening and numbers of endothelial microvilli are reduced after 24 h and blood vessel morphology is normal 7 days after complete ischaemia.[4]

2.3.4 Endothelial Denudation

A long-term response by the endothelium which seems to be exacerbated by recirculation has also been described. After a minimum period of a 2-h ischaemia followed by 2 h of recirculation, endothelial denudation exposing subendothelial tissues occurred in about a third of small arteries after occlusion of the middle cerebral artery of the rat.[34] When arterial occlusion was extended to 6 h, followed by 2 h of recirculation, endothelial denudation was more widespread to the extent that the entire luminal surface of the basal lamina was exposed. This allowed platelet adhesion and fibrin deposition to occur.[34]

2.3.5 Long-Term Changes

Only a small number of studies of microvascular responses to insult to the brain have provided quantitative and long-term temporal analysis. Opening of the BBB may, hypothetically, result either as the result of an opening of the interendothelial tight junctions, an increase in the number of so-called tubulovesicular profiles[28] and pinocytotic vesicles, or shrinkage or swelling of individual endothelial cells resulting in denudation of the basal lamina.[29] Recent work has suggested that the endothelial basal lamina rather than endothelial cells may be the major barrier to passage of extravasated protein.[28] Studies using tracers such as Evans blue (EB), horseradish peroxidase (HRP), lanthanum (La^{3+}), and immunocytochemical marking of intrinsic blood plasma albumin have been used to elucidate this process, but it is also becoming apparent that the pathological changes in blood vessels after ischaemia are not straightforward and may occur in a far wider area of the brain than that in which neuronal death eventually occurs. Earlier work with high molecular weight tracers (EB and HRP) failed to demonstrate opening of the BBB (reviewed in Sampaola et al.).[37] Neither was extravasation of low molecular weight markers (La^{3+}) obtained within 15 min of incomplete continuous ischaemia,[37] but between 30 and 60 min tracer was found in interendothelial clefts and the endothelial basal lamina, in the cytoplasm and mitochondria of perivascular astrocytes, and in the brain extracellular space of the area bordering the pale central zone of the lesion.[37] Thus La^{3+} extravasation occurs not in the ischaemic core where tissue water, Na^{+}, and Ca^{2+} content increase[38] but in the nonoedematous tissue bordering the ischaemic core. In addition, tracer studies using low molecular weight markers[37] and quantitative analysis of pinocytotic vesicle numbers in contusion[22] or head acceleration injury[36] have not provided evidence in support of the concept of increased transendothelial passage. Based upon the above data, the only clear evidence for opening of the BBB in ischaemic and other brain lesions is demonstrated by an opening of interendothelial tight junctions in vessels within the nonoedematous penumbra of the lesion some minutes after the ischaemic insult has occurred. Thus, the relocalisation of ions and water into the ischaemic core may not reflect opening of the

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BBB, but rather compromised membrane pump activity of cells within the core. It would, therefore, be of interest to investigate alterations in membrane pump activity in models of ischaemia using cytochemical techniques. Although it must be acknowledged that recent work does provide good evidence for both opening of interendothelial tight junctions and passage of tracer through the cytoplasm of damaged endothelial cells,[28,29] it must also be noted that present data relate to material only up to 30 min after insult. It will be of great interest to learn what changes occur in long-term survival of experimental animals.

2.4 MICROGLIA

Whereas astrocytes mainly respond to an ischaemic insult by swelling and hypertrophy, microglia, both parenchymal and perivascular, demonstrate a sequence of activation as demonstrated by progressive expression of major histocompatability complex (MHC) class I and II antigens, proliferation, and morphological changes reflecting transformation into brain phagocytes. However, in addition, microglia synthesise and/or release both cytotoxic factors and factors that may promote neuronal survival.[39]

2.4.1 Time Course

There are two factors that provide for variation in the time course of microglial activation: the model of ischaemic injury utilised in any particular study, be it global ischaemia induced by four-vessel occlusion or transient/permanent middle cerebral artery occlusion, or the detection method utilised to demonstrate activation. Nonetheless, there is increasing evidence for a common sequence for activation of microglia[39] even in areas of the brain where there is no loss of neurons.[40] For example, there is relatively rapid MHC class I expression on microglia, possibly mediated by widespread ischaemic neuronal depolarisation and changes in extracellular potassium levels.[41] There is a later expression of MHC class II antigen. However, the significance of this response at the cellular level in the ischaemic brain is as yet unresolved.

In those areas of the brain where there is postischaemic neuronal death, activation of microglia as early as 20 min after ischaemia in the stratum radiatum of the CA1 hippocampus is suggested by lectin histochemistry,[43] together with an upregulation of expression for MHC class I antigens (with most prominent labelling obtained on day 6[44]), CD4, and cell adhesion molecules.[42,45] However, no ultrastructural changes indicative of activation have been obtained until at least 24 h, and most notably 72 h after an ischaemic insult.[42] There is an increase in the transverse dimensions or hypertrophy of microglia and the assumption of an amoeboid or bipolar form similar to but not directly comparable to Nissl's rod cells observed in human neocortex after trauma or under conditions of inflammation.[46] The early activation of microglia has been suggested[39] to be a sensitive indicator of impending neuronal cell damage — for example, in layer 3 of the rat neocortex[47] or in columns of cortical neurons innervated by thalamic neurons.[39]

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At longer postischaemic survivals microglia proliferate during the first 48 h following ischaemia,[41,42] then develop into active phagocytes or foamy macrophages which remove "dark" degenerating neurons (see below) 3 days after global ischaemia. In addition, there is a differential time course of the microglial response between different regions of the brain, for example, after MCAO in the rat. The more rapid response occurs in the primary site of tissue damage and its penumbra within 24 h.[48] However, there is secondary or later activation of microglia as indicated by transformation into fully developed phagocytes in, for example, both ipsilateral and contralateral neocortex, thalamus, and hippocampus five days after insult and in the medullary pyramids and cervical corticospinal tracts four weeks after an ischaemic insult.[48] At 13 days postischaemia there is *de novo* expression of MHC class II antigen in the pyramidal cell layer of hippocampal region CA1. A further increase in immunoreactivity is obtained at 21 days, followed by decreased labelling at 28 days.[44] It has been suggested that the expression of MHC class II antigen, together with expression of leucocyte common antigen and CD4, reflect the activation of microglia for antigen presentation.[44]

2.4.2 Cytotoxic Factors, Growth Factors, and Cytokines

There is a considerable literature based upon *in vitro* studies that microglia release cytotoxic factors, microglial-derived growth factors, and cytokines. However the influence of these factors *in vivo* in the ischaemic brain is unresolved. This is particularly so since there is evidence that there are species differences as to sources, for example, of nitric oxide (NO). Microglia are the main source of NO in rodents while astrocytes are the source in human beings, although activity in the latter cells may be controlled by release of IL-1 by microglia.[49,50] In ischaemia, microglial-produced free radicals, nitric oxide proteinases, and glutamate seem to mediate a crucial role in neuronal damage.[39] However, between one and three days following global ischaemia, TGF-β1 is also induced in activated microglia.[51] This could have several functional implications. TGF-β1 inhibits proliferation of astrocytes[52] and has a downregulating effect on microglia,[8,53] thereby preventing gliosis. Thus, TGF-β1 synthesis by microglia may represent an intrinsic CNS response to ischaemia by serving to limit the extent of tissue damage.[39] Lastly, β-APP may either be synthesised or internally localized in microglia, among other reactive glial cells such as astrocytes and oligodendrocytes. However, the level of expression for β-APP in microglia is generally lower than in other glial cell types and is limited to the immediate vicinity of infarcted tissue.[39] Thus, our understanding of the biological significance of upregulation of cytotoxic factors, cytokines, and growth factors by microglia after ischaemia is limited and requires much further work.

2.5 NEURONS

2.5.1 Neuronal Susceptibility

Transient arrest of cerebral circulation leads to neuronal cell death in selectively vulnerable regions of the brain. However, in ischaemia followed by recirculation

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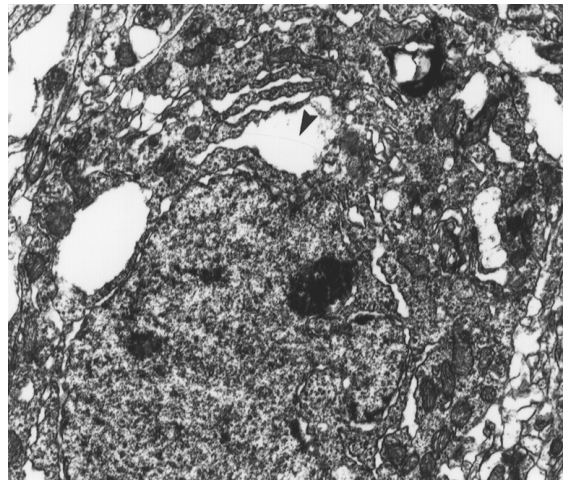

FIGURE. 2.5 A transmission electron micrograph of an ischaemia-susceptible neuron 24 h after constriction of the right middle cerebral artery in the rat. The most marked change is the occurrence of clefts (arrowhead) in the cytoplasm. Mitochondria demonstrate a normal morphology. (Original magnification × 10,500.)

such as occurs after cardiac arrest, it is clear from animal models that there are two different responses by neurons, with increasing length of survival after such an insult. It is also clear that there is a wide sprectrum of susceptibility to ischaemic insult by neurons in different regions of the brain. Neurons of the CA1 region of the hippocampus are particularly susceptible to ischaemic insult. Susceptible neurons also occur in layers 3 and 4 of the cerebral cortex, in thalamic nuclei such as the dorsal medial nucleus and central amydala, and in the globus pallidus and caudate nucleus.

2.5.2 Two Types of Neuronal Response

Morphological responses by neurons are grouped either as the so-called "dark" and "light/pale" types. These neurons may also be termed ischaemia susceptible and ischaemia resistant, respectively. In the former, cresyl violet staining of neurons results in numbers of dark or heavily stained, shrunken cells surrounded by clear halos, with loss of Nissl substance early after resuscitation of cardiac activity. At the ultrastructural level a high proportion of both ischaemia resistant and ischaemia susceptible neurons demonstrate transient swelling of mitochondria. With continued reperfusion this swelling is resolved, but a high proportion of both resistant and susceptible cells are shrunken with the loss of polyribosomes, RER, microtubules, and Golgi apparatus.[54-56] With periods of reperfusion lasting up to 24 h, however, there is a differential response between ischaemia susceptible and resistant cells. In the former there is focal swelling of RER cisterns in a small proportion of cells to form nonmembrane-bound clefts in the cytoplasm[54] (Figure 2.5). Neurons are surrounded by electron-lucent, swollen perineuronal neurite and astrocytic processes.[54,57] With recirculation, cell loss has been noted from 6 h and increasing numbers of neurons assume a spindle shape with pycknosis and vesiculisation of

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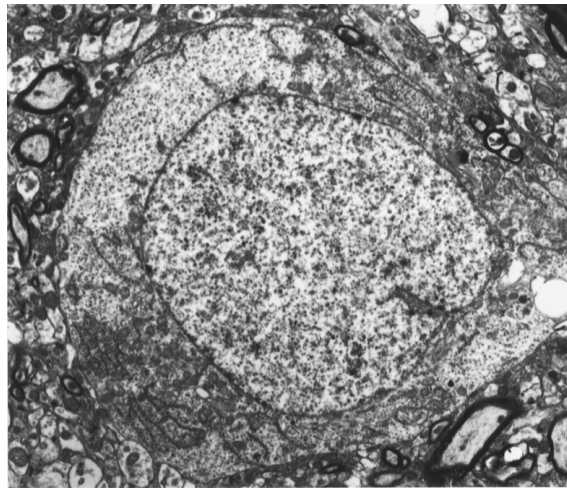

FIGURE 2.6 A transmission electron micrograph of a "light/pale" neuron. There is loss of Nissl substance and a dearth of membranous organelles in peripheral regions of the cell cytoplasm. (Original magnification × 4,000.)

apical dendrite cytoplasm.[58] After periods of recirculation up to 48 h there is a clear differential between ischaemia susceptible and resistant cells. In the former there is "peripheral chromatolysis"[54] with clustering of cytoplasmic organelles around the eccentric and irregularly shaped nucleus in about half the population of CA1 neurons. In a small proportion of cells, however, there is ischaemic cell change,[57] where neurons are shrunken with electron-dense cytoplasm, pyknotic, fragmented nuclei, and disruption of plasma membranes. By 72 h more than 60% of CA1 neurons demonstrate this morphology, but in resistant CA3 neurons there are increasing numbers of recovering normal cells such that 90% of neurons are similar to controls after 72 h of recirculation. Only about 6% of control numbers of CA1 pyramidal neurons are present at 3 weeks, and 13% at 10 months, but at the latter time the thickness of the hippocampal layers is reduced by more than 50%.[59]

In the "light/pale" response there is the impression of an increase in cell size with clustering of organelles around the crenated nucleus, resulting in a lucency of the peripheral cytoplasm in which cytoplasmic organelles are scarce (Figure 2.6). In these cells there is also an increase in the size of the nucleolus. The "light" cells remain in some numbers scattered among intact or normal neurons in long-term survival, up to 10 months, after cardiac arrest. In these cells the nuclear chromatin is finely aggregated, the nuclear envelope is intact, and crenations are absent. The cytoplasm contains aggregates of lipofuscin granules and lysosomes. Ribosomes form aggregates or polysomes. The peripheral rim of the cytoplasm is void, providing the "pale" appearance of these cells.[59] Although the present evidence is still largely anecdotal, there is now consensus in the literature that these "dark" and "pale" changes reflect the incidence of two discrete pathologies in neurons after an ischaemic insult. "Dark" neurons do not occur between 6 and 10 months after transient cardiac arrest,[59] while "pale" cells are still numerous, occurring among

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morphologically intact cells. This has led to the suggestion that morphological changes in "pale" neurons, including both pyramidal and interneurons, are reversible[59] and allow recovery after ischaemia.

2.5.3 Pathological Mechanisms

2.5.3.1 Calcium

As alluded to earlier, long-term damage to cells of the brain has been suggested to result from disruption of Ca^{2+} homeostasis, generation of free radicals, and activation of second messenger systems.[12] In experimental cortical ischaemia, total tissue content of calcium rises during reperfusion, most particularly following prolonged ischaemia.[38,60] Use of the pyroantimonate cytochemical technique has demonstrated accumulation of calcium in swollen mitochondria in neurons showing cytoplasmic vacuolation 30 min after induction of ischaemia,[61,62] but when ischaemia is followed by reperfusion pathological changes are exacerbated. For example, the nucleus has an irregular profile with condensation of chromatin, and mitochondrial morphology is grossly disrupted with the assumption of a spherical shape and almost complete loss of cristae.[61,62] More recently, mitochondrial disruption has been shown in central, myelinated fibres in an *in vitro* preparation of hypoxic injury.[62] In addition to mitochondrial changes in axons there was loss of axonal microtubules and disorganisation of neurofilaments.[63,64]

2.5.3.2 Cytoskeletal Proteolysis

Immunocytochemical techniques have provided evidence of a disruption of the neuronal cytoskeleton after transient ischaemia. Increases in intracellular calcium induced by ischaemia/hypoxia have been suggested to result in increased cytoskeletal proteolysis by means of activation of calpains,[63-66] and fairly recent evidence has demonstrated degradation of structural proteins after just 5 min of hypoxia.[66] This degradation is inhibited by perfusion with calpain inhibitor.[66] However, care is needed in interpretation of these results because it has been shown that there is a differential distribution of neurofilament proteins between different neuronal types or even between different parts of neurons.[67,68] For example, CA2 and CA3 pyramidal neurons are richly labelled by NF-68 but CA1 cells are only weakly so. Dendrites of CA1 pyramidal cells are labelled heavily with antibodies to NF-200 while dendrites of CA3 pyramidal cells are more heavily labelled for NF-68.[68] Within 1 day of ischaemia there is loss of labelling for NF-68, with an almost complete disappearance in the CA1 alveus by 4 days when pyramidal cells in that area have degenerated.[68] At the same time, however, neurons in CA3 are labelled similarly to those in normal brain. Reduction in intensity of labelling for NF-200 occurs more slowly, between 2 and 3 days of ischaemia, with almost total loss by 4 days in the CA1 alveus. Again, labelling in CA3 is similar to normal animals.[68] Thus, although there is therefore increasing evidence that proteolytic events occur rapidly after ischaemia, the direct correlation of proteolysis with cell death between 2 and 4 days is still not possible since proteolysis does not increase *in vitro* beyond initial levels with further oxygenation.[66] However, it may now be suggested that there are differences

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in the postischaemic proteolysis of the cytoskeleton between susceptible and non-susceptible cells after ischaemia and that this difference reflects a differential biochemistry of the cellular cytoskeleton.

2.5.3.3 Membrane Damage

Activation of calpains in ischaemic neurons necessitates uncontrolled influx of calcium through altered membrane function and/or damage. Oxygen free radicals primarily damage lipids in susceptible neurons during recirculation.[71] The Golgi apparatus is a central component of the system that maintains the integrity of the plasma membrane. Both reversible and irreversible alterations in the morphology of the neuronal Golgi have been noted in a number of studies following ischaemia,[54-56] with reappearance of Golgi cisternae in the former and progressive dilation in the latter in selectively vulnerable CA1 hippocampal neurons but not in cortical neurons. Recent evidence has indicated that structural alteration in the Golgi apparatus, in fact, is more marked than disruption of RER.[56] It has been suggested first that alterations in the structure of the Golgi apparatus may be a more important marker of lethal injury than release of ribosomes from the endoplasmic reticulum, and second that this disruption may be correlated with lipid peroxidation.[56] This lipid peroxidation has been suggested to reflect attempts by ischaemic neurons to recycle and repair damaged plasma membrane. It is an attractive hypothesis that a damaged membrane allows influx of toxic levels of ions and molecules. Possibly, the early response by the Golgi apparatus is an attempt by injured neurons to repair membrane structure to overcome such damage. Recent cytochemical evidence has suggested such a response in axons after stretch injury.[72]

2.5.3.4 Excitotoxicity

It has been suggested that a major component of the postischaemic calcium level results from accumulation of endogenous glutamate and aspartate (reviewed by Michaels and Rothman[73]). Microdialysis studies have demonstrated that glutamate is excessively released during transient cerebral ischaemia in the CA1 region of the hippocampus where neurons are susceptible to ischaemic injury.[73-75] Pyramidal neurons in the CA1 field show complete neuronal death after a 5-min ischaemia as a result of occlusion of the internal carotid artery in the gerbil.[75]

There are two discrete sources of glutamate release. With ischaemia lasting up to 5 min, glutamate is released from neuronal elements with small energy stores as a result of energy failure, such as presynaptic terminals and postsynaptic neurons which are either lost or swollen. Glutamate release occurs later from neuronal elements with larger energy stores and only from astrocytes if ischaemia lasts for 20 min or longer.[76] Exposure of neurons and glia to elevated levels of extracellular glutamate results in swollen, electron-lucent presynaptic terminals, dendrites, and perineuronal and perivascular astrocyte processes. Neurons are shrunken and of irregular profile with an electron-dense cytoplasm containing rounded but electron-dense mitochondria, vesicular lucent membranous profiles, but still with discrete cisternae of rough endoplasmic reticulum (Figure 2.7A). The nuclear envelope is

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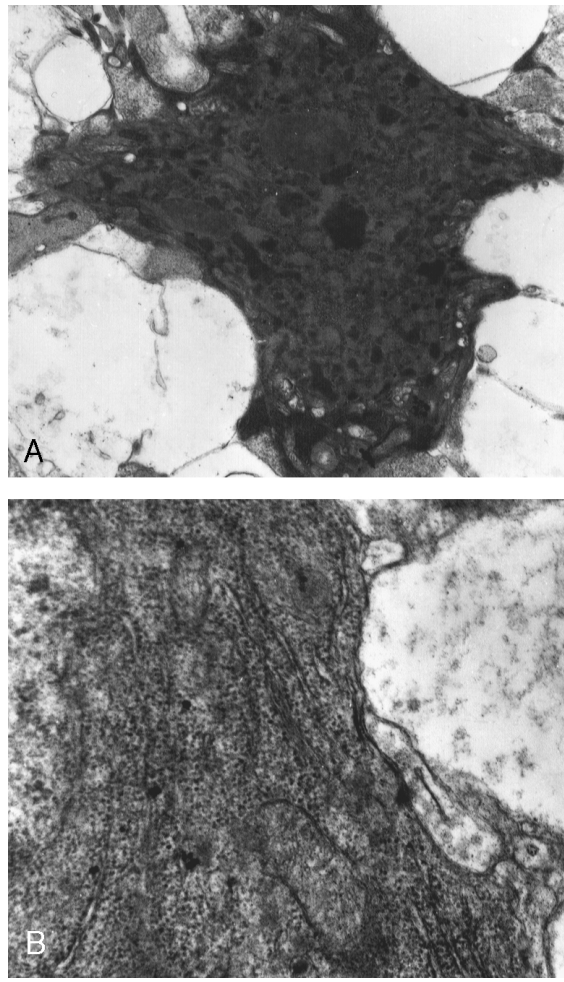

FIGURE 2.7 A/B Transmission electron micrographs of a neuron from a rat which had been exposed to 0.5 *M* glutamate by means of iontophoresis 4 h before being sacrificed. The neuron is electron dense with a crenated, irregular profile surrounded by swollen astrocyte and presynaptic elements. There is no mitochondrial swelling or lucency, but a number of vesicular inclusions occur. Nuclear structure is grossly disrupted. Detailed examination (Figure 2.7B) shows cisternae of rough endoplasmic reticulum and numerous polysomes within the cytoplasm. (Original magnification: **A** × 16,500; **B** × 65,000.)

discrete but the nucleus is crenated and the chromatin forms discrete electron-dense clumps of material.

The description of neurons given above and the comparison with the "dark" cell response discussed previously has been established in the literature for some years.[77] However, detailed comparison of the ultrastructure of "dark" neurons and those that have been exposed to high levels of glutamate result in questioning of a direct correlation

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between the two. For example, ischaemic neurons demonstrate mitochondrial swelling and loss of Nissl substance. Neurons exposed to toxic levels of glutamate do not demonstrate mitochondrial swelling and Nissl substance is still recognisable. Neither is there loss of polysomes (Figure 2.7B). There are also major differences in the fine structure of the chromatin within the nuclei of cells in these two groups (Figure 2.7A).

Thus, it may be suggested that a selective concentration upon a single pathological mechanism, such as postischaemic elevated levels of glutamate or calcium without a major integration of data from other sources, may not be rewarding in the long term.

2.6 OLIGODENDROCYTES

Despite the fact that oligodendrocytes make up some 70 to 90% of all glial cells[78-82] and therefore large numbers may be exposed to an ischaemic insult, the literature relating to these cells is relatively limited. Indeed, early work suggested that oligodendrocytes may be resistant to ischaemia.[80] However, more recent work has documented ultrastructural responses by oligodendrocytes to both irreversible and reversible ischaemic insults.[16]

2.6.1 Light and Dark Oligodendrocytes

There is also a differential response between dark and medium-light oligodendrocytes. The former demonstrate transient dilatation of cisterns of the Golgi apparatus and rough endoplasmic reticulum and an acute, mild cytoplasmic lucency after irreversible neuronal ischaemic injury. In reversible ischaemic injury there is no change in cell size although the cisternae of the Golgi apparatus and rough ER appeared more prominent 24 h after insult.[16] Medium-light oligodendrocytes, on the other hand, demonstrate a significant increase (+16%) in cell size, contracted mitochondria, enlarged rough ER cisterns, and increased content of microtubules and tubulovesicular profiles (Figure 2.8) after irreversible neuronal ischaemic injury.[16] With reversible neuronal ischaemic injury, these oligodendrocytes are mildly swollen with increased cytoplasmic lucency within minutes of injury. They resume an appearance similar to controls at 24 h although their cell size is still significantly larger than in normal animals.[16] Thus the medium-light oligodendrocytes respond to ischaemic insult to a greater degree than dark oligodendrocytes, but to a lesser degree than do astrocytes. In addition, the time course of the oligodendrocyte response is markedly slower than that of smooth muscle, microglia, endothelium, astrocytes, and neurons. However, the generalisation that oligodendrocytes do not respond to an ischaemic insult can no longer be held to be correct.

2.7 CONCLUDING REMARKS

The literature reviewed above demonstrates that all cell types occurring within ischaemic regions of the mammalian central nervous system respond to such an

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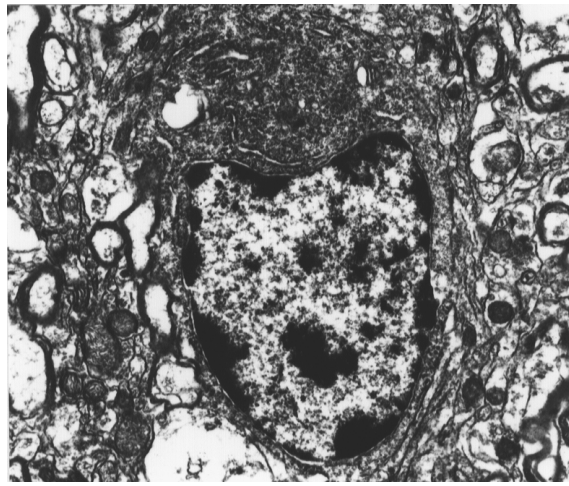

FIGURE 2.8 A transmission electron micrograph of a medium-light oligodendrocyte in the ischaemic boundary zone after constriction of the right middle cerebral artery by application of endothelin-1. There is enlargement of rough ER cisternae. (Original magnification × 5,500.)

insult. This is hardly surprising since ischaemia will necessarily result in a marked reduction of the level of major substrates for energy metabolism throughout the ischaemia area. But it is also clear that there are differences in either or both the rate and degree of response by different cells.

Until recently it has been suggested that astrocytes respond most rapidly. However, this impression seems to have been based upon changes in their morphology. Use of molecular techniques, however, now indicates that microglia and smooth muscle in the tunica intima of parenchymal arteries respond most quickly to an ischaemic insult. In the case of microglia, changes occur in their physiology and biochemistry before there is any ultrastructural indication of a response. Ultrastructural changes in microglia occur in the same postischaemic time frame as responses by astrocytes. At the other extreme, the concept that oligodendrocytes do not respond to an ischaemic episode has not been substantiated. Rather there is a response by medium-light oligodendrocytes within a long-term postischaemic time frame. However, the significance of this response has not yet been elucidated.

It is also clear, and has been established for some time now, that some neurons are susceptible to relatively short periods of ischaemia while others are not. Initially there are changes in the biochemical activity of neurons, in parallel with other cell types, which precede progressive tissue destruction. Although both types of neurons demonstrate similar morphological responses initially, susceptible neurons, perhaps due to differences in the biochemistry of some structural cytoskeletal components, for example, enter a postischaemic pathological cascade culminating in their death between 2 and 4 days and later after the ischaemic episode. However, despite a great deal of work which has attempted to unravel these complex intracellular processes, there is still not a clear and simple overview available. It is also apparent that for

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reperfusion to be beneficial after a period of ischaemia it must be reestablished very early after an ischaemic episode. A delay in the reestablishment of reperfusion may in fact exacerbate degenerative neuronal and other cell sequelae. In addition, although circumstantial evidence exists that other nonsusceptible neurons recover from a short-term ischaemic episode, there is still a lack of good quality, quantitative experimental data to substantiate this impression.

Thus, a number of biochemical/biological factors which certainly differ from neuron to neuron and probably between different subtypes of other cells within the central nervous system, govern the specific ischaemic vulnerability of each cell. Since the interval available for the reversal or inhibition of biochemical processes initiated during the ischaemic episode may in fact be quite short, it is clear that analysis of structural changes will provide little insight but rather will reflect the end-point of such processes. Therefore, it may be more rewarding to therapeutically interfere with the variety of complex biochemical changes that essentially determine the fate of the ischaemic tissue while acknowledging that no single cell population can be analysed in isolation; rather, there is a very complex interaction at the molecular level between all cell types within the central nervous system. This complex interaction is disrupted to differing degrees in different types of cell by an ischaemic episode.

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3 Immune Response and CNS Injury

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CONTENTS

3.1 INTRODUCTION

When considering brain injury or trauma and the immune response, three questions can legitimately be asked. Can injury of the brain generate an immune response, in particular an autoimmune response? Is there any interaction between an immune response and the response to injury? Does injury of the CNS have an effect on the immune system?

Injury of any tissue gives rise to an immediate inflammatory response and it is probable that this initial response is primarily aimed at priming the process of repair. It is likely that adaptive immunity has evolved in the context of tissue damage induced by infection and the production of an immune response is seen to be most efficient in those tissues most likely to be at risk of a primary infection, e.g., the nasal-oral mucosa, the respiratory, intestinal, and genitourinary surfaces, and the dermis. The CNS is not a site of primary infection and the invasion of the CNS by organisms following systemic infection is limited, presumably by virtue of the properties of the brain vasculature, and when it occurs it seems to reflect more the adaptive ability of the infective agent.

The nature of the nervous system is such that its integrity needs to be strongly preserved. The blood-brain barrier (BBB) restricts the entry of solute and is essential for the normal functioning of the CNS; a similar barrier is a general feature of the nervous system of invertebrates as well as vertebrates and of peripheral nerves as well as the CNS. Although the BBB may be highly efficient at limiting access to

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microorganisms, it is unlikely that it represents an adaptation to limit only infection. At the most basic level, the barrier's function must have reference to maintaining an ionic milieu conducive to impulse propagation. However, within the CNS the diversity of molecules used for synaptic transmission and for the modulation of propagation must render even greater significance to the role of the BBB in limiting the entry of molecules, both large and small, which might interfere with neuronal stimulus processing. Thus, the composition of the CSF, which is considered to closely reflect that of parenchymal extracellular fluid, in humans contains only 100 to 350 mg of protein per litre compared to connective tissue extracellular fluid's 28,000 mg/l. Injury of the CNS in mammals is relatively rare, but is likely to be rapidly inimical to survival and so the tissue's response to the event in terms of selection would be trivial, unlike the development of mechanisms for reducing possible damage — consider, for example, the thickness of the ram's skull! The central nervous system's response to injury, however, has gained significance as techniques for life support have improved. Similarly, the immune response after injury of the CNS has gained importance as the grafting of neural tissue, which like any surgery is a controlled injury, is considered as a treatment for some degenerative diseases.

The inflammatory response is triggered by the release into the damaged tissue of substances generally classifiable as proinflammatory agents, e.g., platelet products, prostaglandins, cytokines, and chemokines produced and released by damaged cells and tissue elements such as blood vessels and the leucocytes and platelets released from the damaged vessels. This is followed by invasion of the damaged tissue by the cellular elements of the innate immune system, in particular, actively phagocytic polymorphonuclear leucocytes (PML) and macrophages. Serum proteins leaking into the site of injury include "natural" antibodies such as anti-Ig and, in humans, anti-α-galactoside[1] as well as complement a variety of specialised proteins with particular binding properties, such as lectins. These proteins act to opsonise damaged cells and any invading microorganisms and so serve to direct and encourage their phagocytosis by macrophages and PMLs. This initial inflammatory response is essential for progression from the innate to the adaptive immune response.

The process of adaptive immunity allows specific molecular features of invading microorganisms to be "memorised" in a clonal fashion by specific classes of bone marrow-derived cells, the T and B lymphocytes. Invoking adaptive immunity depends on the transfer of material from the site of injury as soluble antigen or by cells which act as antigen-presenting cells (APCs) to lymphoid tissues where it can be screened for antigenic potential. The antigenic material may reactivate memory lymphocytes or invoke proliferation and activation of those naive cells with appropriate surface receptors which bind to part of the new antigen. The antigen receptors of both T and B cells are heterodimers and the antigen binding region of each polypeptide is derived from the random association of alternative segments of their corresponding genes. This gives rise to a large number of possible structures, and hence of binding specificities, so that any molecule could in theory be recognised as an antigen. During the development of both T and B cells, clones with strong reactivities with self-derived antigens are removed by a process which is only

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partially understood. The recognition of antigen by the T-cell receptor is restricted in that the cognate peptide antigen must be bound to appropriate molecules of the MHC complex when presented on the surface of APCs.

The receptor of B cells, a membrane-bound form of immunoglobulin, will interact with a surface feature of the antigenic molecule without the involvement of any other molecule. B cells can also act as APCs, presenting peptides derived from endocytosed antigen along with MHC class II molecules to T cells. B-cell interaction with a T cell in the presence of cytokines, such as I1-10, can induce a further shuffling of some of the immunoglobulin gene segments and a limited random mutation, giving rise to new combinations of antibody structures which can be further selected for increasing affinity for the antigen. Thus, repeat exposure of B cells to the antigen can in most circumstances lead to the production of immunoglobulin of increasing affinity for the antigen. T cells do not show such an adaptive response — the combinatorial process generating their receptor diversity is limited to the period of their early clonal development. The stimulation of lymphocytes to produce their clonal expansion requires an interaction between cells via a number of surface receptors with both soluble and cell-bound ligands in addition to their interaction with the antigen. Incomplete interaction leads to partial stimulation and apoptosis. It is the presence of these secondary ligands at a sufficient density on the surface of "professional APCs" which characterises the interdigitating and follicular dendritic cells found in lymphoid tissue and which are essential for the initiation of a primary immune response. Tissues such as the skin and the mucosa contain immature dendritic, Langerhans, and reticular cells, which on their activation by cytokines and tissue products phagocytose antigen and migrate to the local lymphoid organ.[2]

With the development of a primary response, newly activated T cells and newly synthesised immunoglobulin will be released into the circulation to reconnoitre the tissues for their cognate antigens. Normally, half of all of the IgG is extravascular and it is likely that a large proportion of circulating T-memory cells are also extravascular.

Should the site of injury contain antigens to which an earlier immune response has been mounted, then tissue macrophages and related invasive cells can present antigen fragments to the relevant activated T cells at the site of injury, so inducing a further response, in particular the release of cytokines and chemokines. Thus, T cells and macrophages will accumulate to initiate or augment any ongoing inflammation (delayed type hypersensitivity or DTH). This process normally becomes self-limiting as antigenic material is removed and there is an increase in the numbers of cells which function to downregulate both the release of proinflammatory cytokines and the antigen-specific cells, thus allowing obvious tissue repair to take place.

With respect to the CNS, it is clear that in considering the first question, "can injury of the brain initiate an immune response?," the problem revolves around whether or not injury of this tissue is followed by an inflammatory response and whether or not potentially antigenic material is relayed to lymphoid tissue in a manner appropriate for initiating an immune response. The further consideration of autoimmunity following tissue injury obviously involves the general problem of the induction of self-tolerance during the development of the lymphocyte repertoire, but in view of the diversity of brain constituents, and its lengthy and late development,

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there may be an increased possibility for the generation of an autoimmune response. The consequences of any preexisting immunity on the injury response depends on whether or not antigenic material becomes more accessible to the surveillance of activated lymphocytes or antibody and whether or not the injury response modifies the outcome of immune recognition. In addition, the effect of brain injury on the hypothalamus-adrenal axis will produce a general stress response leading to suppression of the immune response. It has been suggested that severe brain injury is strongly immunosuppressive so that infection is a serious risk,[3,4] and that this may originate from a specific suppression of CD4 T-cell responsiveness.[5,6] However, the organs of the immune system are not exempt from direct innervation by the autonomic system[7,8] and the sensitivity of immunocompetent cells to neurotransmitters and NGF,[8,9] in addition to the sensitivity of neural cells to cytokines, implies the possibility of extremely complex interactions between the brain and the immune system. This aspect of the relationship between brain injury and immunity, however, is outside the scope of this review and will not be dealt with further.

3.2 PRIMARY IMMUNE RESPONSE TO ANTIGENS IN THE CNS

Early observations relating to the persistence of tumour and tissue allografts[10,11] and of viruses[12] when placed in the brain suggested that this organ was a site of "immune privilege," in which antigens were poorly immunogenic and did not elicit an inflammatory response. To elicit an immune response, antigen must be able to leave the brain, either directly or following endocytosis by a predendritic cell, and arrive at a lymphoid organ. The character of the immune response induced, including the level and type of antibody, is highly dependent on such factors as the amount and nature of the antigen, period of exposure to the antigen, and its mode of presentation. These variables help to define the balance between CD4/CD8 positive cells and between the CD4-positive TH1 and TH2 cells.[13] A primarily CD4 TH1 response is associated with the DTH type of response.

There is some conflict, however, regarding the extent to which the CNS may be a site of immune privilege. Unlike the testis and the anterior chamber of the eye, both well-recognised sites of immune privilege, the brain does not appear to have a positive mechanism for inhibiting T-cell-mediated inflammation. Antigen presented in the anterior chamber of the eye produces a specialised form of response, termed anterior chamber-associated immune deviation (ACAID).[14] ACAID is characterised by a predominantly TH2 type of response involving antibodies of low complement-fixing activity and T cells with a low production of TNF-α and IFN-γ. This type of immune response, plus the intraocular expression of the Fas ligand, means that intraocular DTH responses are suppressed. Similarly, in the testis the expression of the Fas ligand produces apoptosis of invading T cells, CD4 or CD8, and so suppresses the development of inflammation.[15,16] The fact that the primary immune response to antigen in the anterior chamber is typically of a TH2 type suggests a modification in the method of antigen presentation, a significant consideration in this tissue which, unlike the testis and the brain, will be susceptible to primary

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infection. The conjunctival surface of the anterior chamber forms part of the mucosal immune system and is equipped with APCs and connections to the draining submandibular and cervical lymph nodes and so differs fundamentally to the CNS.

3.2.1 Responses to Soluble Antigens

Numerous studies have shown that both T-dependent and T-independent antigens injected into the brain or CSF are very efficient at producing humoral responses.[17-22] It appears that the injection of soluble antigen into the brain parenchyma or into the ventricular space produces a humoral response indistinguishable from that elicited by its extracerebral injection.[20,23] This suggests that soluble antigen can leave the brain and gain access to lymphatics or the blood with a similar efficiency as from any other tissue, even though there is no anatomical lymphatic system within the brain.

Studies of the fate of labelled material injected into the CNS in a number of animals indicate that there is a drainage of the extracellular fluid from the brain to the cervical lymph nodes (CLN), primarily via the cribriform plate in the spaces contiguous with the olfactory nerves, to join with the drainage from the nasal submucosa.[24] The relative significance of this route for the drainage of extracellular fluid from the brain parenchyma does, however, vary between species.[25] In humans there is extensive development of arachnoid granulations over the surface of cerebral hemispheres providing a major route for CSF drainage, though it may be less significant than is the case in the rat and rabbit.[26]

The perivascular space surrounding blood vessels in the brain is considered to be a structural analogue to the lymphatics, providing a form of lymphatic drainage, although they do not have direct structural continuity with lymphatic vessels proper nor do they provide a route direct to a lymphoid organ. These routes, along with drainage along the spinal roots, provide a means whereby antigen can leave the CNS to reach the draining lymph nodes and blood and, hence, the spleen. Antigen administered via the CSF may be even more effective than administration at a systemic site in producing antibody.[20] Such a phenomenon would be compatible with the idea of a bias towards a primarily TH2 response. A higher ratio of IgG1:IgG2 in the rat after intracisternal injection than that following intramuscular injection of antigen, although there is higher complement-fixing activity,[22] also suggests that the CSF injection induces a predominantly TH2 response. A bias towards a predominantly TH2 response, however, may reflect a slow, continuous release of antigen from the CSF compartment compared to the systemic site. A slow release of antigen from the ventricles might also explain the observation of intrathecal synthesis of IgG in these animals, antigen persistence enabling circulating activated B lymphocytes to enter and be held within the CNS. It should be noted that intrathecal synthesis of Ig is a common and well-known phenomenon in many inflammatory neurological diseases and reflects the presence of plasma cells in the CNS.[27] Although the means and circumstances under which activated T cells enter the brain has been studied, there is no information regarding the entry of B cells and it is assumed that they enter by the same or similar mechanisms as apply to T cells.

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3.2.2 Responses to Insoluble Antigens

Although a complete humoral response is evoked following the intracerebral or intraventricular injection of soluble antigen, there is evidence to suggest that insoluble antigen, e.g., tissue grafts or microbes,[28,29] is dealt with differently. This is clearly demonstrated by the experiments of Head and Griffin[30] whose grafting of allogeneic parathyroid tissue into parathyroidectomised hosts allowed the functional status, and hence the level of rejection of the graft, to be assessed continuously. Allografts of parathyroid into the cerebral cortex between Fischer and DA rats were not rejected and remained functional over the 96 days of the experiment. A small number (the same number as found with isografts) failed to become established and so failed to function. Similar grafts placed under the kidney capsule or subdermally became established and initially produced normal serum calcium levels, however, rejection and functional failure was obvious by 13 days.

Experiments using skin grafts between Lewis and DA rats produced similar results to those obtained with grafts of parathyroid tissue. Presensitisation by orthotopic grafting led to failure of subsequent intracerebral grafts. The host's immune responses to the graft were not made permanently anergic by the intracerebral grafts since a later orthotopic graft was rejected and complement-fixing anti-alloantibodies were produced with the same time course as in naive animals, with the concomitant failure of the established intracerebral grafts. Injection of dead microbes (BCG or streptococcus) into the cerebral hemisphere invokes a relatively mild inflammatory response with an early invasion by neutrophils followed by a slow invasion of macrophages and the local breakdown of the BBB.[28,29] The local microglia are upregulated and a few T cells may appear,[28] but this response essentially dies away by 3 weeks. Antigen persists at the site of injection long after the primary response has subsided. A second injection of the microbial antigen into the skin, up to 6 weeks after the primary intracerebral injection, induces a localised DTH response in the brain.[28] These responses were still present at up to 5 months after the second injection, indicating long localised persistence of particulate antigen.

Neural allografts, usually of foetal tissue and into cortical sites, have also been found to be successful, even when sequential allografts are made.[31-33] By comparison, allografts of neural tissues placed in the lateral ventricle have a limited survival time.[34-36] This may reflect the use of a larger block of tissue, but may more importantly reflect a property of the grafting site, the passage of particulate, cellular antigens from the ventricles being more efficient than from the parenchyma. Thus, in the studies discussed above[30,33] it was noted that the site of the graft and the care taken to reduce the degree of damage were important in determining the graft's survival. Penetration of graft tissue into white matter tracts or of the ependyma led to early rejection of grafted tissue.[30] Rejection of allografts would be due to a TH1-based immune response with a subsequent development of a local DTH reaction. A TH1 response is supported by the autocrine production of IL-2, and allografts into the lateral ventricle which are normally rejected can be maintained if the IL-2 receptor is blocked.[36] The low or absent inflammation following optimal grafting into the brain parenchyma is similar to the result of grafting into the anterior chamber

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of the eye, but as indicated above, the end result does appear to be achieved by different means.

Thus, there does appear to be a difference in the way in which soluble and particulate antigens are handled in the CNS and, furthermore, there appear to be differences in the response to particulate antigens depending in which compartment of the CNS they are placed. The bulk of soluble antigens possibly pass to lymphoid organs directly, i.e., not within a macrophage or APC, and this may be responsible for the preponderance of a TH2 rather than of a TH1 response. On the other hand, the transfer of particulate antigen relies on the mediation of macrophages and, more importantly for the generation of a primary response, on dendritic cells. Furthermore, the observations to date suggest differing capabilities for the transfer of particulate antigen from the CNS cortical parenchyma or from the ventricles to lymphoid tissues.

3.2.3 Antigen Presentation to the Immune System

Following injury and the introduction of inflammation-promoting material into the CNS, a localised response slowly develops that is relatively low-key and with a delayed influx of macrophages.[28,37-39] The resident parenchymal macrophages, the microglia, also show an increase in the expression of molecules associated with activation, such as the scavenger receptor, C3R, and MHC class I and II,[40,41] but this response is also slow to develop. The slow clearance of phagocytosed material,[28,42] including the slow clearance of myelin during Wallerian degeneration in the CNS, indicates that neither macrophages nor microglia are as highly activated as similar cells would be in peripheral tissue[42] and do not appear to be very mobile.[43] This low level of activation may also characterise the perivascular macrophages.[44] This poor response of macrophages and microglia appears during postnatal development in the mouse.[39] More significantly from the aspect of the production of a primary response to particulate antigens, veiled macrophages (predendritic cells) are not recruited.[28]

In the skin the Langerhans cells, resident predendritic cells, are activated. Following injury and phagocytosis of possible antigenic material they become motile, and pass to the local draining lymph node to become dendritic cells and act as the professional APCs initiating the immune response. The dendritic cell is probably the major component of the innate immune system, providing a connection with the adaptive immune response. The activation of predendritic cells can involve either receptors for bacterial products[45] or, it is suggested, products released from damaged tissue including free radicals, GM-CSF, and other cytokines.[46] Dendritic cells are particularly important in alloimmunisation and it has been demonstrated that in peripheral allografts the donor dendritic cells probably act as the major APCs in eliciting rejection,[47-50] although host cells do appear to be able to induce immunity.[51] If it is the donor cells which are the most important, then the persistence of skin grafts in the brain would suggest that the “immobilising” influence of the brain extends to the graft dendritic cells, either within the graft or as they leave and come into contact with the brain parenchyma.

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The recruitment and mobilisation of predendritic cells is clearly dependent on the balance of cytokines and other factors. Thus, granulocyte-monocyte colony-stimulating factor (GM-CSF) is a strong activator of Langerhans cells and acts synergistically with TNF-α, IL-1, IL-6, IL-12, and free oxygen radicals.[46] All of these molecules are produced in the brain following injury, but so are the inflammatory suppressive agents IL-10 and TGF-β. TGF-β is particularly relevant since this cytokine inhibits the production of both TNF-α and free oxygen radicals and stimulates the production of extracellular matrix which may increase the adherence of dendritic cells. The immediate source of TGF-β following injury is the platelet, which contains and releases large amounts of pro-TGF-β.[52,53] The initial platelet supply of TGF-β, followed by that produced by the brain and combined with a slow and poor recruitment of macrophages, would contribute to an inhibition of activation of any dendritic cells. In the skin, CGRP released from sensory nerves inhibits the mobility of Langerhans cells,[54,55] and it is possible that in the brain a local release of CGRP and other neuropeptides contribute to the lack of activation and immobilisation of dendritic cells. Thus, a combination of poor recruitment and immobilisation of predendritic cells in the injured brain could explain the failure to initiate an immune response against particulate antigen.

However, it is clear that when an intense inflammatory response is invoked within the brain the balance of cytokine changes and increases the recruitment of macrophages and predendritic cells as well as enhancing the activation of the microglia, although, interestingly, even under these circumstances the clearance of particulate antigen can still be slow.[28] Such an increase in the recruitment and activation of monocytic cells could increase the opportunity for antigen presentation from the brain to the immune system. There is evidence of new T-cell specificities (epitope spreading) arising during chronic experimental allergic encephalomyelitis (CREAE).[56,57] Epitope spreading can involve new epitopes on the priming antigen, intramolecular spreading, or intermolecular spreading involving a new antigen. T-cell activation against the myelin proteolipid protein, following sensitisation against basic protein either by active immunisation with a specific peptide epitope of myelin basic protein (MBP) or by passive transfer of T cells, has been demonstrated[58,59] although this may depend on the demonstration method adopted. Interestingly, anti-GFAP antibodies are generated during acute EAE induced with MBP.[60]

3.2.4 Injury and Autoimmunity

There are no direct indications that injury of the brain is followed by a T-cell autoimmune response, not even against strongly immunogenic antigens from myelin such as basic protein. MBP is released into the CSF and serum following traumatic injury and cerebrovascular damage,[61,62] but there is no evidence that EAE nor its prototype MS follows brain injury. Facial nerve section in Lewis and BN rats has been reported to increase the numbers of MBP-reactive T cells in the superior cervical lymph nodes, and some perivascular T cells were found in the facial nucleus.[42] These effects were more pronounced in the Lewis rat, a strain with a greater susceptibility to EAE, underlining the significance of the genetic background in determining the level of the immune response.

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Autoantibodies, including antibodies reactive with neural antigens, can be found in normal healthy subjects and although of low titre these can be of high affinity.[63] Such antibodies appear to be more frequently seen in patients with a variety of neurological diseases, particularly those of a chronic neurodegenerative type,[64,65] and chronic viral encephalopathy.[66] Antineurofilament antibodies occur with higher frequency in patients with CJD, familial Alzheimer's, and Parkinson's dementia, but also in viral encephalopathies such as subacute sclerotic panencephalomyelitis (SSPE) and acute herpes simplex encephalitis.[67]

In experiments in the rat, lesioning of the hippocampus or anterior olfactory nucleus does not generate antineurofilament antibodies, whereas they are produced after lesioning of the olfactory bulb and the grafting of PC12 cells in all sites,[68] indicating again that the site within the brain strongly determines the immunological outcome. Antineurofilament antibodies are not considered to be pathogenic since the target antigen is intracellular. However, intracellular injection of antibody against the intermediate neurofilament protein into cells of the early *Xenopus* embryo leads to later axonal abnormalities,[69] and an anti-large neurofilament antibody isolated from a case of ALS also reacted with neuronal surface proteins.[70] Previous head injury is a factor in the development of Alzheimer's[71,72] and epilepsy.[73,74] Activation of microglia is found in both of these conditions and autoantibodies are elevated in about 50% of Alzheimer's patients,[64] but at present specific autoimmunity is not considered to be significant in their pathogenesis.

Rasmussen's encephalitis is a possible exception. This is a rare form of focal epilepsy associated with pronounced focal cortical inflammation. The condition may be associated with antibodies to the glutamate receptor type R3[75-77] and it has been suggested that it is triggered by brain damage or infection, these events opening the BBB to the circulating antibodies, although how these arise is not known (it is possible that they represent cross-reactive antibodies generated against bacterial periplasmic amino acid-binding proteins[78]). Interestingly, the lesion is typically unilateral and does not spread to the contralateral hemisphere even after hemispherectomy.[79] Why the lesion does not spread is unclear if all it requires is a break in the BBB subsequent to an activation of microglia and perivascular macrophages. Activation of these cells following unilateral lesioning appears not to spread generally across the midline (personal observations), as is also the case with astrocyte activation.[80] Similarly, following a local antigenic induction of inflammation the response spreads throughout the ipsilateral cortex but not the contralateral.[28,39] It is unlikely that this asymmetric spread of activation is due to either a humoral factor alone or intrinsic neural paths, but may reflect a spread along the ipsilateral vascular tree, possibly through a paracrine mechanism via the perivascular macrophages and microglia.

Since autoantibodies are detectable in healthy subjects it is not possible to say whether or not their increased detection in CNS degenerative and inflammatory disease is a result of a new primary response or the stimulation of preexisting B-cell clones by the macrophage transfer of antigen from the brain. In a chronic inflammatory disease with a suspected autoimmune aetiology such as multiple sclerosis there has been a failure to identify a primary pathogenic autoantigen, but many

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studies have identified immunity against a number of myelin and myelin-related and unrelated antigens with variable incidence.[65,81-85] These again may reflect "epitope spreading" or secondary immune responses arising from damaged tissue in the context of ongoing inflammation or may represent a stimulation of preexisting autoimmunity.[65,83] Overall it must be assumed that the generation of antibrain antibodies following trauma will be limited by the normal mechanisms operating to restrict the production of autoantibodies and a poor T-cell immunisation to brain antigen may further reduce the chances of a humoral response.

3.3 INJURY AND THE ACCESSIBILITY OF THE BRAIN TO THE IMMUNE SYSTEM

In the normal brain the BBB is distinguished by its selective operation against solutes in the plasma, including immunoglobulins, whereas activated T cells pass through the brain postcapillary-venule endothelium as effectively as they do in any other tissue.[86] This is clearly exemplified by the phenomenon of adoptive transfer of EAE by the intravenous injection of activated anti-MBP T cells into naive syngeneic recipients,[87,88] whereas the systemic administration of antimyelin antibody does not lead to demyelination.[89] The entry of T cells into the brain is dependent only on their being activated and does not depend on antigen recognition, and so does not depend on MHC presentation of antigen[86,90] nor is it dependent on specificity for neural antigen.[28] The entry of activated cells into the CNS is quite fast; they are found within 90 min of injection, reaching a peak concentration within 9 to 12 h.[86]

However, although activated T cells appear to be able to reconnoitre the CNS they are not readily seen in the parenchyma of the normal brain.[91] After an intravenous injection of MBP-specific activated T cells they are found to localise in the perivascular space within the brain, and this is followed by a recruitment of more cells from the host, some of which migrate into the parenchyma.[92] It is most likely that under normal conditions all activated T cells passing through the brain are restricted to the perivascular space and later drain into the venous system in the subarachnoid and meningeal spaces.[26,93,94] It is the perivascular microglia/macrophage which fulfils the role of the APC[90,95] and retains and further activates appropriate T cells. It is this aspect of the cellular dynamics in the brain which gives the greatest difference to peripheral tissues, in which activated T cells pass through the tissues' extracellular space to enter the local lymph node via the lymphatics. The recognition by the T cell of its cognate antigen in the context of an appropriate MHC complex on the surface of an APC will lead to its further activation and the secretion of inflammatory cytokines. The ensuing activation of local vascular endothelium leads to the recruitment of more lymphocytes and macrophages. If in the brain it is the perivascular macrophage which acts as the APC, then in passive transfer EAE this will depend on there being a transfer of MBP products from the normal white matter to these cells. Whether or not this is dependent on the amoeboid microglia from the parenchyma or the slow transfer of material through the network of microglial processes[96] is not known. If, as is suggested, the normal turnover of brain

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constituents involves the transfer of cellular material to the perivascular macrophages, then it would seem unlikely that this phenomenon would be restricted to MBP.

The systemic injection of S-100 and myelin-associated glycoprotein-specific T cells also produces perivascular lymphocytic infiltration, even though there may not be clinical signs.[97,98] Thus, although the brain differs from the peripheral tissues with respect to the anatomical dynamics of T-cell surveillance, tissue antigens are not hidden, nor is there an intrinsic mechanism for the suppression of further T-cell activation, as seen in the testis and the anterior chamber of the eye. The same is true for foreign antigens. Thus, the late reexpression of viral antigens in the brain can lead to intense inflammation and tissue damage, as seen in slow virus infections of the brain such as SSPE and Visna.[99-102]

Injury will have two consequences relevant to the subsequent behaviour of autoreactive T cells; firstly the injury would increase the release and availability of self-antigen and the acute local release of cytokines such as IL-1 and TNF-α would upregulate the perivascular macrophages and microglia, increasing their potential to present antigen, and also activate the vascular endothelium thereby increasing the retention and passage of T cells into the "brain". Consequently, cranial nerve damage, which will produce deafferentation and microglial upregulation in the corresponding nuclei and projection fields, intensifies the inflammatory response in these sites if EAE is subsequently induced in the animals.[103] Thus previous injury could act to exacerbate any subsequent autoimmune inflammation.

It is generally stated that the intact BBB prevents the passage of immunoglobulin, along with most other macromolecules, into the brain. However, this restriction is not complete, IgG is found in the normal brain parenchyma in the absence of serum albumin. The levels of IgG increase significantly after injury.[104,105] Preimmunisation against brain antigens increases the amount and the speed of extravasation of IgG but not of albumin.[105] It does, therefore, seem possible that there is some facilitated passage of IgG into the brain even following injury, and that this is not just related to a "leaky" BBB. This increased entry of IgG may not be general since some antibodies have been shown to be able to pass into the brain with considerable ease. Antibodies against the transferrin receptor can pass through the intact BBB by virtue of their reactivity with the receptor on the vascular endothelium and can be used as a vehicle for the transport of other molecules.[106] More intriguing is the anti-b-2 integrin, MAC-1, monoclonal antibody 5C6 which readily passes through the BBB by an unknown mechanism which is independent of its antigen specificity.[107] The occurrence of this antibody does raise the possibility that amongst the normal repertoire of IgG molecules a number of members may not be excluded by the BBB, these accounting for the IgG observed in the normal brain.

Clearly, the BBB presents a problem in the pharmacological approach to treating brain injury and encouraging neuronal repair. Although in the acute phase the BBB is impaired it does recover, making drug access difficult. The use of antibodies which are not excluded offers a means of overcoming this difficulty, and it has already been demonstrated that antitransferrin receptor can be used to transfer NGF into brain tissue.[106] Similarly, if the structural characteristics of McAb 5C6 (see above) which allow it to pass through the BBB can be defined then hybrid antibodies

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carrying these features could be constructed. The F(ab) could carry any specificity desired, for example, to block the activity of a molecule with a putative growth-inhibitory activity.

There is an alternative route by which immunoglobulin may normally gain access to the CNS. Central neurons with peripheral extensions, such as motor neurons, will take up by endocytosis and retrogradely transport IgG, particularly IgG with antineuronal activity.[108-112] Significant differences are also found in the ease of uptake and of retrograde transport of individual antibodies.[113] This route of entry is significant only for those groups of neurons with peripheral extensions and since there is no indication that it will then leave these cells it is unlikely to have general significance in CNS injury. Although a pathogenic role for intracellular IgG has not been demonstrated, it may be significant in some suspected autoimmune neuronopathies. However, once IgG has entered the extracellular space of the injured brain it can be endocytosed by other neurons[114] as well as by astrocytes.

3.4 SUMMARY AND CONCLUDING REMARKS

The brain has a unique relationship with the immune system which is determined by the structural and physiological characteristics of the brain's vasculature. The restricted permeability seen as the BBB and the presence of a perivascular compartment in combination with the brain's small extracellular compartment constrain the functioning of the immune system in the brain. The immune system's surveillance function is compromised by the exclusion of antibody from the brain by the barrier, and although the role of activated T lymphocytes is unaffected, their passage through the tissue is normally restricted to the perivascular space. The extent of the drainage from the extracellular space in the brain into the lymphatic system is variable between species and between different brain regions. This, coupled with the partially suppressed activity of brain macrophages, which is probably partly due to the activity of the BBB, leads to a variability in the degree and the nature of the primary immune response generated against antigens within the brain. A lowered basic activity of brain macrophages and microglia, and importantly their low-key recruitment during a primary inflammatory response, reduces the generation of a primary immune response to particulate antigens and generates the impression of immune privilege. Antigen within the ventricular or subpial space is more efficiently delivered to the immune system so that immune responses are normal. The injury response in the brain leads to a local breakdown of the BBB and a slow recruitment of macrophages and other cells of the innate immune system, but typically this is insufficient to generate a specific autoimmune response, although it may lead to an augmentation of "natural" autoantibodies. The low-grade, slow inflammatory response in the brain may be a consequence of a combination of the production of antiinflammatory cytokines such as TGF-β and a local release of peptide neurotransmitters, but this balance may be overcome in the presence of intense immune-mediated inflammation. The brain is particularly sensitive to inflammatory cytokines and they play a role in the global response of the brain to injury and infection. Whether or not they also play a role in neuronal plasticity in less extreme circumstances is not known. Similarly, although there are no indications that "natural" autoantibodies such as

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those reacting with neurofilament proteins are pathogenic, there is evidence that not all antibodies are equal. It should be borne in mind that in antibody-mediated autoimmune diseases severity is rarely related to antibody titre. This fact, combined with the neuron's propensity to endocytose proteins, including immunoglobulin, may mean that some antibodies could have an influence on neurons following injury and their entry into the brain.

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4 Haematogenous Cell Responses to CNS Injury

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CONTENTS

4.1 INTRODUCTION

The purpose of this review is to address the proposal that haematogenous cells are active participants in the initiation and repair of inflammatory insults in the central nervous system (CNS). Leucocytes are effector cells of the immune response and are normally engaged in the destruction of foreign pathogens. For many years the brain was considered to be an immunologically privileged site protected from the deleterious effects of lytic factors released from activated leucocytes. However, this view has waned in recent times[1] because of the CNS inflammatory responses generated by immune cell reactivity against autoantigens and invading microorganisms.[2,3] To illustrate the involvement of haematogenous cells in CNS injury, we will focus on the contribution of the three main classes of leucocytes, namely, neutrophils to cerebral ischaemia and stroke, macrophages to wound healing and acute tissue injury, and lymphocytes to demyelination.

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4.2 NEUTROPHILS, ISCHAEMIA, AND STROKE

Cerebral ischaemia is responsible for approximately 85% of strokes (cerebrovascular accidents) irrespective of whether they are thrombotic or embolic in nature.[4] Reestablishment of blood flow (reperfusion) in the postischaemic period is often accompanied by further tissue injury that is associated with aberrant microvascular function, damage to endothelial cells, and inflammatory cell exudates.[5,6] In recent years, particular attention has focused upon the contribution of neutrophils to the pathogenesis of the "reperfusion injury" seen in cerebral ischaemia.

In patients with cerebral ischaemia, there is a good concordance between the blood neutrophil count and the size of cerebral infarcts.[7] Neutrophils accumulate in experimentally induced ischaemic regions of the brain[8] and there is a strong correlation between the number of infiltrating neutrophils and the extent of ischaemic brain injury.[9] Depletion of neutrophils in experimental models reduces reperfusion injury,[10] although these findings were not supported by a later investigation,[11] and neutrophil entry into ischaemic areas of the brain is inhibited by the recombinant glycoprotein, neutrophil inhibitory factor.[12]

4.2.1 Adhesion Molecules

The inflammatory response following ischaemic injury consists of two main influxes of leucocytes into the zone of injury. The first is comprised mainly of neutrophils (6 to 24 h) and the second predominantly of monocytes (2 to 10 days).[6] Postischaemic damage may arise from the firm attachment of neutrophils to blood vessels to produce microvascular occlusions[13] or from the infiltration of the cells into the CNS and cytotoxicity towards neurons.[14] Crucial to either of these pathogenic mechanisms is the recruitment of neutrophils to blood vessel walls within ischaemic areas. As outlined in Chapter 6, neutrophil-endothelial interactions are governed by the expression of vascular adhesion molecules on the endothelial surface, for example, intercellular adhesion molecule-1 (ICAM-1) and E- and P-selectins, which bind counterreceptors (leucocyte adhesion molecules) on the surface of neutrophils in a sequential manner.[15,16] Both E- and P-selectins mediate the weak, transient attachment of neutrophils to activated endothelium ("tethering") whereas ICAM-1 promotes strong adherence and transendothelial migration.

4.2.2 Cytokines and Chemokines

In experimentally induced brain focal ischaemia, the upregulation of ICAM-1 and the induction of E- and P-selectin[17-19] is believed to result from the activity of inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), which are produced by the ischaemic brain[20,21] or by the reoxygenation of microvascular endothelial cells.[22] There is a direct association between increased expression of ICAM-1 and E-selectin,[19,23] the appearance of inflammatory cytokines,[20] and the degree of polymorphonuclear (PMN) cell infiltration into ischaemic tissue. Moreover, cultured murine brain microvascular endothelial cells stimulated with IL-1β or TNF-α express E-selectin and ICAM-1 and support neutrophil adhesion.[24]

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The important contribution of adhesion molecules to cerebral ischaemia-reperfusion injury is emphasised by antibody blocking studies. Infusion of antibodies against the leucocyte adhesion molecules CD11b,[25] CD18,[26] or endothelial ICAM-1[27,28] into animals with experimentally induced CNS ischaemia reduces damage at the blood-brain barrier, improves recovery of motor function, and increases CNS blood flow. In a different experimental approach, genetically engineered ICAM-1-deficient mice showed less reperfusion injury than normal animals of the same strain.[29] To our knowledge, inhibiting the expression of selectins in cerebral ischaemia has not been undertaken although antagonists of selectin function are known to reduce lesion loads in animal models of myocardial ischaemia.[30,31] Clinical trials are already investigating whether monoclonal antibodies against ICAM-1 will be of therapeutic benefit for the treatment of stroke. However, the wisdom of administering such antibodies to patients several hours after stroke has been questioned by some workers,[32] particularly as ICAM-1 is expressed constitutively by endothelial cells in all vascular beds.

4.2.3 Neutrophils and Tissue Damage

Both ICAM-1 and E-selectin are upregulated by the action of the inflammatory cytokines TNF-α, IL-6, and IL-1, all of which are present in sites of cerebral ischaemia.[20,21,33] Immediately following reperfusion, injection of IL-1 into rat cerebral ventricles enhances ischaemic brain infarction and oedema formation and increases the number of neutrophils adhering to endothelium and infiltrating ischaemic areas.[34] Protection against ischaemia is afforded by anti-IL-1 antibodies[34] and receptor antagonists of IL-1.[35] Areas of ischaemic brain produce chemokines, such as interleukin-8 (IL-8)[36] and platelet activity factor (PAF),[37] which activate neutrophils to increase the expression of surface adhesion molecules that mediate firm binding to endothelium. Both chemokines also serve as potent neutrophil chemoattractants and the establishment of a chemotactic gradient is a necessary prerequisite for guiding neutrophils to the site of inflammation. Following reperfusion of the ischaemic area, the appearance of IL-8[36] and PAF[37] precedes brain oedema formation and neutrophil infiltration. Neutrophils in ischaemic injury express elevated and persistent levels of receptors for the chemoattractant leukotriene B4, that is associated with the time-course of gliosis.[38] Injection of IL-8 or the cytokine macrophage inflammatory protein-2 (MIP-2) into the murine hippocampus induces a marked neutrophil recruitment that is associated with an increase in the permeability of the blood-brain barrier.[39] When the latter experiment was repeated in leucopaenic animals, the permeability effect was almost abolished, indicating that disruption of the blood-brain barrier is a consequence of neutrophil extravasation.[40] Additional evidence of chemokine involvement in cerebral injury comes from the study of transgenic mice in which the neutrophil-specific chemokine N51/KC was expressed in the CNS.[41] In these animals, a marked neutrophilic infiltration of the brain is accompanied by pronounced neurological symptoms and often by death from severe neuropathology.[41]

Once recruited into ischaemic tissue, neutrophil-mediated injury could arise from the release of free radicals and/or cytotoxic enzymes.[5] Activation of neutrophils

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produces a marked increase in the uptake of oxygen, which is referred to as the respiratory burst. Enhanced oxygen consumption is accompanied by increased glucose metabolism via the hexose monophosphate shunt, and oxygen is rapidly reduced to a series of toxic metabolites, including superoxide anions, hydrogen peroxide, singlet molecular oxygen,[42-44] and the most potent of them all, the hydroxyl radical.[45] Nitric oxide, whose production is increased in thrombotic microangiopathies and in cerebral trauma and ischaemia,[46-48] interacts with superoxide anions to form peroxynitrite which is directly cytotoxic, and also spontaneously decomposes to produce reactive hydroxy radicals[49] that disrupt lipid membranes through lipid peroxidation.[46] Inhibition of nitric oxide synthase reduces infarct size in cerebral ischaemia.[50,51] The reduction in reperfusion injury that occurs in patients receiving heparin with thrombolytic agents poststroke could be due to the antagonism of superoxide production by neutrophils.[52]

Tissue injury by neutrophils may also be induced by the extracellular release of lysosomal enzymes that include elastase, collagenase, and gelatinase.[5] By degrading extracellular matrix proteins,[53] neutral proteases facilitate the extravasation of additional neutrophils into ischaemic sites. Some of the neutral endopeptidases secreted by neutrophils antagonise the effects of natriuretic peptides which protect endothelial cells from neutrophil cytotoxicity.[54] It is conceivable that both lysosomal enzymes and products of the respiratory burst are simultaneously contributing to the lytic activity of activated neutrophils. For example, damage to endothelial cells by elastase is enhanced by xanthine oxidase,[55] killing by oxidants is augmented by cationic proteins and proteases,[56,57] and in ischaemic tissues the neutrophil-derived reactive oxygen species inactivate inhibitors of lysosomal enzymes.[5]

In conclusion, this section has highlighted the potential pathogenic contribution of blood neutrophils to the CNS injury that accompanies the ischaemia-reperfusion injury of stroke. From experimental models of this disorder, it appears that the second wave of tissue damage is induced either by neutrophil-mediated vasoocclusion or by the infiltration of neutrophils into the ischaemic tissue with concomitant release of lytic factors. Antagonising both neutrophil attachment to endothelium and the transendothelial migration of these cells at the level of the blood-brain barrier is likely to be of clinical benefit to cerebral ischaemia-reperfusion injury. Consequently, it is anticipated that a further unravelling of the mechanisms that promote neutrophil interaction with cerebral vessel walls will lead to the introduction of a more specific therapeutic intervention for the treatment of stroke.

4.3 MACROPHAGES AND TISSUE INJURY IN THE CNS

In terms of functional activities, macrophages are probably the most versatile effector cells of the immune system. They are phagocytic, present antigen to T lymphocytes, and, upon activation, effect intracellular killing of pathogens and lysis of tumour cells. Macrophages have a potent secretory repertoire as illustrated by the release of proteolytic enzymes (including lysozyme, neutral proteases, and acid hydrolases), α-2-macroglobulin, chemotactic and angiogenic factors, complement components, arachidonic acid metabolites, reactive oxygen species, and cytokines. Some of these factors have opposing actions. For example, elastase and collagenase are antagonized

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by α-2-macroglobulin whilst the proliferation of CD4-positive T lymphocytes is enhanced by IL-1 but inhibited by transforming growth factor-β (TGF-β). Macrophages possess the mediators necessary to degrade pathogens (e.g., lysosomal enzymes and reactive oxygen species) and this same armoury is also used in the initial remodelling phase of tissue repair and in tissue destruction itself. Thus, macrophages may either initiate or exacerbate the inflammatory response. Let us consider if some of these characteristics apply to macrophages in the CNS.

4.3.1 Contribution of Microglia

Microglia are regarded as the resident phagocytic cells of the CNS. During neonatal life they appear to be derived from infiltrating monocytes and it is generally agreed that microglial cells originate from bone marrow, particularly as they express the common leucocyte antigen CD45 which is present only on cells of marrow origin.[58] Microglia are distributed throughout the CNS and are considered to be integral components of the blood-brain barrier.[59] Their morphology, origin, and distribution is adequately reviewed elsewhere,[60] but little is known of the function of microglia other than that they protect the CNS from infection and injury, remove cellular debris following experimentally induced traumatic injury, participate in tissue modelling, and phagocytose apoptotic cells.[61,62] Microglial defence against infections may be supported by other macrophages strategically placed at sites where pathogen entry is most likely to occur, e.g., the choroid plexuses and leptomeninges.

Most acute inflammatory responses are characterised by infiltrates of polymorphonuclear cells (PMNs), whereas leucocytes entering sites of CNS injury are mainly comprised of monocytes which have migrated across an intact blood-brain barrier.[63] An increase in macrophage numbers at areas of tissue damage outside the CNS depends upon the recruitment of circulating monocytes, since blocking monocyte extravasation delays tissue repair.[64] Similarly, reducing the number of macrophages at sites of injury in the CNS impedes debris clearance, astrogliosis, and neovascularization.[65] From the study of microglial responses in experimental models of hippocampal deafferentation,[66] ischaemia,[67] nerve axotomy,[68] and demyelination,[69] it appears that an expansion of the macrophage population is dependent upon both monocyte recruitment and proliferation of local microglia. Microglia are believed to be instrumental in promoting axonal growth within the CNS[70] by providing lipids needed for synthesising membranes of growing axons and by secreting nerve growth factor.[71] The cells are also sources of TGF-β, platelet-derived growth factor, epidermal growth factor, basic fibroblast growth factor, insulin-like growth factors, and neurotrophins, all of which may be necessary for the development and function of neurons and glia.[72-74] Co-culture of microglia with neutrophils promotes proliferation and phagocytosis[74] and these findings are in accord with the rapid clearance of cell debris at sites of tissue insult.

Microglial activation and proliferation are early and sensitive signs of injury in the CNS and they are associated with changes in cell morphology and phenotype, particularly the upregulation of receptors for the cytokines macrophage-colony stimulating factor (M-CSF) and granulocyte/macrophage-colony stimulating factor (GM-CSF).[75] These cytokines and also IL-4[76] induce proliferation of microglial cells

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in culture[77] and injection of colony stimulating factors into the CNS stimulates microglial mobilisation.[78]

Microglia are important sources of inflammatory cytokines, such as IL-6 and TNF-α.[79,80] Secreted IL-1 promotes astrogliosis,[81] and Fc receptor expression on cultured rat microglia is upregulated following activation with IL-1, TNF-α, IFN-γ, or LPS.[82] The combined expression of Fc and C3b receptors[83] is in keeping with a scavenger role for microglia, as shown by the modulation of nerve endings in the posterior pituitary.[84]

4.3.2 Microglia and Antigen Presentation

Experimentally induced lesions resemble those of chronic neurodegenerative diseases in that there is induction of microglial MHC class II expression with few infiltrating polymorphonuclear leucocytes.[85] Following facial nerve axotomy, the extent of microglial activation that occurs around the affected neurons closely parallels the time course of their TGF-β1 mRNA expression.[86] In this experimental lesion, IL-6 appears to initiate the early phase of microglial stimulation, which in turn leads to the cells releasing TGF-β1 that acts as a negative feedback signal to limit further microglial activation.

Several events are involved in the presentation of antigen by macrophages to CD4-positive T lymphocytes. First, the exogenous protein that has been phagocytosed by macrophages undergoes extensive intracellular processing in which it is enzymatically degraded into small peptides (antigens) that are translocated to the cell surface in "antigen binding pockets" of class II molecules. Co-stimulatory signalling molecules, such as IL-1 and binding proteins, are involved in promoting T-lymphocyte interaction with the macrophage, but it is the combination of class II molecule and antigen that is vital to recognition by the specific T-cell receptor. Although MHC class II molecules are absent from the surface of resting microglia, their expression is induced upon stimulation with IFN-γ[87,88] and activated microglia cells present antigen to lymphocytes *in vitro*.[89] In addition, antigen presentation could be effected by astrocytes whose ubiquitous distribution within the parenchyma and intimate connections with blood vessel walls[90] makes them ideally situated for this role.

4.3.3 Cytotoxic Activity of Microglia

Stimulation of microglia by IFN-γ also induces cytotoxicity towards tumour cells[89] and activated microglia produce reactive oxygen species[91] and nitric oxide,[92] both of which are highly cytotoxic. Indeed, the generation of oxygen radicals by microglia could contribute to the formation of amyloid deposits[93] in Alzheimer's disease and Down's syndrome.[94,95] Identification of myelin debris within microglia at the edge of MS lesions[96] and their secretion of cytotoxic agents that induce death of neurons and demyelination of oligodendrocytes[97,98] has advanced speculation that the cells are actively participating in a number of CNS disorders. Microglia expressing MHC class II molecules are readily identifiable in the CNS of animals with experimental allergic encephalomyelitis (EAE)[99] and of patients with MS,[100] and the demonstration that depletion of macrophages in EAE severely impedes disease progression[101]

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provides persuasive evidence that microglia occupy a prominent position in the chronic inflammatory process.

From current evidence, it seems that microglia are actively involved in the formation of brain cytoarchitecture and resolution of acute injury, but paradoxically, they also promote chronic nerve damage by exacerbating demyelination. Such functional dichotomy, which is also expressed by macrophages in non-CNS tissue, may be dependent upon the nature of the stimulus that activates the cells. For example, in response to traumatic injury, the reparative properties of microglia could be stimulated by M-CSF or GM-CSF released from endothelial cells, resident cells of the CNS parenchyma, or from infiltrating monocytes. In contrast, microglial participation in demyelination could be generated by stimuli that induce phagocytosis[102] and cytotoxicity,[91] or by T-lymphocyte-derived cytokines, such as IL-4, which promotes microglial proliferation, and IFN-γ which induces cell activation and the expression of MHC class II molecules necessary for neuroantigen presentation to autoreactive T lymphocytes. It is the latter effect that might be central to the initiation and progression of immune-mediated pathological disturbance.

4.4 BLOOD LYMPHOCYTES AND DEMYELINATION

The classic example of demyelination of the CNS is multiple sclerosis (MS), in which a chronic inflammatory lesion is characterised by a sharply demarcated plaque containing preserved axons denuded of myelin. Demyelination also occurs in infectious diseases such as progressive multifocal leucoencephalopathy and acute disseminated leucoencephalitis, but it is the disseminated focal form of MS that will be addressed in this review article.

Multiple sclerosis is often considered to be an immune-mediated disease because cellular and soluble components of the immune system are found within lesional areas, abnormal distributions of lymphocyte subsets, cytokines, and immunoglobulins occur in the blood and cerebrospinal fluid, and because it has pathological and clinical similarities to experimental allergic encephalomyelitis (see below). Compromisation of the blood-brain barrier leads to an increased permeability that produces extensive local tissue oedema and passage of blood mononuclear leucocytes into the CNS.[103] Such manifestations may result from an abnormal immune response initiated within the CNS or from a systemic immunoregulatory disturbance that permits the entry of autoreactive T cells directed against myelin antigens. At present it is unclear as to whether myelin loss is due to its "stripping" from the myelin sheath itself or dysfunction of myelin production by the oligodendrocyte.

4.4.1 Pathological Features of MS Lesions

Cellular plaques or lesions are distributed throughout the CNS and range in size from a few millimetres in diameter to confluent areas involving most of the cross section of the spinal cord, brain stem, or large areas of the cerebral hemispheres.[104] The lesions usually encircle a venule or small vein and extend for considerable distances along the course of individual vessels.[105] An early lesion appears as a perivascular area of hypercellularity with infiltrating lymphocytes and monocytes

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and activated microglia expressing MHC class II molecules.[104,106] At this stage, there is no ingestion of myelin by phagocytic cells as judged by staining of conversion products (neutral lipids) with oil red-O (ORO). When demyelination occurs, the lesion is termed "active"; it develops centripetally and macrophages with internalised myelin debris acquire a characteristic "foamy" morphology. Activated lymphocytes and macrophages expressing MHC class II molecules abound in the lesion and they are often surrounded by deposits of immunoglobulins (including oligoclonal IgG) and complement. At the centre of the active lesion there is a loss of oligodendrocytes as the activated microglia and macrophages complete the phagocytosis of myelin debris. Even when the inflammation subsides, the peripheral rim of lesions retain MHC class II macrophages that are positive for ORO. The inactive lesion is comprised of a sharply demarcated area of demyelinated axons accompanied by local astrocytosis, little evidence of lipid destruction, and a few HLA-DR-positive macrophages and microglia.[107]

The majority of small lymphocytes in early MS lesions and in the hypercellular edge of plaques extending into normal white matter are CD4-positive T lymphocytes.[108-110] T lymphocytes of the CD8 phenotype are present in early lesions but this population predominates in later lesions.[111] Examination of the distribution of T-lymphocyte subsets in MS plaques of different activity has led to the proposal that CD4-positive cells are responsible for the development and expansion of lesions, whilst the CD8-positive subset controls their local activity.[110,112]

4.4.2 Experimental Allergic Encephalomyelitis (EAE)

Unravelling the contribution of T lymphocytes to the demyelinating process has benefited from the study of the animal model of MS: experimental allergic encephalomyelitis (EAE).[113,114] The model is induced by myelin proteins and their immunogenic peptides or by adoptive transfer of sensitized CD4-positive T lymphocytes to naive, syngeneic animals.[115] Depending upon the species and strain of the animal, a monophasic, acute or spontaneous relapsing-remitting form of the disease is induced. Disease progression is inhibited by treatment with antibodies to MHC class II molecules,[116] to CD4-positive cells,[117] and by "vaccination" with disease-producing T lymphocytes.[118]

4.4.3 T Lymphocytes and Recognition of CNS Autoantigens

Organ-specific autoimmune diseases are triggered either by loss of self-tolerance to a tissue antigen or by sensitisation to self-antigen following an encounter with an infective microorganism (e.g., molecular mimicry and cross-reactivity, release of sequestered autoantigens, or T-cell activation by superantigens).[119] Since T cells that recognise myelin-specific proteins (e.g., myelin basic protein, proteolipid protein, and myelin oligodendrocyte protein) circulate in MS patients and healthy subjects, it appears that lymphocytes which recognise CNS autoantigens belong to the "normal" T-cell repertoire.[120] In MS blood there is an increased prevalence of myelin-specific CD4-positive lymphocytes[121] which, upon entering the CNS, could be activated

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by myelin products released as a consequence of CNS inflammation, viral infection, or by the recognition of epitopes common to pathogens and autoantigens.[122] Sequence homology exists between myelin proteins and several viruses,[123,124] but T lymphocytes that recognise both viral and myelin epitopes have yet to be demonstrated. Lymphocyte reactivity against myelin proteins is often confined to selective immunodominant determinants in EAE,[119] and shifts in epitope recognition by clones of T cells in MS could contribute to the relapsing-remitting phase of the disease.

The T-cell receptor (TCR) binds antigen in association with an MHC class II molecule. Extensive heterogenicity exists in the structure of such receptors on different lymphocytes so as to accommodate the vast array of potential antigens that the immune system is capable of recognising. In T lymphocytes that infiltrate the CNS of animals with EAE, there is considerable conservation of the genes (known as V region genes) that encode for the specific regions of the αβ TCR suggesting that they might be reactive against CNS antigens. Indeed, EAE is inhibited by the administration of antibodies directed against TCR Vβ variable sequences[125] and by DNA vaccines encoding variable regions of the TCR.[126] However, evidence for restricted V region gene usage in MS is controversial[127,128] despite the beneficial application of specific TCR blocking peptides to patients with this disease.[129]

Regardless of antigen specificity, it is activated lymphocytes that cross the blood-brain barrier, but only T lymphocytes that recognise CNS antigens persist in the parenchyma and induce inflammation.[130] An increase in the number of lymphocytes entering the CNS, therefore, is more likely to depend upon activation events in the periphery rather than infection and injury in the brain. Demyelination may arise from the direct or indirect activity of T lymphocytes. Multiple sclerosis plaques in areas of acute demyelination[131] and at margins of chronic lesions[132] contain T cells which express γδ TCRs. These unusual T cells recognise peptides of prokaryotic origin including heat shock proteins and attack and destroy oligodendrocytes *in vitro*.[133] Oligodendrocytes are also susceptible to "bystander" damage during T-cell-mediated reactions within the local microenvironment. Cultured oligodendrocytes are lysed by T-lymphocyte-derived TNF-β[134] (also known as lymphotoxin), and by perforins, produced by activated cytotoxic T cells.[135] The generation of pore formation by perforins leads to intracellular calcium influx and cell death that resembles the cytopathic effects of the membrane attack complex of complement.[136] These observations suggest that it is the oligodendrocyte rather than the myelin sheath which is particularly susceptible to T-lymphocyte-mediated damage.

4.4.4 T Lymphocytes, Cytokines, and Macrophages

Recognition of neuroantigens by sensitised T lymphocytes within the CNS could result in the release of cytokines and the activation of resident cells such as microglia which then induce demyelination. Based upon the profile of secretory cytokines, CD4-positive lymphocytes are subdivided into the TH1 and TH2 populations. Cytokines characteristic of TH1 cells include IL-2, IFN-γ, TNF-β, and hence these cells are deemed to be proinflammatory. Interleukin 2 is vital for the survival of activated T cells, IFN-γ enhances the phagocytic, cytotoxic, and antigen-presenting properties of macrophages and microglia,[137] and TFN-α lyses oligodendrocytes,[138] induces

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demyelination *in vivo*[139] and, at the level of the blood-brain barrier, it may recruit and activate leucocytes. All three cytokines are readily identifiable within inflammatory cell infiltrates in MS lesions[140] and it is the TH1 cells that are responsible for the passive transfer of EAE.[141] On the other hand, TH2 cells secrete TGF-β and interleukin 10 (IL-10) and are assigned an antiinflammatory function. Interleukin 10 impairs indirectly the activities of TH1 cells,[142] TGF-β promotes the healing phase of inflammation,[143] and both suppress the proinflammatory activities of macrophages.[144,145] Disease remission in EAE is associated with an expansion of TH2 cells[146] and an inability to switch from a predominantly TH1 to TH2 response may underlie the demyelinating events of EAE. No doubt, information will shortly be forthcoming concerning the distribution and function of TH1 and TH2 cells in MS.

Macrophage-mediated demyelination is implicated in both early and late MS lesions. The initial stages of myelin destruction are rapid[147] and the oligodendrocyte or myelin sheath or both may be targeted in the primary disease process.[148] Macrophages are held responsible for the majority of demyelination by releasing toxic factors such as TNF-α, proteinases, free radicals, and nitric oxide.[149,150] The cells appear to lyse and then internalise myelin lamellae around myelinated axons until they become engorged with myelin debris.[115] This debris becomes attached to clathirin-coated pits on the macrophage surface before internalisation in a process termed "receptor-mediated phagocytosis."[151]

In summary, considerable evidence implicates the T lymphocyte as occupying a pivotal role in the pathogenesis of demyelination in MS. Following recognition of myelin antigens, it is most likely that these cells release cytokines which trigger a cascade of events that results in leucocyte extravasation, activation of infiltrating inflammatory cells and resident macrophages, and loss of myelin. As the lesion ages, recruitment of γδ T cells could result in further myelin destruction. It is therefore understandable that considerable efforts are in progress to devise methods that will antagonise either the entry of T lymphocytes across the blood-brain barrier or their recognition of myelin antigens.

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5 Role of Macrophages and Microglia in the Injured CNS

Wolfgang J. Streit

CONTENTS

5.1 INTRODUCTION

The normal adult CNS contains roughly as many microglia as it does neurons[1] and based on this numerical relationship alone one could argue that microglia must be extremely important for maintaining a healthy CNS. In contrast, macrophages are generally absent from the mature CNS parenchyma unless there is some pathological event resulting in neuronal or glial cell death which calls for the removal of degeneration debris by phagocytic cells. Since microglia are ubiquitous and can readily transform into phagocytic cells, they are *the* primary source of brain macrophages. This is true especially in those pathological situations where the blood-brain barrier remains undisturbed, or where (auto)immune mechanisms do not play a role. When the blood-brain barrier is disrupted, or when there are specific immune responses, blood-borne as well as meningeal and perivascular macrophages contribute to form a heterogeneous brain macrophage population at the lesion site. A variety of interesting cellular interactions are likely to occur between brain macrophages, neurons, and macroglial cells in the lesion microenvironment. For the most part these interactions

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are beneficial, and are designed to clean up and reconstruct the wounded brain or spinal cord area. Understanding these cellular interactions will improve the design of strategies to enhance the limited regenerative power of the CNS.

5.2 RELATIONSHIP BETWEEN MICROGLIA AND BRAIN MACROPHAGES

This has been the subject of intense controversy for the past few decades, due in part to the terminology used, but also due to the different schools of thought which are sometimes dogmatic and inflexible. Thus, from the outset, we would like to provide a few simple definitions which may help facilitate a more meaningful discussion of the sources and functions of brain macrophages.

A *brain macrophage*, simply put, is a macrophage in the brain. The normal brain parenchyma is devoid of macrophages; they appear only when scavenger cells are needed. There are a number of potential sources of brain macrophages and these include CNS resident microglial cells, blood-borne monocytes, and perivascular and meningeal cells.[2]

A *microglial cell* is a resident cell of the CNS. This means that it is distributed ubiquitously throughout the parenchyma and that it is in contact with other macroglial cells and neurons. Resident microglia in the normal CNS are also called ramified, or resting microglia, and these cells are normally not phagocytic (they are resting from phagocytosis). They transform into phagocytes only if degeneration occurs and debris needs to be cleared. Because microglia are present everywhere in the CNS and are the first to encounter any degenerative changes in the parenchyma, they constitute *the* primary source of brain macrophages. Microglia have the potential to renew their cell pool via mitosis; once they have become activated and transform into microglia-derived brain macrophages their ultimate fate is one of programmed cell death.[3]

Mononuclear phagocytes is a generic, descriptive term for a major cell population of the immune system capable of phagocytosis. These cells are found in every organ, and are often referred to as tissue-specific macrophages. Examples of these include Kupffer's cells of the liver, alveolar macrophages of the lung, and monocytes of the blood. Although there is little doubt that tissue-specific macrophages are ontogenetically related and possibly of the same lineage with a common primordial precursor cell, in the case of microglia it is unlikely that they are continuously being replenished by monocyte-like precursor cells from the blood stream, as claimed by the central dogma of the mononuclear phagocyte system (see below).

A *monocyte* is a mononuclear phagocyte in the blood. Monocytes have free access via fenestrated capillaries to most organs in the body where they can become tissue-specific macrophages. The CNS represents an exceptional organ in that its capillaries are nonfenestrated and the endothelial cells form tight junctions which, in essence, constitute the blood-brain barrier. Thus, all blood leukocytes, including monocytes, are normally prohibited from entering the CNS.

A *perivascular* cell is a mononuclear phagocyte closely associated with the brain vasculature. Perivascular cells are components of the blood vessel wall and they

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occupy the perivascular space, an area covered by the vascular basement membrane that separates the CNS parenchyma from non-CNS tissue. Perivascular cells are thus not contained within the CNS parenchyma.

Perivascular microglial cells, also known as a *juxtavascular microglial cells*,[4] are true CNS parenchymal cells like all microglia. They get their name because of their location, i.e., they are microglia located in the immediate vicinty of cerebral blood vessels. Perivascular microglia often contact the perivascular basement membrane with their cytoplasmic processes.[1,5,6]

Fetal macrophages is a term used to describe some of the earliest hematopoietic cells which have differentiated along the macrophage lineage during embryonic development.[7] In rodents, they colonize the developing neuroectoderm near mid gestation.[8] They possess a high proliferative potential,[7] and they are likely to be immediate precursors for microglial cells.[9]

In the following, possible interrelationships between these closely related cell types will be discussed separately in the developing and in the adult brain.

5.2.1 Microglia and Macrophages in the Developing Brain

5.2.1.1 The Normal Developing Brain

The immature CNS contains considerably more brain macrophages than the mature CNS because large numbers of cells undergoing developmentally regulated cell death are being eliminated. All macrophages and microglial cells can be stained with the B_4-isolectin from *Griffonia simplicifolia*,[8-10] a marker whose binding specificity for microglia/macrophages is independent of developmental age (Figure 5.1). This is in contrast to other surface markers such as the OX-42 antibody which recognizes the CR3 complement receptor, a molecule that is not expressed on most microglia until the second postnatal week.[11,12]

Around mid gestation, possibly earlier, lectin-reactive fetal macrophages are found in small numbers throughout the developing neuroectoderm.[8-10] As the developing brain increases in size, so does the number of the lectin-binding fetal macrophages that soon develop a process-bearing morphology. However, the morphology of these early, process-bearing cells is not quite the same as that of adult ramified microglia. Their cytoplasmic processes are shorter and thicker, and not as extensively ramified as those of fully differentiated microglial cells (Figures 5.1 to 5.3). We prefer using the term *intermediate* microglia[9] for these early process-bearing cells to emphasize that their morphology is in between that of ameboid and ramified microglia. During the perinatal stage of development both intermediate and ameboid microglia are abundant, the latter being found aggregated in ameboid cell clusters. These clusters, which are most conspicuous during the early postnatal period, are found in characteristic locations such as the supraventricular corpus callosum, the cavum septum pellucidum, and other developing white matter regions.[9,11,12] It has been shown by using a fluorescent marker, rhodamine B isothiocyanate, that ameboid microglia differentiate into ramified microglia during postnatal development.[13] Accordingly, the idea of developmental metamorphosis from ameboid to ramified

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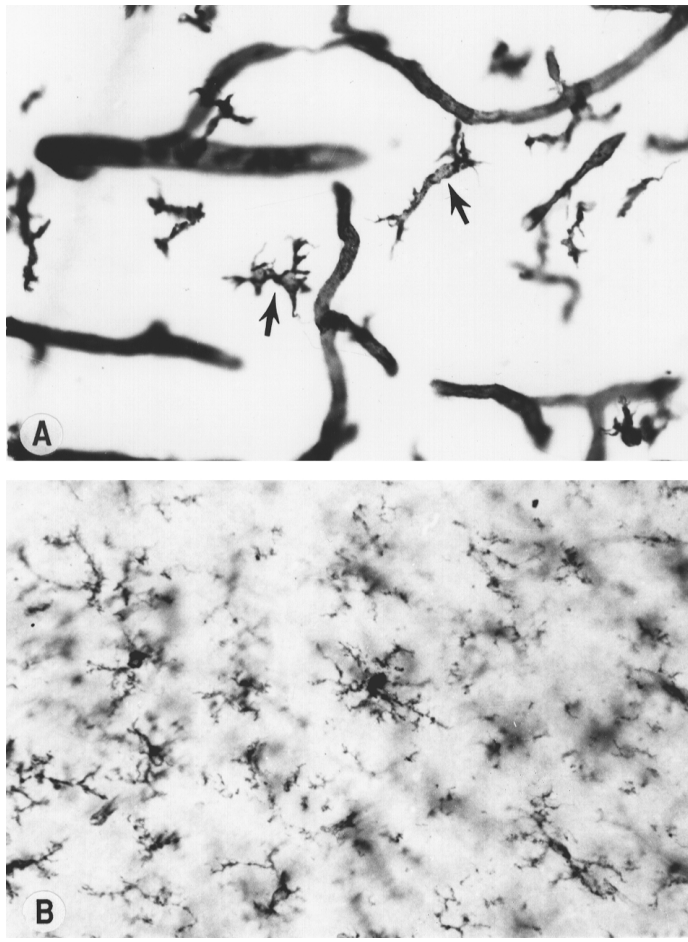

FIGURE 5.1 A/B Vibratome sections of midbrain from an embryonic day 18 rat (**A**) and from an adult rat (**B**) stained with the *Griffonia simplicifolia* B_4 isolectin (GS I-B_4-HRP) to visualize microglial cells. Young microglia in the developing brain have a morphology intermediate between ameboid and ramified with stout cell processes (arrows in **A**). Note the strong staining of developing vascular channels. Fully ramified microglia are abundant in the adult CNS (**B**). (Magnification × 400.)

is widely accepted, and leaves one wondering only whether this is true for all ramified microglia.

A somewhat more controversial issue concerns the origin and function of ameboid microglia. Specifically, do monocytes give rise to ameboid microglia, and how do they get into the brain? Monocytes are differentiated blood-borne mononuclear cells and members of the mononuclear phagocyte system (MPS). According to the central tenet of the MPS, blood-borne monocytes invade all organs of the body and give rise to tissue-specific macrophages.[14] Thus, if one were to regard microglia as tissue-specific macrophages of the CNS, according to the MPS hypothesis there

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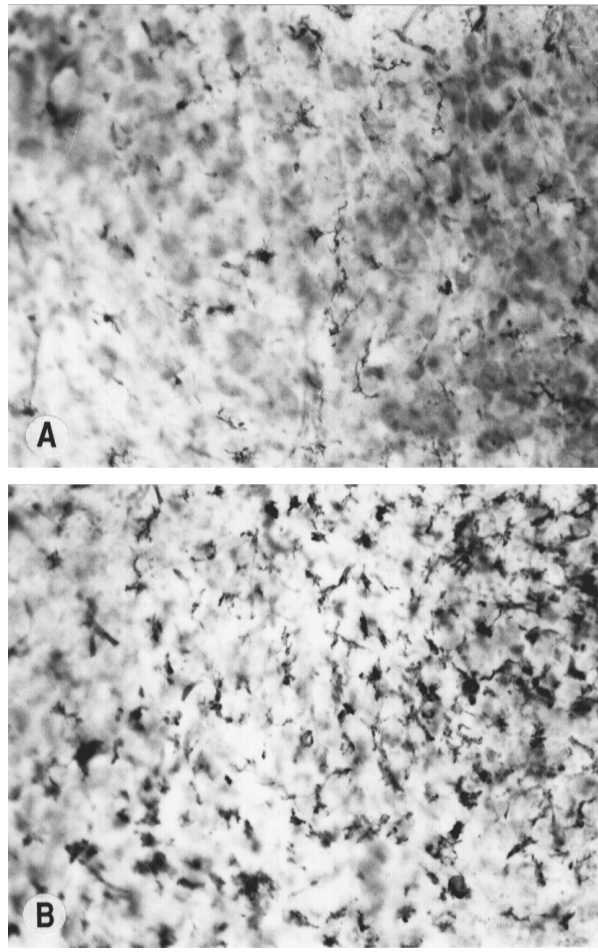

FIGURE 5.2 A/B Staining of microglial cells with GS I-B_4-HRP in vibratome sections after unilateral facial nerve axotomy in neonatal rats (cresyl violet counterstain). Panel **A** shows microglial cells scattered throughout the unoperated facial nucleus on postnatal day 5. The contralateral axotomized nucleus (**B**) reveals activated microglia responding to axotomy-induced neuronal cell death 5 days after nerve transection. Microglia are hypertrophied and clumped in phagocytic clusters. (Magnification × 200.)

would be continuous replenishment of microglia by monocytes over the lifetime of the organism. However, blood-borne mononuclear cells are normally excluded from the CNS, and invasion of the CNS by mononuclear cells is rarely observed histologically under nonpathological conditions. Moreover, during the perinatal and early postnatal periods when the rodent brain undergoes its most dramatic growth, one would expect to see a massive influx of blood monocytes to account for the large number of microglia that are present in the CNS at that time. Particularly, those white matter regions showing perinatal clusters of monocyte-like ameboid microglia would be expected to show histologic evidence of monocytes migrating through

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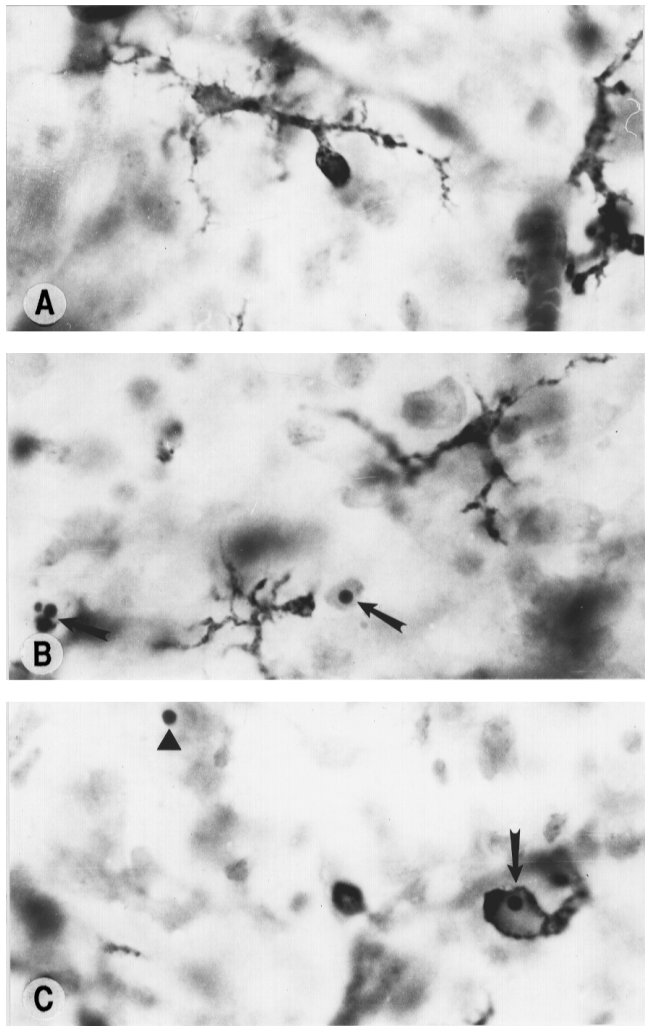

FIGURE 5.3 A/B/C Staining of microglial cells with GS I-B_4-HRP in vibratome sections of brainstem from a postnatal day 5 rat (cresyl violet counterstain). Panel **A** shows two young microglial cells which are undergoing differentiation into ramified microglia. Panel **B** shows two primitive ramified microglial cells in the vicinity of two apoptotic cells (arrows). The apoptotic cell on the left shows several nuclear fragments. In **C**, a pyknotic nucleus has been engulfed by a microglial cell process which is identified by positive lectin staining (arrow). The triangle indicates another apoptotic cell which has not yet been engulfed. (Magnification × 1000.)

blood vessels which, at this stage, are developing interendothelial junctions.[15] This histologic evidence simply does not exist. So if not from monocytes, where do the ameboid microglia come from and why do they collect in clusters?

It is our hypothesis[9] that fetal macrophages (primitive myelomonocytic cells), which are present in the developing CNS prior to the onset of brain vascularization[8-10]

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and prior to the presence of monocytes in the body,[16] differentiate into intermediate and ameboid microglia, with the latter aggregating in characteristic white matter sites, such as the supraventricular corpus callosum. The reasons for the distinctive homing of ameboid cells to white matter are unknown, but probably not related to the presence of developmentally regulated programmed cell death which is more common in the cortex than in the corpus callosum.[17] Instead, the reasons are more likely to be found in mechanisms that underlie white matter tract development and/or in chemotactic phenomena.[18,19] Interestingly, while ameboid microglia do show macrophage characteristics ultrastructurally, including numerous lysosomes that are positive for acid phosphatase and periodic acid-Schiff,[20] they do not contain prominent phagocytic inclusions such as the ones commonly seen in microglia-derived macrophages that appear after experimentally induced neurodegeneration.[21,22] Therefore, the role of ameboid microglia during development is not simply one of a scavenger cell. The cells are more likely to participate in the promotion of axonal growth and in the guidance of growing axons.

5.2.1.2 The Pathological Developing Brain

At the time of birth the CNS contains large numbers of microglial cells, most of which are of the intermediate type with stout cellular processes (Figures 5.1 to 5.3). These young microglia, like their adult ramified counterparts, are exquisitely sensitive to neuronal death and are quickly activated following CNS injury. In fact, most microglia in the developing CNS are in an activated state as judged by their morphology and phagocytic activity. Intermediate microglia are active in the phagocytic removal of apoptotic cells throughout the developing CNS (Figure 5.3). When in addition to naturally occurring cell death there is experimentally induced neurodegeneration, such as after peripheral nerve lesions, activation of immature microglia involves changes in cell immunophenotype. These phenotypic changes, which reflect altered expression of cell surface receptors, are similar to those seen during activation of adult microglia after a CNS lesion. When activated by neonatal sciatic nerve transection, immature microglia upregulate the expression of immunomolecules, such as major histocompatibility complex (MHC) and lymphocytic antigens.[23] Cells expressing these antigens constitutively in the neonatal CNS are rare, and are primarily perivascular cells. As sciatic nerve axotomy causes scattered death of motoneurons throughout affected spinal cord segments, phagocytic clustering of activated microglia around such dying motoneurons is a common occurrence and facilitates the histopathological identification of these sites of neurodegeneration. Interestingly, proliferation of microglia, which is robust in adult animals after facial nerve axotomy,[24] does not appear to occur as intensely in neonatal animals since mitotic figures are uncommon. Although microglial density is increased in the axotomized neonatal facial nucleus, this is due largely to cellular hypertrophy (Figure 5.2). On the other hand, the demonstrated presence of microglial mitogens at E20 does suggest ongoing microglial proliferation during the perinatal period.[25,26] Further studies to quantitatively assess microglial proliferation after neonatal nerve section are needed.

Clearly, one of the major functions of intermediate microglia in the developing CNS is the continuous removal of cell debris resulting from naturally occurring cell

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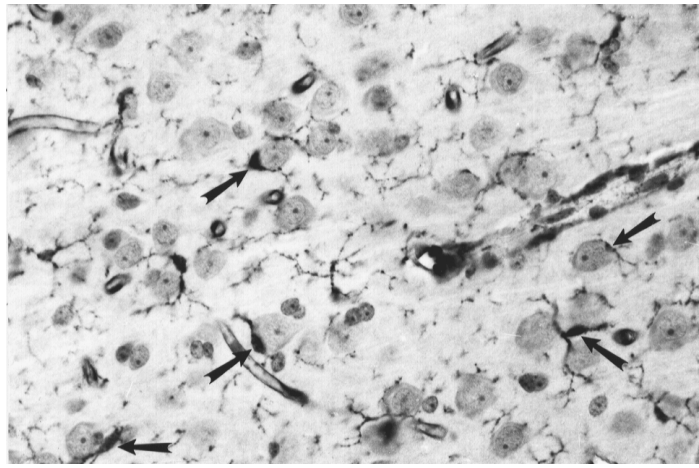

FIGURE 5.4 Paraffin section of normal adult monkey cortex stained with the lectin from *Ricinus communis* to visualize microglial cells. Several microglial cells are seen in close association with neurons (arrows). (Magnification × 400.) Note: Thanks goes to Dr. Amyn Rojiani for providing the sections of monkey brain used to produce this figure.

death. We consider these intermediate microglia to be in a heightened phagocytic state compared to ramified microglia, that is, they change into brain macrophages much more readily than ramified cells, and thus resemble the activated microglia encountered after CNS lesions in the adult. Conceptually, intermediate microglia may be viewed as a transitional stage, both morphologically and functionally, between immature ameboid cells and the fully differentiated ramified cells of the adult (Figures 5.3 and 5.5).

5.2.2 Microglia and Macrophages in the Adult Brain

5.2.2.1 The Normal Adult Brain

Ramified microglial cells populate all areas of the CNS, and depending on the type of histochemical stain used and the species examined, there may be considerable regional differences in microglial density.[1,27,28] Generally speaking, microglial densities tend to be somewhat higher in gray matter regions. Notwithstanding these regional variations, microglia are ubiquitous in every part of the CNS suggesting that they are essential for maintaining normal homeostasis. What may be the specific functions of this widespread glial cell population? The ubiquity of resting microglia, together with the fact that they are often present as perineuronal satellites directly contacting many neurons[29] (Figure 5.4), suggests ongoing neuronal-microglial interactions. Evidence which could help elucidate the nature of such interactions comes from studies showing that microglia produce neurotrophic factors *in vitro*.[30-35] It is therefore conceivable that when neurons are experiencing metabolic stress, perhaps related to increased activity during normal brain functioning, microglial satellite

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cells provide support in the form of neuronal growth factors. This type of microglial-neuronal interaction is likely to occur continuously during the life of an organism, particularly when toxic or other disturbances place neurons in need of increased trophic support. For example, following axotomy of adult motoneurons perineuronal microglial satellites proliferate and rapidly ensheath the axotomized cells. Since adult motoneurons regenerate their severed axons, production of growth factors, such as transforming growth factor-β, by activated microglial cells during perineuronal ensheathment could play a pivotal role in facilitating successful axon regeneration.[36-38] In this context, it is intriguing to note that perineuronal ensheathment by microglia is absent after axotomy of rubrospinal neurons, intrinsic cells of the CNS which do not regenerate after axon transection.[39,40]

The notion that microglia form a network of immunocompetent cells in the CNS also derives from both *in vivo* and *in vitro* observations.[41] Numerous histological studies have investigated microglia phenotypically using an array of antibodies directed against immunomolecules, such as MHC, lymphocyte, and macrophage antigens, most of which show enhanced expression after CNS injury.[4,19,28,42] In the normal noninjured CNS, particularly in the human, there is constitutive expression of MHC class II antigens by microglia[28,43,44] as well as by perivascular cells.[45] Such constitutive and selective MHC class II expression has been critically important for developing the idea that microglia and perivascular cells are antigen-presenting cells of the CNS.[5,46,47] Additional support derives from studies conducted *in vitro* which demonstrate that cultured microglial cells can indeed function as antigen-presenting cells by stimulating lymphocyte proliferation,[48-50] and that they produce a number of cytokines, including those required for functional antigen presentation.[4,19,51,52] A question arises as to whether this antigen-presenting function occurs in the normal nonpathological brain, or only during pathological states. Since lymphocytes are virtually absent from the normal CNS, it is unlikely that there is any significant interaction between microglia and lymphocytes that occurs constitutively. However, the idea that small numbers of activated lymphocytes continuously patrol the normal CNS is an interesting one[53] and raises the possibility for a mechanism by which the peripheral immune system may become sensitized to CNS antigens. The fact that one of the most antigenic CNS molecules, myelin basic protein (MBP), is present in white matter, together with the observation that microglia in white matter constitutively express MHC class II molecules, could mean that microglia in white matter process MBP and present encephalitogenic peptide fragments to patrolling lymphocytes, particularly after white matter damage. In the same vein, processing of myelin components might be a constitutive activity of microglia that is necessary for the recycling of membrane components.

This discussion has been limited to possible roles of ramified microglia, and not macrophages, in the normal adult brain. This is because full-fledged macrophages, like lymphocytes, are virtually absent from the healthy brain parenchyma, and are normally found only in the perivascular and meningeal spaces. However, under pathological conditions macrophages can become prominent cellular elements in the CNS, exerting critically important functions related to the rebuilding effort at the injury site.

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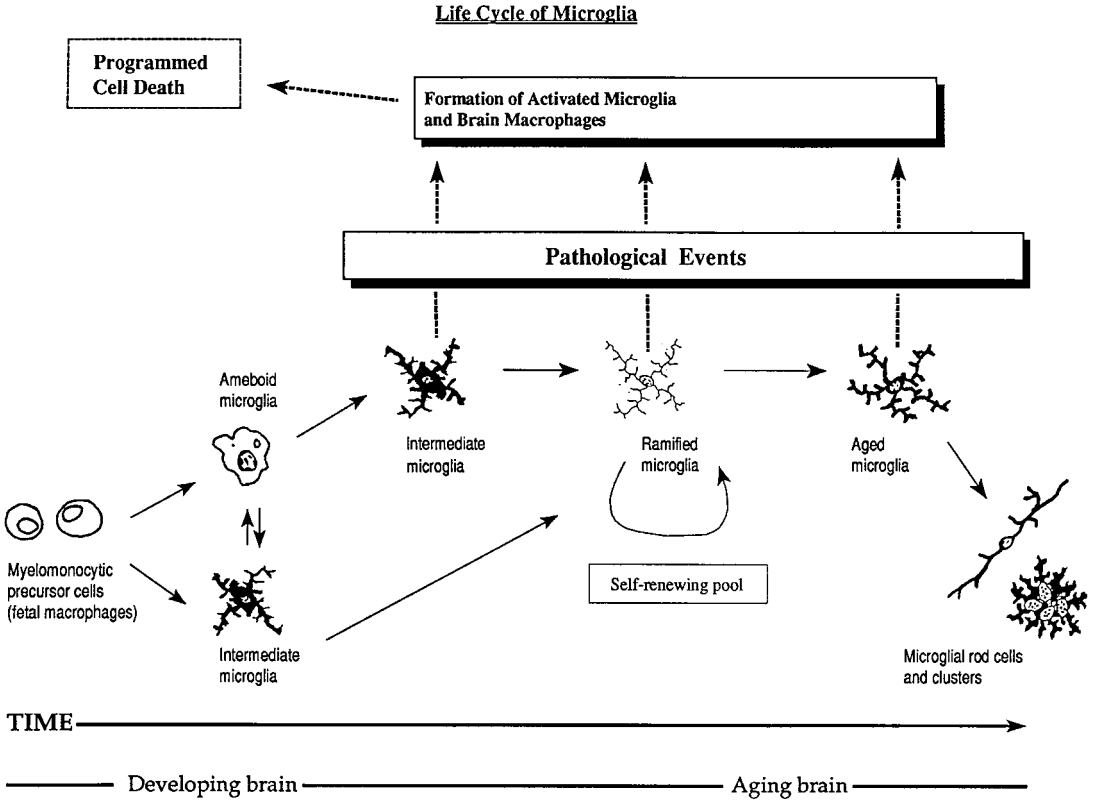

FIGURE 5.5

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5.2.2.2 The Pathological Adult Brain

As pointed out, microglia, due to their widespread presence in all corners of the CNS, are *the* primary source of brain macrophages when the need for phagocytic cells arises. That microglia can, in fact, undergo transformation into brain macrophages was shown conclusively using intraneural injections of toxic ricin.[21] Thus, the majority of brain macrophages in areas of neurodegeneration are derived from microglia. When there is a breach of the blood-brain barrier, such as after a traumatic injury, blood-borne monocytes contribute significantly to the brain macrophage population at the lesion site, however, these monocytes do not differentiate into microglia.[54] Studies in recent years using bone-marrow chimeras have provided additional strong support to the idea that renewal of the microglial population does not occur via replenishment by blood-borne monocytes, even under conditions of brain inflammation.[55-57] Instead, it is primarily the perivascular and meningeal macrophages which are being replaced by blood-borne monocytes.

The literature concerning microglia and macrophages in the pathological adult CNS is enormous and, at times, controversial. Since there are several recent reviews on this subject,[4,19,42,58-60] we will limit this discussion to a few salient concepts and observations. First there is the concept of *microglial activation*. Microglial activation refers to the transformation of resting microglia into activated microglia. *In vivo*, this process involves morphologic and phenotypic changes which occur in microglia responding to pathologic stimuli. The transformation of resting into activated microglia involves retraction of finely branched microglial processes into short and stout ones, as well as the upregulation and/or the *de novo* expression of cell surface molecules, e.g., MHC antigens. In pathologic situations which are accompanied by cell death, activation of microglia results in the formation of brain macrophages which remove cellular debris. In addition to changing their morphology and antigenic profile, microglia are readily induced to proliferate after CNS injury.[24] Thus, microglial activation *in vivo* is characterized by major changes affecting cell number, cell morphology, and cell surface antigens (immunophenotype).

The *in vivo* observations are complemented by studies examining microglial activation *in vitro*. Tissue culture studies have revealed an important microglial property which changes as a result of cell activation, namely, microglial secretory activity. Numerous studies have reported that cultured microglial cells can produce

FIGURE 5.5 Schematic illustration of key stages of the microglial life cycle. Primordial macrophage precursor cells invade the developing neuroectoderm early in development and differentiate into ameboid and process-bearing (intermediate) microglia. The possible interconversion between ameboid and intermediate microglia is indicated by the bidirectional arrows. Mature ramified microglia represent a cell population which is capable of self-renewal via mitosis, but this is likely to be true for intermediate and ameboid cells as well. With increasing age, microglia undergo subtle morphological changes and show enhanced MHC antigen expression.[73] At an advanced age, microglia become rod-like and tend to aggregate into small clusters. If pathological changes occur in the CNS, microglia become activated and may transform into brain macrophages which then undergo programmed cell death. The possibility remains that, once pathological changes subside, activated microglia may convert back to the resting state.

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various cytokines and growth factors following stimulation with bacterial lipopolysaccharide (LPS), interferon-γ (IFN-γ), or other immunostimulatory agents.[30-35,51,52,61,62] However, given the fact that these results are obtained in culture using nonphysiologic stimuli, the *in vitro* data must be confirmed by *in vivo* studies examining the secretory activity of activated microglia in the CNS. It is necesssary at this juncture to point out an important difference in the use of the term "activated microglia" between *in vivo* and *in vitro* studies. As stated, resting microglia are the highly ramified cells that can be visualized in sections of the normal, nonpathologic CNS. In response to a pathologic stimulus these resting cells become activated as judged by the criteria listed above, i.e., cell number, morphology, and immunophenotype. In contrast, purified microglial cells *in vitro* which are isolated from mixed whole-brain cell cultures, are already in an activated state using these *in vivo* criteria. This is because in the process of preparing primary whole-brain cultures, tissue trituration and enzymatic digestion generate large amounts of debris which is phagocytized by microglia present in these primary cultures. Accordingly, the microglia that are isolated from primary cultures have already become macrophages, and they do not at all resemble resting microglia as defined *in vivo*. Upon isolation of brain macrophages from primary mixed brain cultures, they are typically stimulated with LPS, or other agents, and are subsequently referred to as "activated brain macrophages". From this, it is obvious that microglial activation *in vitro* respresents a level of activation quite different from that observed in most *in vivo* paradigms. Accordingly, we suggest using the term superactivated to set apart those cells activated with immunostimulatory agents in culture.

Microglial activation can occur very rapidly after brain injury.[63] In light of the aforementioned close relationship between neurons and perineuronal microglial satellites, it would seem that any normally occurring communication between neurons and microglia is altered after CNS injury in such a fashion as to stimulate microglial activation. The nature of the chemical or electrical signals exchanged between neurons and microglia is unknown, however, one important clue might lie in the peculiar nature of microglial ion channel patterns.[64]

Microglial activation seems to occur in a stereotypic pattern, that is, the cells undergo similar changes after different types of lesions. This suggests a programmed cellular response which follows a standardized set of genetic instructions. The genetic switches which control microglial activation are unknown and much work remains to be done in this area. It is likely that the first step in this intracellular signalling cascade involves an inciting stimulus coming from injured neurons.

Microglial activation shows regional specificity in that it is limited to those regions which contain injured or dying neurons. Our own studies using trimethyltin intoxication have underscored this specificity of the microglial response, since trimethyltin selectively affects some neurons in certain CNS regions.[22] The regional specificity of microglial activation adds further support to the idea that certain injury signals are being produced by affected neuron populations.

Microglial activation is reversible. A classic example for this can be found, once again, in the facial nucleus paradigm where microglia are rapidly activated, remain in an activated state for several weeks, and then return to the resting state again. At

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least, this is what appears to be the case. We should caution that reversibility from the activated to the resting state has not been proven experimentally, and that programmed cell death after activation has been shown to occur (Figure 5.5).[3]

5.3 WHAT COMES FIRST — MICROGLIAL ACTIVATION OR NEURODEGENERATION?

Axotomy models have shown that neuronal death is not necessary to activate microglial cells.[39,59,65,66] Conversely, they have also shown that microglial activation does not result in neuronal death, but instead coincides with neuron regeneration. A particularly fascinating aspect of the microglial response to axotomy of motoneurons is the rapid ensheathment of axotomized neurons by microglial cell processes. Not only does this result in "synaptic stripping,"[59] but it also brings the microglial cell membrane into direct contact with the neuronal membrane. This is a situation which could clearly enhance any neuronal-microglial interactions, such as the transfer of neuronal injury signals, as well as any transfer of growth factors from microglia to neurons. At the same time, the ensheathment of axotomized neurons by microglial cell processes is very reminiscent of a phagocytic engulfment, and one could argue that if, in fact, a neuron was not going to recover from axotomy and was going to die, microglial cells would be situated perfectly to proceed with the removal of the dying cell. Thus, from a neuronal point of view, there may be a very fine line between receiving neurotrophic support and being eaten up, and any signals emitted from injured neurons should be sufficiently specific to enable this life or death decision. Since neurotoxicity is a well-documented property of cultured microglial cells,[67-69] one wonders how and when microglial neurotoxicity comes to bear *in vivo*. Obviously, this dark side of the microglial cell must be under strict control in acute injury situations, since microglial activation *in vivo* does not result in additional neurodegeneration in the immediate vicinity. However, it is likely that some of this inhibition is lost in certain chronic conditions, such as Alzheimer's disease or HIV infection, and that in those circumstances the disinhibited microglia may indeed be the cause of neuronal damage.

5.4 CAN MICROGLIA AND MACROPHAGES PROMOTE CNS REGENERATION?

We would like to answer this question with a definitive "yes". Although the evidence has been somewhat slow in arriving, recent studies are providing clear indications that one of the major functions of microglia and brain macrophages after CNS injury is not just to clean up, but also to reconstruct the wound site. We have already mentioned studies which show production of neurotrophic and proregenerative peptides by these cells (see above). Other investigations have shown production of extracellular matrix molecules by macrophages/microglia, such as thrombospondin and laminin, which serve as excellent substrates for growing neurites.[70,71] Perhaps the most direct indication of the benefits of microglia and macrophages in promoting

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regrowth of injured CNS axons comes from experiments showing enhanced neurite growth after transplantation of these cells.[71,72] With these recent insights, a new chapter has been opened for advancing strategies in the treatment of CNS injuries.

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6 Cellular Trafficking

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CONTENTS

6.1 INTRODUCTION

Inflammation often arises from the untoward activity of blood leucocytes whose extravasation into tissue depends upon attachment to endothelial cells, passage across vessel walls, and migration along a chemotactic gradient. Lymphocytes constantly recirculate between blood and tissues in their active pursuit of antigens expressed by foreign pathogens.[1] Very few lymphocytes are present in normal brain[2,3] and this observation combined with the demonstration of poor lymphatic drainage, little MHC class II expression, and incomplete rejection of foreign tissue grafts has led to the proposal that the brain is an immunologically privileged site.[4-8] Leucocyte entry into the central nervous system (CNS) is restricted by the unique properties of the blood-brain barrier (BBB). This custodial protection of the brain is necessary since it is more vulnerable than other tissues to immune or inflammatory-mediated

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damage by virtue of an integrated network of neurons with a low regenerative capacity and little connective tissue support.

The BBB consists principally of concentric layers of endothelial cells, pericytes, basal membranes, and tightly apposed astrocytic end-feet,[7] but it is the endothelium that is predominantly responsible for restricting the entry of leucocytes into the CNS. Several features of the CNS endothelium may contribute to its unique barrier function: the cells are interconnected by tight junctions, are relatively devoid of pinocytotic vesicles and fenestrations, carry an unusually high net negative surface charge,[24] and have a low or inappropriate expression of surface adhesion molecules that support leucocyte attachment.[21,25-27] Of the small number of leucocytes present in the normal brain, the majority are lymphocytes.[3] When the BBB is compromised serious pathological manifestations may ensue as illustrated by multiple sclerosis (MS) in which the features of encephalomyelitis are attributed to the CNS entry, perivascular sequestration, and local activation of sensitized lymphocytes and other blood mononuclear leucocytes.

This review will focus on the current understanding of mechanisms controlling the migration of lymphocytes across the BBB in MS and its animal model, experimental allergic encephalomyelitis (EAE), because these demyelinating disorders have attracted more investigation than any other inflammatory disease of the brain. It is likely that results generated from these studies will impact upon lymphocyte trafficking in several pathological disorders of the CNS.

Multiple sclerosis is characterised by demyelination and inflammatory cell infiltrates in the CNS, particularly in the periventricular white matter and the cervical region of the spinal cord. Selective destruction of the myelin sheaths with sparing of the axons produces diminished nerve conduction and neurological defects. Disease onset and perpetuation are believed to be immune-mediated,[9-12] with CD4-positive lymphocytes and macrophages ascribed prominent roles in lesion progression and the demyelination process, respectively.[13,14] Experimental allergic encephalomyelitis (EAE) is a T-lymphocyte-mediated autoimmune demyelinating disease of the CNS with clinical and histopathological similarities to MS.[15-17] It is generally induced in rodents by the injection of either whole brain/spinal cord homogenate or purified myelin proteins (e.g., myelin basic protein, MBP and proteolipid protein, PLP) in complete Freund's adjuvant. The disease is passively transferred to syngeneic recipients by activated CD4-positive T cells that are specific for myelin antigens.[18] Irrespective of whether neuroantigen-reactive T cells are generated in lymph nodes draining the site of injected CNS proteins or from passive transfer, it is proposed that upon entering the CNS the recognition of myelin antigens by the cells[19,20] results in the release of cytokines which promote the entry of other lymphocytes and monocytes whose activity leads to demyelination and the clinical signs of EAE.[21-23] Chronic relapsing EAE (CREAE) is an acute paralytic disease followed by a fluctuating clinical course that parallels the intensity of demyelination. Central to the tissue damage in both EAE and CREAE is the homing of lymphocytes to the CNS and their migration across the BBB.

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6.2 MOLECULAR BASIS OF LEUCOCYTE-ENDOTHELIAL INTERACTIONS

In order to understand how blood leucocytes adhere to cerebral endothelium prior to extravasation across the BBB, it is necessary to consider in general terms the mechanisms by which blood leucocytes recognise and bind to molecules expressed on the luminal surface of vascular endothelial cells. Leucocyte-endothelial interaction is a multistep process[28] in which families of cell adhesion molecules regulate different stages of adhesion that culminate in migration across the vessel wall (see Table 6.1).

TABLE 6.1
Distribution and Ligand-Binding of Integrins

Name	Ligands	Distribution
	β1 Integrins	
VLA-1 (a1β1) (CD49a/CD29)	LM, CO	Activated lymphocytes, fibroblasts
VLA-2 (a2β1) (CD49b/CD29)	LM, CO	Activated T cells, fibroblasts, endothelial cells, epithelial cells, platelets
VLA-3 (a3β1)	LM, CO, FN	Fibroblasts, epithelial cells (CD49c/CD29)
VLA-4 (a4β1) (CD49d/CD29)	FN, VCAM-1	Lymphocytes, monocytes, large granular lymphocytes
VLA-5 (a5β1) (CD49e/CD29)	FN	Thymocytes, T cells, fibroblasts, epithelial cells, endothelial cells, platelets
VLA-6 (a6β1) (CD49f/CD29)	LM	T cells
	β2 Integrins	
LFA-1 ($aL\beta_2$) (CD11a/CD18	ICAM-1, ICAM-2	All leucocytes
Mac-1 ($aM\beta_2$) (CD11b/CD18)	ICAM-1, C3bi, FB, LPS	Neutrophils, monocytes, macrophages, Some B cells, NK cells
p150.95 ($ax\beta_2$) (CD11c/CD18)	C3bi	Activated lymphocytes, granulocytes, monocytes, macrophages
	Cytoadhesins	
gp IIb/IIIa ($a11b\beta_3$) (CD41/CD61)	FN, FB, vWF	Platelets
Vitronectin receptor ($aV\beta_3$) (CD51/CD61)	VN, FB, TSP, vWF	Endothelial cells
β4 Integrins	?	Epithelial cells
β5 Integrins	VN, FN	Epithelial cells, fibroblasts
β6 Integrins	?	Lymphocytes

Note: CO — collagen; FB — fibrinogen; FN — fibronectin; LM — laminin; LPS — lipopolysaccharide; TSP — thrombospondin; VN — vitronectin; vWF — von Willebrand factor.

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6.2.1 Integrins

The integrins are a large family of aβ heterodimeric cell surface glycoproteins that govern the firm adhesion, migration, and extravascular localisation of leucocytes.[29] There are several subfamilies, summarised in Table 6.1, and each has a common β subunit which associates with a specific subunit. Most integrins bind to more than one ligand in a cation (Ca^{2+} or Mg^{2+}) dependent reaction, for example, VLA-4 (CD49d) recognises vascular cell adhesion molecule-1 (VCAM-1) and the Arg-Gly-Asp (RGD) sequence found within extracellular matrix proteins such as fibronectin.[30]

6.2.1.1 The β_1 Subfamily

The β_1 integrins are also known as the very late antigen (VLA) subfamily because they were first identified on lymphocytes several days after activation.[31] At present there are six members, all of which bind to proteins of the extracellular matrix (see Table 6.1). Unlike the other molecules, VLA-4 (CD49d) is found on the majority of unstimulated T lymphocytes, its expression being higher on memory than naive cells.[32] It contributes to T-cell adhesion to inflamed endothelium by interacting with VCAM-1 and, as we shall see later, VLA-4 facilitates T-cell infiltration across the BBB in EAE.

6.2.1.2 The β_2 Subfamily

This family is often referred to as the leucocyte integrins. There are three main groups: CD11a/CD18 is present on virtually all leucocytes, whereas CD11b/CD18 and CD11c/CD18 are mainly confined to phagocytic cells (see Table 6.1). Anti-CD11a antibodies prevent the increased adhesion of leucocytes to cytokine-treated endothelium, and in experimental models of non-CNS inflammation, antibodies against CD11a, CD11b, or CD18 impede the entry of leucocyte infiltrates.[33-35] Regulation of integrin expression and binding efficiency is linked to haemopoietic maturation[36] and to the state of cell activation. Associated with the transition from naive to memory T cells is an increase in CD11a expression, and it is the memory T cells that preferentially migrate to inflammatory lesions.[37,38]

6.2.2 Immunoglobulin Superfamily of Adhesion Molecules

Members of this superfamily are characterised by the presence of two or more domains similar to those found on immunoglobulins, each of which possesses a disulphide-bridged loop. Three members of the immunoglobulin superfamily, ICAM-1, ICAM-2, and VCAM-1, act as ligands for leucocyte integrins.[39]

6.2.2.1 Intercellular Adhesion Molecules-1 and -2

Intercellular adhesion molecule-1 (ICAM-1, CD54) and ICAM-2 (CD102) are constitutionally expressed on cultured endothelial cells and both serve as ligands for CD11a/CD18.[40,41] ICAM-1 possesses five extracellular Ig domains:[42] the first and third are recognised by CD11a and CD11b, respectively. Because ICAM-2 only has two Ig domains it binds CD11a but not CD11b. The endothelial expression of ICAM-1

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but not ICAM-2 is upregulated by the action of inflammatory cytokines (e.g., interleukin 1 [IL-1], tumour necrosis factor α [TNF-α] and interferon-γ [IFN-γ]).[43]

6.2.2.2 Vascular Cell Adhesion Molecule-1 (VCAM-1)

Although absent from the surface of resting endothelial cells in culture the expression of VCAM-1 is induced by IL-1, IL-4, and TNF-α but not IFN-γ.[44-46] Endothelial VCAM-1 supports the adhesion of lymphocytes and monocytes (but not neutrophils) through interaction with VLA-4,[45,47] and this recognition mechanism could lead to the accumulation of mononuclear cells during the transition from acute to chronic inflammation. It appears that VCAM-1 also underlies eosinophil and basophil adhesion to activated endothelium.[48]

6.2.3 Selectins

The term selectin was introduced to describe three adhesion molecules whose function and expression were highly selective and which possessed a terminal lectin domain. The nomenclature for each molecule relates to the cell on which they were first described: E-selectin (endothelium), L-selectin (lymphocyte), and P-selectin (platelet). All three share similar structural features: (1) an extracellular amino terminal carbohydrate-binding (i.e., lectin-like) domain that requires Ca^{2+} for activation; (2) an epidermal growth factor-like domain; and (3) repeated domains with homologies to complement-regulatory proteins.

6.2.3.1 L-Selectin (CD62L)

On the surface of blood lymphocytes, L-selectin serves as a "homing" receptor for secondary lymphoid tissue. It directs trafficking of lymphocytes by recognising CD34 or the vascular addressin GlyCAM-1 on high endothelial venules in peripheral lymph nodes[49,50] and another addressin, MadCAM-1, on mucosal endothelial cells.[51] L-selectin is also constitutively expressed by neutrophils, monocytes, and other myeloid cells,[52] and at sites of inflammation the molecule mediates a weak, transient leucocyte binding ("tethering") to walls of the local microvasculature.[53,54] Following leucocyte activation, L-selectin is rapidly shed from the cell surface, an event that is thought to permit cell detachment and allow rolling along the endothelial surface.[55,56]

6.2.3.2 P-Selectin and E-Selectin

Stimulation of cultured endothelial cells with thrombin, histamine, or H_20_2 results in a rapid (within minutes) translocation of P-selectin (CD62P) to the cell surface from secretory granules known as Weibel-Palade bodies. In contrast, induction of endothelial E-selectin (CD62E) expression is dependent upon *de novo* synthesis following stimulation with cytokines such as IL-1a and TNF-α.[57,58] P- and E-selectin are only transiently expressed on the cell surface, during which time they bind to the sialylated Lewis X antigens of neutrophils and monocytes.[59-61] Both selectins are implicated in leucocyte extravasation associated with the acute inflammatory response.[62,63]

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TABLE 6.2
Chemokines and Leucocytes

Chemokine	Responding Cells	Ref.
Macrophage chemoattractant protein-1 (MCP-1)	Monocytes	65
Interferon-γ inducible protein-10 (IP-10)	Monocytes and activated T lymphocytes	66
Interleukin-8/neutrophil activating protein-1 (IL-8/NAP-1)	Neutrophils, basophils, and T lymphocytes	67
Macrophage inflammatory protein-1 (MIP-1)	Neutrophils, eosinophils, and T lymphocytes	68, 69, 70

6.2.4 Lymphocyte Migration Across Blood Vessel Walls at Inflammatory Lesions

Selectins control the initial attachment of leucocytes to endothelium. This "tethering" of the leucocytes allows them to roll along the vessel wall and come into contact with chemokines which are chemoattractive and activate specific leucocyte classes[64-70] (see Table 6.2). Chemokines are secreted by diverse cell types in response to stimuli that include bacterial lipopolysaccharides (LPS), IL-1, TNF-α and IFN-γ. By binding to heparin, chemokines become localised to glycoconjugates on the endothelial cell surface and this positioning ensures their presentation to tethered leucocytes.[71] Engagement of the chemokine receptors leads to activation of the β integrins which then mediate the second stage of leucocyte adhesion.[70] Consequently, a combined increase in CD11a expression[36] and change in phosphorylation-linked conformation increases the affinity of CD11a for its counterreceptors ICAM-1 and ICAM-2.[72] This firm adhesion is further strengthened by cognate interaction of VLA-4 with VCAM-1 whose expression is induced by inflammatory cytokines. Once firmly bound to the endothelial surface, the lymphocytes flatten and migrate across the blood vessel wall in response to chemotactic factors.

Having penetrated the endothelium the final obstacle to lymphocyte passage across the blood vessel wall is the subendothelial basement membrane[73] which is composed of type IV collagen, laminin, nidogen/entactin, and heparin sulphate.[74] All of these extracellular matrix molecules bind to the β1 integrins whose expression and binding affinity is greatly enhanced following T-cell activation.[32,75,76]

In summary, the process of leucocyte extravasation, from initial attachment to endothelium to the final entry into the surrounding tissue, involves the sequential utilisation of an array of cell surface adhesion molecules. The important features of these adhesion molecules are that whilst some are constitutively expressed, others are induced, upregulated, or converted to a fully functional form only upon activation of endothelial cells or leucocytes. At present, it is not clear whether the arrival of distinct populations of blood leucocytes to an inflammatory lesion depends upon an initial recognition on the endothelial surface of a combination of known adhesion

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molecules, or the expression of as yet unidentified molecules. The next section of this chapter considers the factors which are believed to govern lymphocyte migration across the BBB and lead to CNS demyelination.

6.3 VASCULAR ADHESION MOLECULES IN THE CNS

In both the acute and chronic relapsing forms of EAE an increase in leucocyte infiltration is associated with an upregulation of ICAM-1 on CNS vessels.[26,77-79] This adhesion molecule is reported to be present on most microvessels isolated from MS brain[80] and on a third of blood vessels in active MS plaques.[81] Blood vessels expressing ICAM-1 may not necessarily indicate sites of lymphocyte extravasation since ICAM-1-positive cerebral vessels appear in normal human brain,[81] and in the active clinical disease of EAE the molecule is expressed on several vessels in uninvolved CNS tissue.[26] By inference, other factors are required to support lymphocyte extravasation and hence disease progression. Human cerebral blood vessels normally express very little VCAM-1, but in MS high levels appear within lesions,[80] especially in older, active plaques where there is concomitant expression of VLA-4 on nearby lymphocytes.[82] The expression of ICAM-1 and VCAM-1 on nonendothelial cells of CNS blood vessels, e.g., astrocytic foot processes of smooth muscle cells, may also contribute to the migration of lymphocytes across the BBB and to the perivascular lymphocyte cuffing that is a characteristic pathological feature of MS.

An upregulation of ICAM-1 and VCAM-1 on the surface of endothelial cells is generally attributed to the activity of inflammatory cytokines such as TNF-α, IL-1, IL-4, and IFN-γ.[43,83-86] All of these cytokines are readily identifiable in the CNS lesions of MS,[87-90] particularly in close proximity to blood vessel walls.[82,91] In EAE, disease activity is exacerbated by TNF-α,[92,93] antagonised by specific neutralising antibodies,[94] and injection of TNF-α into rat spinal cord induces mononuclear cell infiltrates.[95] However, several of the cytokines identified in MS lesions are also present in the CNS of normal and noninflammatory disease controls[91] and therefore caution must be applied in interpreting cytokine expression and localisation to a particular phase of demyelination.

Lymphocyte recirculation between blood and secondary lymphoid tissue is dependent upon homing receptors (e.g., L-selectin) on the lymphocytes recognising distinct ligands (vascular addressins) expressed on specialised vessel walls known as high endothelial venules (HEV). When antiaddressin antibodies were applied to CNS sections from rats or guinea pigs with CREAE they showed strong reactivity in perivascular lesions, particularly in relapsing disease,[77,79] but these findings were not confirmed by others in a mouse EAE model.[96] Attempts to identify HEV-like vessels and their associated addressins in MS brain have been disappointing. One study reported that addressins were present in the plaque and periplaque areas of only one of six MS tissues examined[23] and in our laboratory we have failed to identify HEVs in any of eight MS brain tissue samples examined. Unlike other chronic inflammatory diseases it seems that lymphocyte entry into the CNS of MS patients is not dependent upon recognition of addressins on HEV.

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Some adhesion molecules also exist in a soluble form, and high levels of soluble ICAM-1 (sICAM-1) in blood are thought to be indicators of endothelial cell damage or hyperactivity.[97] In MS, increased levels of circulating sICAM-1 are associated with the active phase of the disease[98] and are directly related to serum concentrations of TNF-α and the degree of BBB damage.[99] Similarly, raised levels of soluble VCAM-1 in the blood of MS patients with active disease correlate with dysfunction of the BBB.[100] Whether these soluble molecules originate from T cells, macrophages, endothelial cells, or glial cells[101] is not known, but by binding to ligands on the surface of leucocytes they could either interfere with recognition of endothelial ICAM-1 or VCAM-1 and impede attachment to cerebral vessels[102] or induce cell activation and promote endothelial interaction.

6.4 CHARACTERISATION OF LYMPHOCYTES THAT MIGRATE ACROSS THE BLOOD–BRAIN BARRIER

Based on the premise that the initiation and progression of demyelination is dependent upon the activity of infiltrating blood lymphocytes, this section will address the question of whether migration across the BBB is a feature of a particular subpopulation of lymphocytes.

Let us first consider the proposal that, in MS, the myelin sheath and/or oligodendrocytes are the targets for an immune-mediated attack led by infiltrating T lymphocytes whose recognition of specific neuroantigens initiates the cascade of inflammatory events that results in demyelination. In the adoptive transfer of chronic relapsing EAE to recipients, labelled neuroantigen-sensitised lymphocytes migrate rapidly across the BBB and remain within the perivascular area where they appear to participate in the recruitment of nonspecific inflammatory cells.[21] For neuroantigen-sensitised T lymphocytes to enter the CNS they must first bind to the endothelial cells of the BBB. This raises the question of whether such cells are present in MS blood and, if so, whether they are more likely than other lymphocytes to cross the BBB. Evidence against a leading pathogenic role for CNS-reactive lymphocytes comes from the demonstration that lymphocytes specific for myelin proteins and associated peptides are present in the blood of both MS patients and healthy subjects,[103] the lack of support for the proposed T-cell receptor restriction in MS,[104] and that only a minority of T cells in inflammatory CNS infiltrates express myelin specificity.[21,22] Thus, most blood lymphocytes recruited into CNS lesions do not display reactivity for neuroantigens. However, it must be borne in mind that very few antigen-specific T lymphocytes — less than 1% of the total mononuclear cell infiltrate — are required to initiate the typical delayed-type hypersensitivity reaction.

Once activated by antigen, T cells will release cytokines and other inflammatory factors that encourage the arrival of nonspecific lymphocytes and monocytes. Even if these events were to occur in the CNS, there still remains the question of what factors promote the early migration of sensitised T cells across the BBB. Vascular adhesion molecules are expressed on normal cerebral vessels but there is no evidence to suggest that they are more favourably recognised by myelin-specific lymphocytes in relation to other leucocytes. Perhaps entry into the CNS is afforded by neuroantigen

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recognition at the level of the BBB. MHC class II molecules are expressed by brain endothelial cells during the progression of EAE[17] and they themselves may function as adhesion molecules.[105] Such a mechanism could maintain the entry of antigen-specific lymphocytes into the CNS, but it is unlikely to be responsible for the first passage of these cells across the BBB since MHC class II molecules and myelin peptides are absent from the walls of the normal cerebral microvasculature. If autoreactive T lymphocytes are to occupy a pivotal role in the pathogenesis of MS then their migration across the BBB must be preceded by perturbations within the CNS. For example, microbial infections could generate the inflammatory sequelae necessary to upregulate the expression of adhesion molecules and chemokines on blood vessel walls that will be recognised by both myelin-specific and nonmyelin-specific lymphocytes.

Alternatively, lymphocyte entry into the CNS may be governed by the status of cell activation rather than antigen-specific homing mechanisms. Activated lymphocytes are highly adherent to endothelial monolayers[106] and increased numbers of these cells are present in the blood of MS patients.[107-109] The demonstration that activation of T cells facilitates their passage across the BBB[19,110] in experimental models led to the proposal that, irrespective of antigen specificity, activated rather than quiescent lymphocytes enter the CNS. Should such infiltrates consist of T cells autoreactive for CNS antigens, then it increases the likelihood that an inflammatory reaction will ensue.[21,23,111] In addition, the observation that circulating activated T lymphocytes disseminate to several organs[19,20] casts further doubt on the established view that the BBB is less impervious to leucocyte penetration than other vascular sites.

Identification of factors that govern the tissue specific migration of lymphocytes has benefited from application of the *in situ* frozen section technique which was introduced by Stamper and Woodruff.[112,113] These workers demonstrated that lymphocytes overlain onto tissue sections of rat lymph nodes preferentially bound to walls of the exposed high endothelial venules (HEV) — an observation that was to advance the understanding of the pathways of lymphocyte recirculation. Since then, the technique has demonstrated the high degree of specificity with which different classes of lymphocytes[114,115] at various stages of development[116] and activation[117,118] adhere to HEV in distinct anatomical sites. We have applied the frozen section assay to the study of lymphocyte adhesion to blood vessel walls in cryostat sections of human brain.[119] Of the main leucocyte classes, lymphocytes were the most adherent for transected cerebral blood vessels in normal brain tissue devoid of HEV; their binding was enhanced by activation with IL-2, and high adhesiveness was a particular property of CD4-positive T cells.[119] These observations are compatible with the demonstration that lymphocyte migration across the BBB is increased by prior activation[19,111] and that most of the lymphocytes that enter the normal CNS express the CD4 phenotype.[3,120,121] We also find that blood vessel walls in lesions of MS brain that contain intense leucocyte infiltrates are several fold more adhesive for overlain lymphocytes than vessels in uninvolved sites or in normal brain tissue sections.[122] It is anticipated that the frozen section assay will advance our understanding of the adhesion molecules that promote lymphocyte attachment to blood vessel walls within CNS lesions in MS.

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Memory T lymphocytes exhibit a higher binding affinity for endothelium at extra lymphoid sites[37] and, in common with other organs, inflammation within the CNS results in the preferential recruitment of memory rather than naive T lymphocytes.[120,123,124] The differentiation of naive into memory lymphocytes is associated with the induction, upregulation, and affinity changes of several surface adhesion molecules including the β1 and β2 integrins. An increase in the proportion of T lymphocytes bearing β1 integrins in MS blood[125,126] could represent an expansion in a cellular population that is predisposed for migration into sites of inflammation. Our studies show that CD4-positive T lymphocytes adhere in greater numbers to monolayers of endothelial cells treated with TNF-α[126] and to blood vessel walls in sections of human brain[119] than other leucocytes. Moreover, lymphocytes from patients with MS, particularly those with evidence of a recent clinical relapse, are more adherent to TNF-treated endothelium than lymphocytes from normal subjects and patients with other CNS disorders, with rheumatoid arthritis, or with psoriasis. This finding suggests, that in contrast to other chronic inflammatory disorders, the CD4 population of MS patients has an increased proportion of cells that bind to inflammatory endothelium. The interaction between CD4-positive lymphocytes and endothelial cells is highlighted by the demonstration that migration across monolayers of cultured endothelial cells is a particular property of a subset of memory CD4-positive lymphocytes.[127]

6.5 ANTIBODY BLOCKING STUDIES

If demyelination is dependent upon the activity of infiltrating CD4-positive lymphocytes then abrogating their passage across the BBB should produce disease amelioration. Multiple administration of CD4-depleting antibodies during the induction phase of EAE was shown to inhibit development of paralysis[128] and in the chronic relapsing form of EAE disease progression was inhibited by one i.p. injection of a CD4-depleting but not CD8-depleting antibody prior to or during disease onset.[129] However, in the latter study anti-CD4 antibodies did not impair the clinical course of the disease once demyelination was established, suggesting that not all of the pathogenetic CD4-positive T lymphocytes are removed by this treatment. A more specific therapeutic approach is targeting antigen-specific T cells. Expression of the protein OX-40 is restricted to recently activated CD4-positive T cells[130] and this determinant is transiently upgraded on MBP-specific T cells in lesional sites of EAE.[131] When anti-OX-40 antibodies conjugated to immunotoxin were injected i.p. prior to and on the day of EAE onset, they bound exclusively to myelin-reactive T cells and reduced clinical signs.[132] The suggestion that these findings form the basis of an immunotherapy for MS patients may be premature in that the treatment has not been applied to the chronic relapsing form of EAE and that OX-40 was not expressed on blood or CSF lymphocytes from three MS patients studied.

Antagonising the expression of adhesion molecules is another means of impeding lymphocyte migration across the BBB. This has been addressed by two approaches: first, inhibiting the expression of adhesion molecules on circulating lymphocytes, and second, interfering with the expression of adhesion molecules on vascular endothelium. In EAE, there are conflicting reports regarding the effectiveness

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of anti-CD11a antibodies upon disease outcome, in contrast to the successful application of these antibodies in other experimental models of inflammation such as uveoretinitis.[34,133] One study reported that neither anti-CD11a or anti-CD18 antibodies modified disease activity in the adoptive transfer (passive) of EAE,[134] whereas another study, using the same model, found that anti-CD11a antibodies enhanced disease severity with subsequent induction of early mortality.[135] Blocking the expression of ICAM-1 has also produced variable results. Anti-ICAM-1 antibodies advanced the onset of passive EAE, but in myelin-induced (active) EAE the antibodies delayed disease initiation and suppressed its severity.[136] The disease-modifying activity of anti-ICAM-1 in the passive but not active model of EAE may be due to its inhibition of antigen presentation rather than T-cell migration across the BBB, particularly as ICAM-1 is expressed on activated T lymphocytes and antigen-presenting cells.[137]

Although it is highly likely that the clinical effectiveness of anti-CD11a and anti-ICAM-1 antibodies in EAE will depend upon features of experimental design, e.g., method of disease induction, and the stage and route of antibody administration, the overall impression from the current literature is that CD11a and ICAM-1 are not major participants in the CNS extravasation of lymphocytes. It also appears that anti-L-selectin antibodies do not affect the clinical course of EAE.[138]

The most convincing evidence of direct association between disease amelioration in EAE and inhibition of adhesion molecule expression comes from the study of VLA-4. A single i.p. injection of antibodies two days after the induction of passive EAE stopped paralysis development in most animals (75%) and reduced its severity in the remainder.[139] These findings were complemented by *in vitro* experiments in which antibodies directed against VLA-4, but not those against other β1 and β2 integrins, inhibited lymphocyte attachment to blood vessels exposed in brain sections from animals with EAE. Further support for a pathogenic role for VLA-4 was the demonstration that encephalitogenic clones of T cells bearing a high expression of VLA-4 readily entered the CNS, and that antibodies directed against VLA-4 and its ligand VCAM-1 delayed disease onset but not severity.[140]

6.6 FUTURE STRATEGIES

This review has emphasised that the migration of lymphocytes across the BBB is fundamental to the demyelinating process. From studies of lymphocyte entry into the CNS let us now consider what future therapeutic strategies may emerge for MS. Most attempts to interfere with lymphocyte extravasation into the CNS of animals with EAE have focused upon either depleting potential pathogenetic cells from the circulation or preventing their binding to endothelial cells of the BBB and hence impeding entry into the CNS. Disease progression in EAE is attenuated by CD4 depletion, but even if this treatment was to successfully modify the course of MS disease, such a Draconian intervention may immunocompromise the patients and lead to opportunistic infections. Rodent CD4-positive lymphocytes consist of two subpopulations which, based on their profile of secreted cytokines, are termed TH1 and TH2. The TH1 cells release IFN-γ and IL-2 and are deemed to occupy a proinflammatory role, whereas the TH2 cells release cytokines such as IL-10 which

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antagonise the inflammatory response.[141] Should the intensity of an inflammatory reaction be governed by a balance between TH1 and TH2 cell activity then selectively antagonising TH1 entry into the CNS could generate clinical benefit.

There is currently no phenotypic marker of TH1 and TH2 cells and their existence in humans has yet to be demonstrated convincingly. Although these populations are regarded as having opposing activities, this convenient functional segregation may prove to be of short-term convenience, for additional subpopulations may be contained within each subset. This view is illustrated by the TH2 cells which release cytokines (IL-10) that impede TH1 activity, and by inference suppress inflammation, and others (e.g., IL-4, IL-5) whose stimulation of B-cell proliferation could promote autoantibody production. Furthermore, cytokine activity in MS may differ from that in its animal model counterpart as demonstrated by the administration of IFN-γ which suppresses disease activity in EAE but induces disease exacerbation in MS.[142,143] Thus, further functional delineation of T-cell subpopulations is required before considering T-cell-depleting antibodies as therapeutic protocols for MS. An alternative approach is to antagonise the CNS entry of neuroantigen-specific T lymphocytes, but this introduces the controversial question of whether the immunopathologic features of MS are "driven" by neuroantigens. Nevertheless, based on successful EAE investigations[144] clinical studies are underway in which patients with MS are being orally tolerised with myelin antigens.

In non-CNS experimental models of inflammation, suppressing the expression of leucocyte adhesion molecules impairs leucocyte-endothelial cell interaction and reduces leucocyte numbers in inflammatory infiltrates. However, blocking CD11a/CD18 expression in EAE has not been successful in abrogating demyelination. Of the β integrins it is only VLA-4 which promotes lymphocyte interaction with the BBB in EAE and, based on the clinical effectiveness of anti-VLA-4 antibodies in this model, phase I studies are currently assessing the potential use of anti-VLA-4 therapy for MS patients. Hopefully, a successful outcome will not only provide clinical benefit but will also generate additional information concerning the contribution of VLA-4 in mediating lymphocyte attachment to endothelial cells of the BBB and of the general vasculature. A possible drawback of this form of immunotherapy is that VLA-4 is present on other leucocytes (e.g., monocytes and dendritic cells) and hence the infusion of specific antibodies could have physiological repercussions.

Rather than antagonising adhesion molecule expression on leucocytes, would it not be more advantageous to interfere with the expression of their counterreceptors on cerebral vessel walls? It appears that endothelial cells adjacent to sites of infection/inflammation in the CNS are just as likely to express increased levels of ICAM-1, VCAM-1, and P- and E-selectins as are similarly perturbed endothelial cells in other organs (e.g., skin, heart, and lung). Inhibiting the expression of these vascular adhesion molecules may impair lymphocyte trafficking across the BBB, but unfortunately it may also predispose to infection.

Such problems could be circumvented by targeting specific CNS endothelial adhesion molecules which have so far eluded identification. That such molecules exist gains credence from the demonstration that "addressins" in lymph nodes are different from those in the mesentery and that lymphocyte trafficking through the

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spleen is probably mediated by novel lymphocyte recognition molecules on vessel walls, since both HEV and addressins are absent from this organ. If endothelial cells within different lymphoid tissues possess distinct surface molecules for circulating lymphocytes then why not endothelial cells in the CNS, particularly as the migration of circulating memory T lymphocytes into the normal cerebral spinal fluid appears to be due to immune surveillance rather than a pathological manifestation.[124] Furthermore, CNS endothelial cells may possess characteristic phenotypic determinants that reflect the unique barrier properties of cerebral vessels. Such distinctive markers could serve as targets for constructs of divalent humanised antibodies directed against both CNS endothelial cells and vascular adhesion molecules known to support lymphocyte extravasation. The search for such markers needs to be encouraged because its successful outcome holds significant promise for the introduction of specific therapeutic intervention for MS and other related CNS disorders at the level of the BBB.

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7 Microglia-Mediated Prevention of Traumatic Neurodegeneration

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CONTENTS

7.1 HISTORY, ORIGIN, AND DISTRIBUTION OF MICROGLIA IN THE NORMAL RETINA

The retina represents an early evagination of the neural tube that remains outside the cranium after cytodifferentiation; it has its own twofold blood-retinal barrier (BRB)

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and possesses further unique features. Concerning the first recognition of phagocytotic cells within neural tissues, one misses a mention of Stäbchenzellen (rod cells) in Frank Nissl's[1] descriptions in the retina. However, it is worth mentioning his views on both the origin of these cells and their role in CNS pathologies because of the common features shared by brain and retinal microglia. Nissl considered Stäbchenzellen to be involved in neurodegenerative diseases and ascribed to them both phagocytic and migratory abilities similar to those of circulating leukocytes in other tissues. Ramon y Cajal[2] analyzed the degenerating optic nerve, among other areas, and recognized phagocytic cells within areas of traumatically induced degeneration, but grouped these cells as neuroglia and migratory leukocytes, although he agreed that his "third element" was of mesodermal origin and distinct from true glia and neurons. The name *microglia* was introduced and established by del Rio-Hortega,[3,4] who stained these cells with the weak silver carbonate technique. Since then cellular elements within the CNS, which differ morphologically and biochemically from so-called neuroglia (i.e., astrocytes and oligodendrocytes) and vascular wall cells, have been termed microglia (argyrophilic, nonneuronal, nonneuroglial, and nonvascular brain cells). del Rio-Hortega[3,4] observed cells corresponding to this definition in normal, undamaged CNS (resting microglia) and in CNS with traumatic or inflammatory damage (activated, progressive, or reactive microglia). He not only noted certain morphological differences between resting and reactive microglia, but was also able to describe many transitional forms in his comparative light-microscope studies and was therefore convinced of their identity (reviewed by Theele and Streit[5]). The coincidental appearance of microglia with the development of the vasculature and meninges during late embryonic and early postnatal life forced del Rio Hortega[3] to consider the meninges as the port of entry of microglia. With these descriptions, he initiated the long-lasting debate on the origin of microglia, while simultaneously describing the various stages of their metamorphosis by using stab lesions. The findings of these later experiments mentioned above, though not always conclusive, cast doubt on del Rio-Hortega's concept of a uniform cell population of resting and reactive microglia.[6-8]

The historical controversy concerning retinal microglia and their origin did not significantly differ from that in other areas of the brain, although the presence of microglia in the various retinal layers became accepted relatively late. The earliest studies[9,10] date from a few years after the first description by del Rio Hortega[4] and point to the technical difficulties in staining these capricious cells. However, the unique extracerebral location of the retina, its vicinity and relation to the vitreous body, and retina-specific diseases like dystrophies and proliferative vitreoretinopathies, raised the question as to whether mononuclear phagocytes and microglia play a pathogenic role in idiopathic and inducible proliferative events.[6]

Microglia form a regular mosaic across the entire retina, although the exact distribution is species specific. In the monkey retina, for example, microglia are described within the OPL, ONL, IPL, GCL, and the OFL (see Glossary, page 142), where the cell's shape is adapted to the course of retinal axons.[11,12] Characteristic phenotypic microglia which express MHC II antigens are also found in the fetal and adult human retina.[13] Their distribution is similar to that in the monkey retina, where

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their association with perivascular elements is obvious. In the cat retina, microglia are evenly distributed within all layers.[12] By using different histological techniques, Kohno et al.[14] identified rat microglia mainly within the GCL and IPL. In the normal retina, typical cells contain EM dense particles like lipofucsin granules, narrow endoplasmic reticulum cisternae, lysosomes, and Golgi apparatus. They are associated with RGC and vascular walls.[14] Quantification of fluorescently labelled microglia within the GCL and IPL reveals an almost even distribution from the peripapillary region to the ora serrata.[15] There is a density decrease according to eccentricity, with the highest frequency of microglia located in the central retina. The cells within the GCL and IPL show a territorial distribution without substantial dendritic overlapping, whereas the territorial patterns of the IPL and GCL are shifted laterally, displaying a staggered pattern when viewed in the whole-mounted retina.[15]

Interpreted in terms of a functional significance of the distribution patterns, contact inhibition most likely accounts for this typical pattern of territorial arrangement. Similar numbers and distributions of microglia are described in the rabbit retina labelled with the lectin from *Griffonia simplicifolia*.[16,17] The density ranges of microglia reported in the maturing and adult rabbit retina are similar to those found in the rat. By using enzyme-histochemical methods, Schnitzer[18] and Schnitzer and Scherer[19] showed microglia distributed within the GCL and the deeper retinal layers in the adult rabbit. The distribution of microglia across the retinal surface is almost uniform with the vascularised visual streak having the highest density. As expected from their nature and origin, microglia in the rabbit,[18-20] rat,[15] and human[13] retina do not react with immunohistological stains which detect astrocytes and Müller cells of macroglial origin.

Navascues et al.[21] investigated the intriguing issue of the origin of microglia in the avascular quail retina. The avian retina includes a structure called the pecten, a richly vascularised organ projecting into the vitreous body from the optic disc. Microglia and their precursors are identified using the monoclonal antibody QH1. These results show that amoeboid macrophages migrate out from the pecten along the ILM to peripheral areas in the retina. Subsequently, macrophages migrate from vitreal to scleral layers to reach the IPL and OPL, where they differentiate into ramified microglia.

7.2 METHODOLOGICAL ASSESSMENT OF MICROGLIA

Although the retina is a well-exposed part of the brain, its microglia have been described later than those in intracerebral parts of the CNS — a task made difficult by their remarkable abilities to migrate and transform morphologically. Early phenotypic criteria could only discriminate between neurons, large neuroglia, and Nissl's small rod-like elements that later turned out to represent the microglia. The weak silver carbonate method allowed del Rio-Hortega[3,4] to distinguish between oligodendroglia and microglia, but the technique is laborious and not specific for microglia. These methods allowed differentiation between fully differentiated "ramified" and "amoeboid" forms of microglia.

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7.2.1 Enzyme Histochemistry

Enzyme histochemistry gained popularity in the 1960s[22] with the advent of the phosphatase methods dominating the characterisation of microglia. NDPase activity was demonstrated in various brain areas[23] and in the retina,[18] and TPPase activity detected in the plasma membrane of microglia.[24] In addition to high concentrations of NDPase and TPPase, microglia contain glucosaminidase and peroxidase.[23] These enzymes make it possible to distinguish microglia from Müller cells which also contain carbonic anhydrase. Adenosine disphosphatase is a histochemical marker for the retinal microvasculature and for cells of vascular origin, like macrophages and microglia. With this method, retinal neuroglia, neurons, and pigment epithelium cells remain unstained.

7.2.2 Plant Lectins

D-Galactose is selectively recognized by plant lectins[16,17,25] which bind preferentially onto the surface of both ramified and amoeboid microglia. Lectins also coat some vascular structures, neuronal substructures, and meningeal elements, but astrocytes, Müller cells, neurons, and oligodendrocytes remain unstained. Labelling of peripheral nerve macrophages indicates that high D-galactose titres are common to these types of cells. This fact limits applicability of lectin labelling to specifically discern between resident microglia and immigrating cells under certain experimental conditions.

7.2.3 Antibody Techniques

The advent of monoclonal antibody techniques have decisively contributed to a better characterisation of the brain[26] and retinal microglia. In particular, rat, chick, and mouse antigens[27,28] have been characterised with antibodies raised against macrophages.[21,29-31] It is therefore not surprising that most of these antibodies preferentially label activated microglia at phagocytic stages. Very effective antibodies are those directed against CR3 and label both the rat (OX-42) and mouse (MAC4) molecules. Human macrophages and microglia can be stained with EMB/11.[32]

7.2.4 Pinocytosis

Pinocytosis has been used to study microglia both *in vitro* and *in vivo*.[33] After intravenous injection of tracers like neutral red, India ink, and the interruption of the BBB and BRB, the tracers are ingested by local microglia. Metabolic staining of microglia is assessed with ^{3}H-thymidine.

7.2.5 Phagocytosis-Dependent Labelling

After induced microglial responses to injury[34,35] phagocytosis-dependent labelling is a relatively new approach with several future perspectives. The fundamental advantage of function-dependent staining is that a number of questions about the

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role of microglia in neurodegeneration can be answered by studying their interaction with prelabelled, degenerating neurons *in situ* (Figure 7.1, A to H). Applied to RGC, such dyes result in function-dependent tracing of the nonneuronal cell scavengers which are the resident microglia. This convenient technique takes advantage of the neurons' ability to internalise and transport material either from the terminals or from transected axons back to their cell bodies. Among the variety of dyes available for this purpose, the lipophilic carbocyanine group, originally used for membrane motility studies,[36] turned out to be applicable in various experimental models including the retina (Figure 7.1). The use of dyes of different excitation/emittance wavelengths (Figure 7.1, I to K), enables double labelling such as in the developing and traumatised retina of rats.[34,35] Dyes can be applied in the form of solid crystals anywhere within the nervous system and have the advantage of ensuring fast, vital labelling of the corresponding neurons (Figure 7.1, A to H). Long-term distribution of microglia can be examined both in the whole-mounted tissue (Figure 7.1) and in retinal sections processed for immunohistochemistry.[36]

Ultrastructural investigation is performed after photocovering the fluorescent material in the presence of diaminobenzidine and processing for electron microscopy. Double immunofluorescence can be assessed by using dyes with different wavelengths or by monitoring autofluorescence (as is done in the model of photoreceptor dystrophy with accumulation of lipofuscin), in addition to loading microglial cells with a fluorescent carbocyanine dye. The major advantage of such staining is the selective detection of the subpopulation of resident microglia actually involved in the clearance of fluorescent neuronal debris. The selectivity in labelling only phagocytosing cells may be used in other neurodegenerative models as well, as microglia have been associated with human diseases like multiple sclerosis (MS), viral infections, and Alzheimer's disease (AD). The major disadvantage of the function-dependent staining technique may be its high selectivity, as it only allows labelling of subsets of microglial cells, namely, those that have ingested fluorescent material, leaving resting microglia unlabelled and thus undetected, for instance, those in deeper layers of the retina.

7.2.6 *IN VITRO* STUDIES

Microglia can be cultured in a variety of media containing brain astrocytes or bone marrow-derived growth factors like CSF-I. The variety of techniques available to date permit one to carry out a host of studies in culture that are applicable to various areas of the brain and to various animal models according to the scope of each study. Since they retain their principal features of antigenicity and phagocytosis, retinal microglia derived from either pathologically altered or normal retina may become a key tool for the characterisation of some of their features. In addition to dissociated microglia, an alternative culture method is that of the retinal organ culture.[37] Lesioned retinal pieces are cultured in a chemically defined medium to observe histotypic retinal tissue maintenance, cell survival, and outgrowth of RGC axons. As *in vivo*, pretreatment of the lesioned retina in culture with either MIF or PI enhances the outgrowth of axons, indicating that microglia co-determine the success of regeneration.[37]

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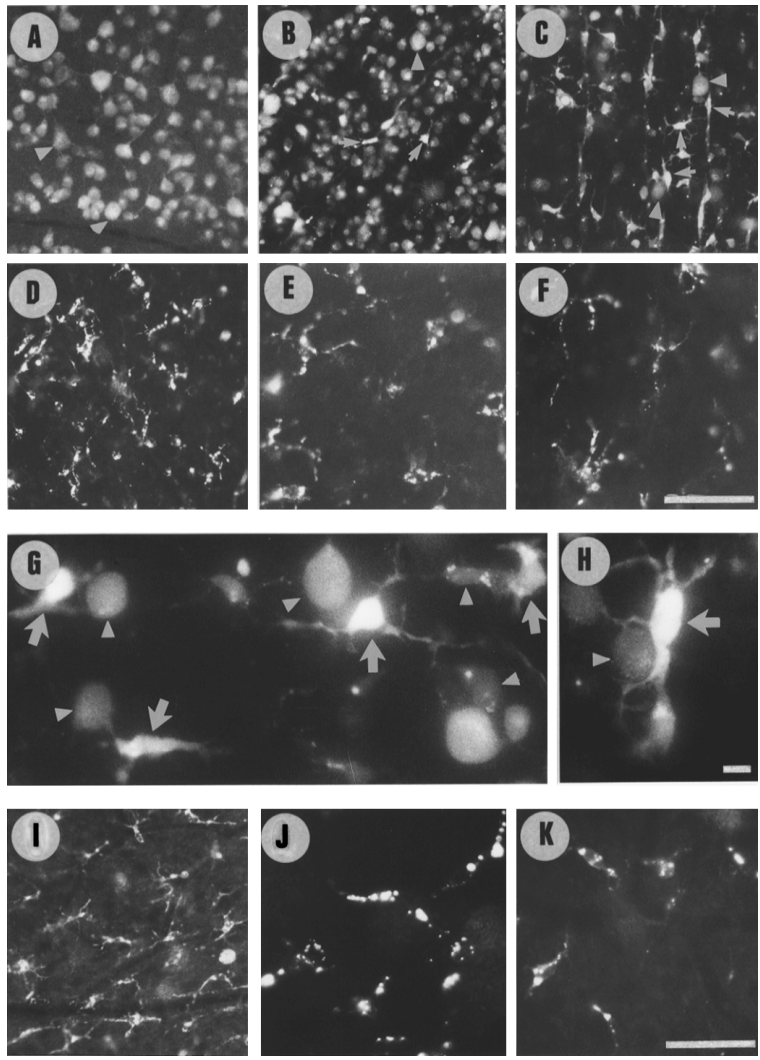

FIGURE 7.1 A-K Function-dependent labelling of retinal microglia. **A** to **F**: fluorogold-prelabelled RGC (**A**) consecutively disappear at the 2nd day (**B**), 9th day (**C**), end of 2nd week (**D**), end of 3rd month (**E**), and end of 6th month (**F**) after optic nerve cut. The RGC (some indicated with arrowheads) are replaced with phagocytosing microglia which become labelled (some indicated with arrows) and reside within the retina over the time period of observation. **G** and **H**: higher magnification of fluorogold-stained RGC (arrowheads) and of their microglial "partner cells" (arrows) during the process of removal. Microglia appear brighter as they may phagocytose more than one RGC. **I** to **K**: examples of other fluorescent dyes that label microglia like the green dye 4Di-l0ASP (**I**), the red DiI (**J**), and the green DiO (**K**). Note the different images of labelling, probably due to the different physicochemical features of the dyes. Scale bars: 100 µm for **A** through **F**, **I** through **K**, and 10 µm for **G** and **H**.

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7.3 FUNCTION DURING DEVELOPMENT AND NATURAL APOPTOSIS

The physiological reduction of the initially overproduced neurons is accepted as a functionally significant regulatory process of retinal morphogenesis.[14,38] Genesis of new nerve cells, creation and remodelling of connections with target neurons, final differentiation and function-dependent consolidation of stable synaptic contacts within the CNS, are accomplished by natural disposal of substantial numbers of neurons (reviewed by Raff et al.[39]). As microglia represent the intraretinal form of phagocytic cells, it is very likely that they are related to developmental events such as network formation, synaptic reorganisation, and PCD that reduces the initial population of RGC by almost 35%.[32]

As part of the CNS, the retina has unique properties in comparison to other parts of the body. There is no replacement of lost neurons due to the absence of neurogenesis in the mature retina, accounting for irreversible loss after deleterious diseases. The retina is relatively isolated by virtue of the inner and outer BRB. This means that only selected groups of small substances have restricted access to the retina. Theoretically, the BRB naturally prevents the passage of cellular elements, and hence mononuclear cells, into the retinal parenchyma. This is the case in the adult retina, but apparently not in the developing retina where the BRB appears relatively late, after the excess of neurons has begun to decrease.[40] Immigration of microglia is observed prior to the onset of the neuronal death[16,17] and continues throughout postnatal life coinciding with maturation of the BRB. The observation that microglia reside within the embryonic tissue before the bulk of PCD does not contradict their role in PCD, but further implicates their regulatory role in earlier regressive events as well. The fact that the speed of cell removal is higher during the phase of rapid cell decay also suggests the intimate relationship between PCD and activity of the phagocytic cells.[40] With the considerable production of retinal cell debris during PCD, a regular system of clearance must be established to protect healthy cells from damage initiated by release of the products of cell death. It becomes more and more apparent that neurons undergoing PCD "attract" monocytes or microglial precursors, perhaps from the circulation when endothelial cell tight junctions, which represent the BRB, are immature. Although not experimentally shown, it may be that coincident maturation of BRB and closure of tight junctions traps the invading microglia within the retinal parenchyma. They then become an integrated part of the neural tissue and individual cells turn into the ramified form that is seen in adult retinal tissue, creating a uniform resident population of microglia permanently available throughout the animal's life.

PCD may play a major function of building up a uniform and very regularly distributed microglial system. This mechanism, together with the intrinsic ability of microglia to inhibit their immediate neighbours, is probably the reason for their staggered arrangement within the GCL and IPL.[35] In theory, three prerequisites suffice to create a geometrically optimal, functionally highly efficient, locally very specific and nonredundant system for the permanent surveillance of the neurons which survive PCD. Firstly, there is uniform death of each second or third embryonic

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neuron. Secondly, equal numbers of microglial precursors are stoichiometrically attracted from the peripheral circulation under an optimal one-to-one relationship. Thirdly, contact inhibition operates between neighbouring microglia. Indeed, these conditions seem to be phenomenologically met, although there are no conclusive experimental data available to validate this theoretical frame.

To study the fate of phagocytosing microglia involved in PCD, a double-labelling approach has been established using dyes of different wavelengths applied at two different ages. Either 4Di-10ASP or DiI deposited into the SC of newborn rats is taken up by RGC terminals and retrogradely transported to the parent cell bodies. Among the labelled RGC population, the dye is taken up by cells destined to die during PCD over the first two weeks of life. The microglia which phagocytose the fluorescent degradable lipophilic debris become fluorescently labelled themselves. Double labelling of this resident population of microglia is achieved in the following way: in the rat retina, microglia are labelled with the red dye, DiI, at postnatal stages; RGC are subsequently axotomised when the rats mature and RGC become labelled with a second green dye, 4Di-l0ASP, placed in the optic nerve; double labelling occurs since the death of back-filled green-labelled RGC results in microglial uptake of the second stain.[35] This technique provides experimental evidence that the same resident population of microglia is capable of repeated phagocytosis. However, the evidence does not exclude the possibility of additional microglia, deriving from either invasion from the periphery or by replication of resident cells.

The particular emphasis given to microglia in relation to PCD has enhanced the view of an association between microglia and the immune system. Expression of markers which are typical for immunocompetent cells, including the MHC I and II antigens,[26,41] Fc receptors, and CR3,[42] suggest a close association with immune functions. However, despite microglia being obvious candidates for an immune capability in the retina, such a conclusion is difficult to retrieve from experimental data. Microglia have been shown to express MHC antigens even when an immune response does not overtly occur, such as in peripheral nerve lesions. Furthermore, some other glial cells, such as astrocytes and the intramural pericytes, are reported to induce immune-like responses *in vitro*.

Microglia in the adult retina may also participate in synaptic remodelling, based on the fact that they accumulate in both PL, where retinal neurons make synaptic contacts, but are excluded from nuclear layers devoid of synapses. Murabe and Sano[43] showed that microglial processes often encircle synapses in the rat cerebral cortex, where they may be involved in the release of neurotransmitters.[22,43] Ramified microglia could act as highly efficient clearing systems in neuronal-rich regions. Microglia could favour axonal growth by producing thrombospondin during development, as this extracellular matrix protein promotes neurite outgrowth in cultured neuronal cells. The expression of thrombospondin in relation to the distribution of microglia has been investigated in the developing rat brain using immunocytochemistry.[44] A cell population displaying the morphology and distribution of brain macrophages is labelled during early postnatal life, as are ramified microglia. Little is known about a potential role for microglia in neuronal and glial support. Elkabes et al.[45] showed that microglia express neurotrophins in a region-specific manner and that within any region only subpopulations elaborate trophins. Immunohistochemical

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studies on normal human retinas[46] indicate that similar populations of microglia are immunoreactive to CD45, MHC I, and MHC II, while relatively few microglia (about 10%) are immunoreactive for human macrophage (S22) antigens, supporting the view of microglial heterogeneity.

7.4 RETINAL DISEASES ASSOCIATED WITH MICROGLIA

Inherited neurodegenerative diseases are characterised by progressive disappearance of neurons due to either intrinsic metabolic deficits or malfunctioning within neuronal networks. Our understanding of the mechanisms involved in hereditary degeneration relies substantially on studies using animal models, like the RCS rat strain, which have a mutation in the RPE cells affecting their phagocytic ability,[47,48] leading to insufficient clearance of photoreceptor outer segment debris produced during phototransduction. Degeneration of the photoreceptor outer segments, and later of the entire cells, commences at the end of the second week (period of eye opening), progressively resulting in a complete loss of the photoreceptor cell layer at the end of the third month of life.[47,49]

Long-term prelabelling of microglia (monitored for up to 12 months with 4Di-l0ASP *in vivo*) helps the detection of intraretinal migration of microglia in RCS rats. RGC of the RCS rat strain are prelabelled by applying 4Di-l0ASP at the cross-sectional area of the cut optic nerve at postnatal day 50. This was done to determine if phagocytosing microglia either remain within the RGL as occurs in normal rats (albino Sprague-Dawley and pigmented rats) or move into the photoreceptor layer where degeneration is occurring. In RCS rats, fluorescent microglia translocate into the photoreceptor layer during the weeks after optic nerve transection and label microglia within the GCL and IPL. The results of this experiment confirm the predicted responsiveness of microglia to degeneration-derived stimuli,[42] although they do not shed light on the nature of signals mediating microglial attraction. Nevertheless, the findings demonstrate double phagocytosis, since the same microglia that have ingested fluorescent RGC can respond to photoreceptor degeneration by migrating to the photoreceptor layer, where they accumulate and actively participate in the degradation of photoreceptors.[15]

A fundamental feature of axotomy-induced TCD within the retina consists of a series of changes in the morphology of the perikarya, finally resulting in the decay of neurons. TCD is not considered a physiological event within the mature retina unless an external insult like axotomy, or pathological conditions like stroke, ischaemia, infections, and toxic agents force the neurons to break down and die. Isolated mechanical axotomy, close to or distant from the cell body is a less dramatic intervention which leads to Wallerian degeneration, disconnecting the cell from its natural target and target-derived trophic factors. The optic nerve macrophages, as detected with ED1-positive immunostaining[50] and electron microscopy,[51,52] are activated to remove the lesion-derived debris.

Vitreoretinopathic diseases are usually the result of perforating lesions and of manual or laser-assisted vitreoretinal surgery.[53] Rhegmatogenous damage of the ILM

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severs neuroglia and disrupts Müller cell end-feet, the integrity of adjacent astrocytes, and parts of the inner retinal vasculature. As a consequence, physiological wound healing processes are initiated which frequently develop into a proliferative retinopathy with the involvement of neuroglial epiretinal scars and activation of mononuclear cells.[53] The origin of the phagocytic cells is not yet clear.[54] The mechanisms leading to so-called idiopathic vitreoretinopathy are very similar because these diseases also develop as a result of retinal detachment, thus leading to the same effects on the cellular elements in the vitreoretinal interface. Retinal microglia identified by different staining methods seem to be involved in both types of vitreoretinopathy.[55] The prevalence is higher in idiopathic than in traumatic vitreoretinopathy, and is insignificant in diabetic forms of the disease.[56] The simultaneous activation of astrocytes and microglia indicates their interaction during the development of the disease. Microglia may activate astrocytes with mitogens,[57] whereas astrocytes may reciprocally activate microglia with IL-3.[58] In effect, treatment of the disease will not be different assuming a causal contribution of microglia in traumatic retinal degenerations and subsequent scarring.

Both microglia- and macrophage-associated diseases include the late stages of retinitis pigmentosa, macular degenerations, viral infections leading to chorioretinitis, and multiple sclerosis frequently leading to optic nerve demyelination. Apart from neurological diseases involving the retina, age-related brain dystrophies like Parkinson's and AD[59-61] have strong connections with microglial cells. We shall discuss one of the most prominent cases of retinal pathology, namely HIV-chorioretinitis and the optic nerve neuritis in the frame of MS, whose pathogenesis and relation to the activation of microglia is still being debated.

HIV-I is known to replicate in the CNS of AIDS patients primarily in microglia. In 50 to 80% of patients, encephalitis, low myelopathy, and retinopathy occur, often resulting in dysfunction and tissue damage.[62] On histological examination, a typical finding is microglial nodules in the neural tissue of AIDS patients, frequently seen in the white matter with immunostaining of the gp4l viral envelope antigen. Although the pathogenetic cascade is still obscure, the primarily affected microglia seem to play a key role.[63] These cells may either encode for neurotoxic proteins,[57] or alter the microenvironment, causing retinal damage by retina-derived neurotoxins, or modify the BBB and BRB to permit both the entrance of haematogenous cells and leakage of proteins.[29] Most likely, a combination of these three mechanisms can be assumed, although isolated components like the envelope glycoprotein gp12O[62] have been shown to cause neuronal cell death in culture. Besides the question as to how the virus exerts its pathology, the mechanism of infection remains unknown. The most likely pathway is through antibody-mediated uptake.[63]

Microglia are capable of antigen detection and specifically display Fc receptors.[64] This demonstrates that transfection may occur via Fc receptors or via the CD4 antigen, which is a viral receptor expressed on microglial cells.[42] Access to retinal and brain tissue may occur after breakdown of the BRB and BBB.[63]

MS is a multifocal disease frequently starting with ocular symptoms like diplopia when it becomes established in the brain stem, or a reduction of visual acuity, or disturbances in colour vision and pain when established as optic nerve neuritis. The typical plaques are initial sites of demyelination, whereas inflammatory elements

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including myelinophagic microglia appear at early stages of the disease.[65] Due to the lack of myelination within the retina, the disease becomes established within the optic nerve. At early stages, leukocytes pass the permeated BBB, and astrocytes become reactive to later form typical gliotic scars. The appearance of microglia in and around the plaques indicates their involvement in the cascade of plaque formation. The favourable model of EAE, however, shows that inflammation and cytokine production accompany the disease. Although the exact cascade of inflammation and myelinolysis is not elucidated, microglial IL-6 seems to play a key role in stimulating lymphocytes.[66] In particular, B-cell stimulation can result in autoantibody production, although it remains to be shown whether this production is involved in the pathogenesis of the disease. The direct involvement of microglia in demyelination is experimentally induced in IL-3 transgenic mice.[67] The patterns of demyelination produced in these mice are very reminiscent of both human MS and HIV encephalopathy.[67]

7.5 HETEROGENEITY OF MICROGLIAL ACTIVATION

The ubiquitous appearance of microglia throughout the brain and among all species investigated so far, based on clinical and experimental evidence, assigns a crucial role to these cells in various events of development and pathology.[68,69] The cell is responsible for clearing the debris of cells erroneously connected, synaptic stripping of cells that are injured, phagocytosing dying cells after injury, releasing substances that can prevent cellular degeneration, and PCD. Accurate analysis of the chronological sequence and topological complexity of the cell-cell interactions during microglial activation are prerequisites for developing therapeutic strategies.

In vitro, microglia secrete a number of substances, many of which may regulate some cellular responses *in vivo*. The substances cover a broad spectrum ranging from neurotrophic factors such as NGF and bFGF,[70] cytokines (IL-l, IL-3, and IL-4), and TNF-α.[63] Production of TNF-α is associated with the expression of class Ia+ antigen after treatment with IFN-γ and induction of microglial cells; the latter become cytotoxic to tumor cells.[58] However, the cytotoxic effects of TNF-α are not limited to tumor cells, as the factor has been shown to damage myelin-forming oligodendrocytes *in vitro*.[71] TNF-α mRNA expression is inhibited in human fetal microglia by antibodies specific to IL-6, IL-l0, and TGF-β,[72-74] showing that these cytokines are likely to modify the beneficial and harmful effects of TNF-α within the developing brain. A novel finding is the expression of TNF-α in microglia. Appel et al.[75,76] have shown that LPS increases the expression of TNF-α protein and mRNA, although the former is detected in untreated microglia, too. TNF-α mRNA is observed only between 4 and 16 h of LPS incubation. TNF-α is involved in TGF-β production as anti-TNF-α antibody significantly inhibits LPS-stimulated TGF-β production.[72-74] These findings suggest an autoregulation of microglial TNF-α production by TGF-β which may limit inflammation-associated brain injury.

Microglia-derived interleukins have been shown to have effects on microglia and other glial cell types. IL-l is a pluripotent and multifunctional molecule that mediates lymphocyte activation, fibroblast proliferation, and endogenous pyrogenesis.[77] Following treatment with IL-l, cultured astrocytes proliferate, indicating that

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the substance is an astroglial mitogen. Cultured microglia also produce IL-3[78] and alpha-2 macroglobulin.[79] Furthermore, the regulation of both microglial proliferation and transformation into phagocytes seem to be controlled by the cytokines of the GM-CSF group.[80]

In addition to cytokines, microglia have been shown to release a number of toxic metabolites including proteases and amino acids[81] such as glutamate,[82] which mediate cytotoxic effects through NMDA-receptor channels. The release of excitatory amino acids has been linked to microglial responses in various nervous system insults ranging from ischaemic lesions to the HIV-1 infection.[57,83] Oxygen radicals[84,85] and nitrogen intermediates (NO)[86] are further substances produced by microglia that exert toxic effects.

The microglia-derived signals which induce PCD are either oxidants or stimulators of oxidative metabolism. Oxidative stress, which refers to the cytotoxic consequences of oxygen radicals, may be induced by free radicals themselves (O_2, OH, H_2O_2, and NO).[87-89] Alternatively, free radicals may either elicit reactive oxygen intermediates, i.e., oxygen species with unpaired electrons, or they may extract electrons from molecules. There is an increasing amount of experimental evidence that oxidative stress causes cell death during development, implying that similar mechanisms of cytotoxicity may be induced in several neurodegenerative disorders, e.g., trauma, stroke, and seizures. There is common agreement that the association of oxidative stress with excessive activation of glutamate receptors is a final common pathway for cell vulnerability in the brain. The agreement on the importance of oxidative stress includes those pathological situations which unavoidably lead to neuronal death. However, the current experimental evidence does not limit the reduction of the complex cascades of cell death to only glutamate-gated receptors and oxidative stress because, firstly, there is a gap in the knowledge concerning the relation between oxidative stress and glutamate receptor activation. A second gap exists between our understanding of the arbitrary and variable activation of glutamate-gated channels and a constellation of associated intracellular metabolic processes.

Apart from secreting cell-modulating substances, microglia display a complex pattern of cell-associated receptor expression that images the multifunctionality of these cells within the eye.[90] While neither bacterial polysaccharide (LPS), IFN-γ, nor IL-1 seem to induce microglial proliferation,[91] they do trigger cell activation, expression of MHC II,[58,92] production of NGF and MCSF LPS,[70,93,94] and release of the urokinase-type plasminogen activator. The plasminogen activator both degrades extracellular matrix proteins and may also facilitate the migration of microglia through the retinal parenchyma, as shown in the RCS rat[15] and the developing bird retina.[21] Colony-stimulating factors like IL-3, GM-CSF, and MCSF influence the proliferation of microglia *in vitro*.[80] Proliferation is then associated with activation of phagocytosis, expression of histocompatibility antigens, lysosomal enzyme production, and release of lysozyme and cystatin-C. Out of the cytokines/growth factors tested, GM-CSF and M-CSF are not only the strongest microglial mitogens, but their withdrawal significantly enhanced rates of microglial cell death by DNA fragmentation. Thus, microglial growth factor expression may be instrumental in controlling steady states of microglia in the injured nervous system.[95]

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Neuronal cell death induced by kainic acid in the hippocampus is accompanied by an accumulation of activated microglia as demonstrated by positive OX-42 and ED1 staining, and also by their affinity for (^{125}I) CGP 42112, which binds to a nonangiotensin binding site.[96] This expression of a novel (^{125}I) CGP 42112 binding site on activated microglia may be of importance in the process of neuronal death and tissue repair. Investigation on the regulation of the rIL-3Rβ (subunit of the IL-3 receptor) mRNA expression of microglia *in vivo* shows an upregulation 4 h after the application of LPS,[75,76] and after occlusion of the middle cerebral artery in rats, where the induction is slower. Hence, induction of rIL-3Rβ mRNA in brain microglia is a very early marker for their activation *in vivo*.

7.6 CONCEPTS OF MICROGLIAL DEACTIVATION

7.6.1 Antimetabolites and Protease Inhibitors

A potential for endogenous excitotoxin produced in the CNS via tryptophan metabolism has been localised to microglia.[97] Microglia contain indoleamine-2,3-dioxygenase (the first enzyme in this pathway), which converts tryptophan to kynurenine, and which is induced in microglia and macrophages by IL-l and infections.[98] Inhibitors of quinolinic acid production such as 4-chloro-3-hydroxyanthranilate and *m*-nitrobenzylalanine[99,100] could probably be of therapeutic value.

Cultured microglia exert neurotoxic effects on co-cultured neurons by producing NO,[81,87] proteases,[83] and nonprotease neurotoxic agents.[57] A fundamental principle of apoptotic cell death appears to be the proteolytic disabling of key homeostatic and repair processes as well as the obvious structural dismantling of the cell. At the heart of the cell death process are proteases (Table 7.1) related to the mammalian ICE, present in large amounts in microglia.[101] Of interest in this regard are the recent reports that the invertebrate cell death gene, ced-3, produces a cell death protein CED-3 which is a homologue of ICE and encodes a cysteine protease (cited by Wood[102]). The essential role that these proteases play in mammalian cell death has been well substantiated by protease inhibitor studies, e.g., using the cowpox virus serpin (CrmA) and the baclovirus p35 protein, which are very potent inhibitors of ICE. ICE/CED-3-like proteins may be an attractive and tangible point for therapeutic intervention.[103] Similarly, microglial proteases have been implicated in several pathological processes occurring in the CNS, including neurodegeneration.

CB is a cysteine proteinase that plays a role in both normal and abnormal intracellular protein turnover and is a useful cytochemical marker to follow activated microglia and astrocytes after epileptogenic stimuli.[104] Ryan et al.[101] observed that the murine microglial CB consisted of two major single-chain species of 32 and 34 kDa, observed extracellularly after LPS activation. Wood[102] found that BV-2 murine microglia possess a significant basal level of CB activity, but that cellular activation results in dramatic and rapid induction of pro-CB mRNA and rapid autoprocessing of translated pro-CB. Concomitant with the posttranslational processing of the proform, the active enzyme is secreted by microglia.[101] Of interest in this regard are the observations that calpain inhibitors, which also inhibit CB, are neuroprotective

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TABLE 7.1
Intravitreally Injected Substances which Rescue Retinal Ganglion Cells from Degeneration

Substance (Stability)	Biological activity		Effective concentr. (µg/ml)	Concentration used (µg/ml)	Total amount injected (µg/ml)
Pepstatin	Pepsin		0.01	35–105	0.01–0.03
(Moderate)	Remo		0.005		
	Cathepsin		4.5		
Leupeptin	Plasmin		70	60–180	0.01–0.05
(Low)	Trypsin		2		
	Chymotrypsin		>500		
	Kallikrein		>500		
	Papain		0.5		
	Lysosomal cathepsin	A	1680		
		B	0.4		
		C	109		
Aprotinin	Trypsin		17–51		0.005–0.015
(High)	Kalliketin		17		
	Plasmin				
	Chymotrypsis				
N-Neuraminidase inhibitor			14–20	14–20	0.870–2.610
(High)					
E-64	Calpain and other cysteine proteases		1–3 µ*M*		0.01–0.003

Note: Protease inhibitors are freshly prepared from frozen stock solutions (–20°C) containing 1.4 mg/ml pepstatin, 2.4 mg/ml leupeptin, 1. 7 mg/ml, aprotinin and 2.9 mg/ml N-neuraminidase inhibitor (all obtained from SIGMA). Final concentrations are mixed in sterile 0.1 *M* PBS (pH 7.4) and 5 µ1 of the final mixture injected through the sclera into the vitreous body of operated eyes with a glass capillary (tip aperture diameter l0 to 20 µm). The effective concentrations of the inhibitors are calculated according to the data and bibliography presented in Reference 131. The concentration of the irreversible inhibitor of calpain is calculated according to references cited by Mehdi[132] and Wang.[133]

against ischaemic brain damage in rats. Cystatin, an endogenous cysteine PI, prevents the processing of the 37-kDa pro-CB to the 34-kDa CB species, which is also partially affected by leupeptin,[101] a nonspecific cysteine, and serine PI (see Table 7.1).

Consistent with the aforementioned results is the fact that retinal proteases, which are partly produced by microglia, are directly involved in the cascade of regressive events initiated by optic nerve transection. Monitoring neuronal cell survival after injection of selected inhibitors of a wide spectrum of proteases (serine proteases, cathepsins, cystein proteases, and neuraminidase) reveals a significantly better survival of RGC.[34,35] Figure 7.2 shows that quantification of neurons and microglia is simple if using fluorescent dyes which permit visualisation of both cell types simultaneously in retinal whole mounts. The distinctive morphology of labelled

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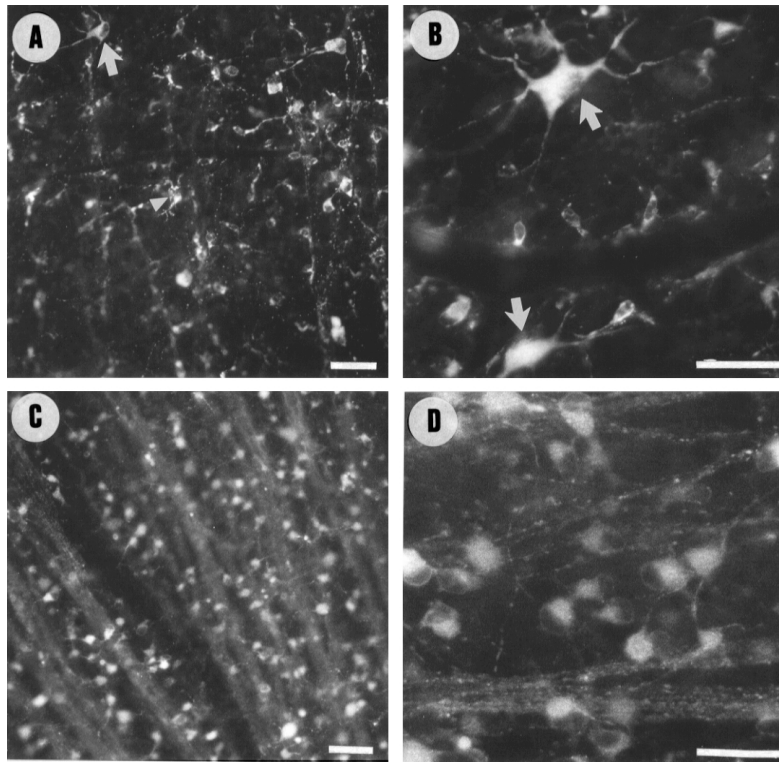

FIGURE 7.2 A-D Fluorescence photomicrographs of flat-mounted retinas with 4Di-l0ASP-labelled RGC (arrows) and phagocytically labelled microglia (arrowheads). **A**: degenerating retina without treatment 14 days after optic nerve cut with most of the RGC having disappeared and intense labelling of microglia. **B**: higher magnification showing two surviving RGC (arrows) and a number of microglia 14 days after cut. **C** and **D**: 14 days after cut of the optic nerve and intravitreal treatment with PI. Most of the RGC are still alive, whereas there is virtually no labelling of microglia due to reduced phagocytosis. Scale bars 50 µm.

microglia and neurons (Figure 7.2, A and B) and the feasible way of quantifying the effects of neuroprotectants (Figure 7.2, C and D) enable a profound analysis of such substances. As expected, significantly more RGC survive after optic nerve lesion-induced proteolytic degradation under conditions of PI (Figures 7.2, C and D and 7.3, A to D). Individual PI, like the calpain inhibitor E64, have limited effects (Figure 7.3, A), whereas those like α-2 macroglobulin and the tetrapeptide Tuftsin (microglia stimulating factor) elevate the speed of traumatic cell death (Figure 7.3, A) labelling of microglia (Figure 7.3, B). Combined treatment with cocktails of PI and other immunosuppressing drugs like MIF or steroids significantly suppress microglia activity (Figure 7.3, D) and delay the nerve cell disappearance (Figure 7.3, C). The *in vivo* observations and cell counts (Figures 7.3, A to D and 7.4, A) are substantiated by the results of *in vitro* organ culture experiments which show that pretreatment with proteolytic blockers rescues large numbers of vital RGC which then express regenerative capacities (Figure 7.4, A and B).

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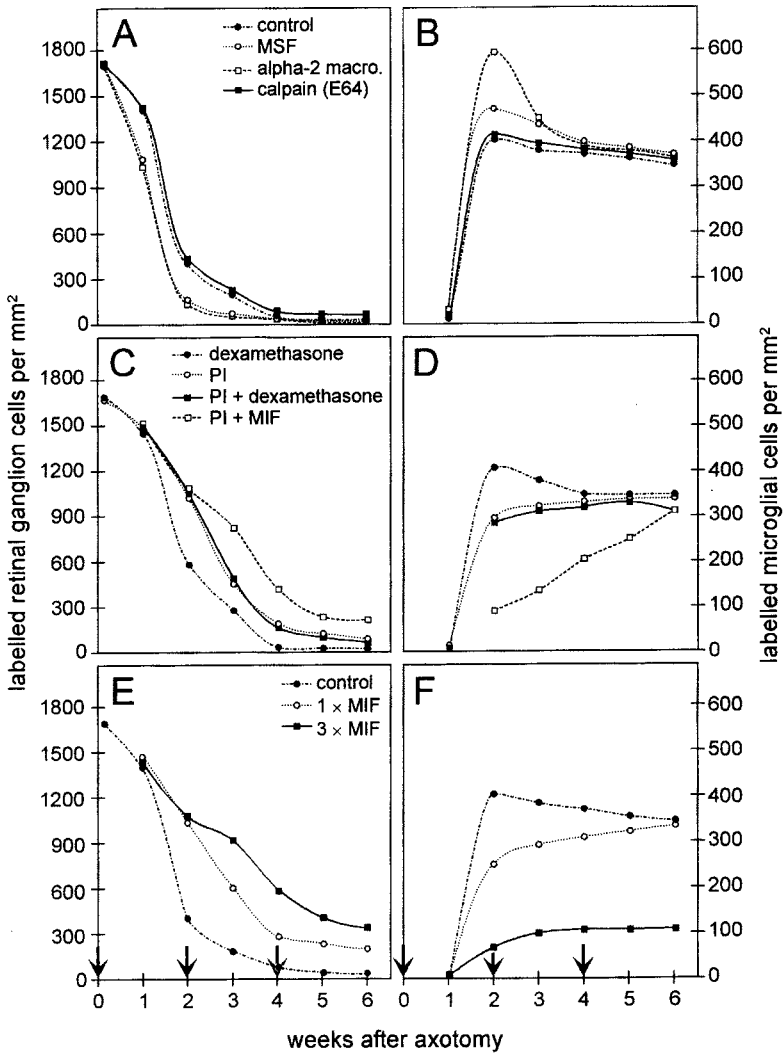

FIGURE 7.3 Graphs representing the course of RGC death and microglial labelling under the different experimental conditions. **A** and **B**: RGC death and parallel microglial labelling can be influenced with MSF, α-2-macroglobulin and E64. **C** and **D**: steroids like dexamethasone induce faster cell death that can be neutralised with PI, whereas MIF has clear surviving effects. **E** and **F**: the effects of MIF are transient and can be prolonged with multiple injections of MIF at every 2nd week (arrows on abscissa). Microglial labelling is greatly reduced when MIF is injected 3 times (**F**).

The major implication of these experiments is that proteases are involved in destruction of vital neurons. Since at least some of the inhibitors are of microglial origin, these cells probably use such bound or releasable proteases to interact with lesioned neurons mediating neuronophagic activities. These data do not distinguish

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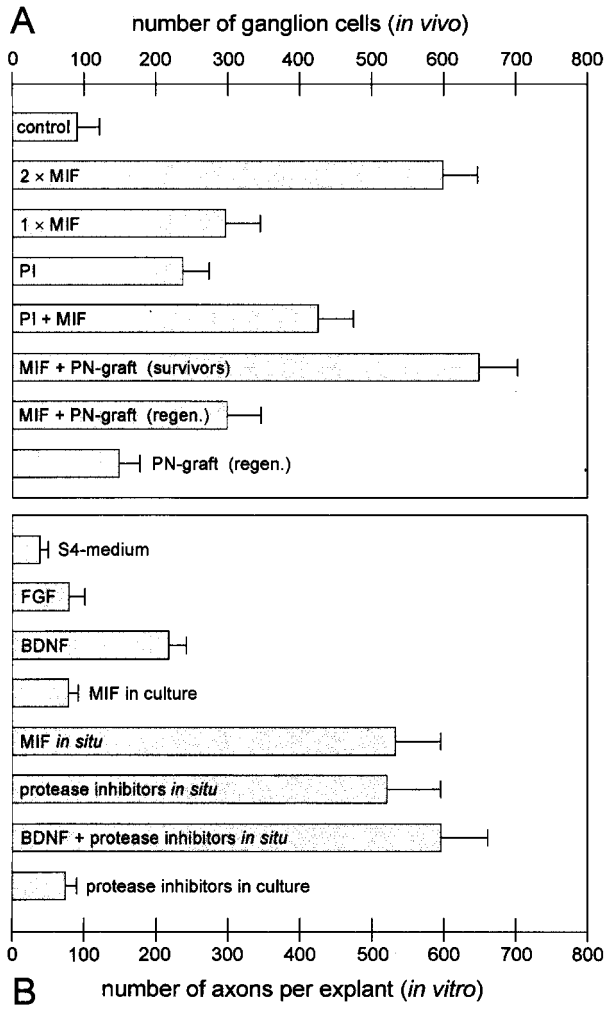

FIGURE 7.4 **A**: quantification of the effects of various microglia-targeted neuroprotectants *in vivo*. Counted cells were determined with retrograde staining and the substances were injected into the vitreous body at the time of optic nerve cut. MIF and PI elevate the number of RGC by a factor of 2.5 to 3. The substances have additive effects which become more pronounced when the rescued RGC have the opportunity to regrow their axons as occurs within a peripheral nerve graft. About half of the rescued RGC send axons within the graft (lower three columns). **B**: effects of MIF and comparison with neurotrophins (BDNF and FGF) *in vitro*. Best results are obtained when PI and MIF are injected into the vitreous body at time of optic nerve crush. Both groups of neuroprotective drugs have no effects when applied to the culture medium, indicating the lack of neuritogenic potential.

between secretable and cell-associated substances, although probably both groups may be involved. It is also possible that intraganglionic lysosomal activity can be blocked, leading to a retardation of cell death. It is less likely, although not impossible, that PI

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are potent neurotrophic substances acting directly on RGC.[34] An alternative explanation for the site of action of these inhibitors is that they block intracellular, lysosomal proteases which digest already degraded and endocytosed material. The combination of proteases and neurotrophic factors with antiinflammatory substances in the promotion of axonal growth *in vivo* is of considerable importance for regeneration.

7.6.2 Antibodies to Microglial Antigens and Cell Inhibitors

One of the experimental strategies to assess the role of microglia during neuronal degeneration is to interfere with their activity[104,105] by assuming their contribution to TCD. One elegant means of inducing apoptosis of microglia is by injection of anti-CR-3 antibodies which cross the BBB. Contemporary pharmacological destruction of the spinal cord microglia *in vivo*[83] rescues ischaemic neurons from death. Blockade of microglial activation in the retina of adult rats makes it possible to investigate the relationship of these cells to axotomy-induced RGC degeneration.[34-37] MIF[106] is a Tuftsin-derived tripeptide sequence of the Fc-chain of human immunoglobulin G[106] which acts on cells of monocytic lineage,[107-109] alters the morphology of microglia from the ramified to the amoeboid form,[37] reduces their neuronophagic activity and results in higher viability (Figure 7.3, E to F) and efficiency of regeneration (Figure 7.4, A and B) of axotomised RGC.[35] In contrast, the tetrapeptide Tuftsin, or MSF, which has only one more amino acid than MIF, stimulates microglia (Figure 7.3, B). PI and MIF display additive effects on surviving neurons (Figures 7.3, C and 7.4, A), whereas the activity of MIF seems to act transiently as repeated injections result in better survival than single applications of the drug (Figure 7.3, E). Further additive effects are observed after the application of a combination of protease inhibitors with BDNF or MIF, whereas the regenerative ability of neurites is significantly enhanced both *in vivo* and *in vitro* (Figure 7.4, A and B). When injected into the vitreous body of newborn RCS rats, MIF also reduces the number of migratory microglia and delays the disappearance of photoreceptor cells.[110] This inhibition strategy attempts to minimise cytodestructive cascades by suppression of microglial activity. The outcome of such a strategy in the dystrophic rat retina is shown in Figure 7.5. The small-molecule MIF has macrophage-modulating properties, a striking effect on the morphology of identifiable microglia, and also reduces their migratory propensity. Probably, the direct effect on the microglial phenotype limits the microglial contribution to the destruction of photoreceptors. Although its mechanism of action is obscure and difficult to assess *in vivo*, the tripeptide most likely modulates the metabolic status of microglia by interference with the intracellular cascades of activation after receptor engagement and internalisation. The resulting effects of immobilisation and reduced phagocytosis, which seem to prevent the death of neurons, are thus considered crucial for the prolonged survival of axotomised RGC.[34,35] The absence of microglial cells enables sick photoreceptors to remain morphologically intact longer, although debris that cannot be phagocytosed by pigment epithelium cells accumulates in the outer segment layer. Both experimental models are consistent with a causal link between microglial activation and speed of neuronal degeneration, indicating that these cells may be

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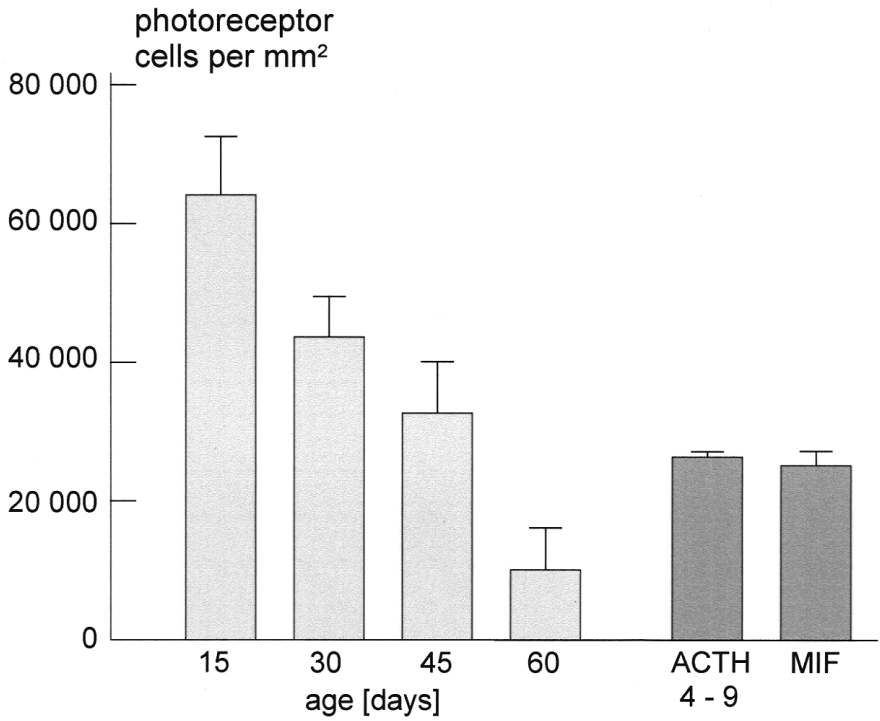

FIGURE 7.5 Decline of photoreceptors in the RCS retina between 15 and 60 days of life and the effects of MIF and ACTH-peptide 4-9 injected into the vitreous body. The rescue effects are significant in both cases. The counts were obtained at 60 days after birth (from Siebert[123]).

directly involved in the recognition of sick neurons as well as in their active destruction and phagocytosis.

7.7 NEUROPROTECTIVE IMPLICATIONS DURING NEURONAL REGENERATION AND LOCAL REPAIR

7.7.1 NEUROTROPHINS

The coincident migration of microglia into the zone of photoreceptor degradation at early stages of the disease before structural changes become detectable is consistent with the capability of these cells to respond to degeneration-derived stimuli.[42,68,111] Retardation of photoreceptor degradation observed in MIF-treated rats is reminiscent of reports on intravitreal or subretinal instillation of the heparin-binding neurotrophic factor bFGF. However, the target cells of bFGF have not been identified.[112] The relationship of microglia to dying neurons is certainly very complex, and the action of injectable factors can be interactive, since some of them may induce or inhibit others or themselves. The investigation of additive effects of various factors

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therefore requires sophisticated strategies of treatment. One of the successful approaches may be the application of neurotrophic factors like bFGF, CNTF, BDNF, and other neurotrophins.[112] Interestingly, BDNF reduced the incidence of macrophages in a light-damaged retinal model,[112] indicating that in addition to its neurotrophic potential it may interfere with macrophages, too. It is therefore not clear whether this factor acts via direct or indirect pathways in the various models tested so far. When tested on explanted retinal strips, BDNF has stronger effects than bFGF (Figure 7.4, B) and additive effects to that described for PI (Figure 7.4, B).

7.7.2 Immunosuppressing Drugs

Further groups of substances with neuroprotective activities are to be found among the steroids and nonsteroidal immunomodulating drugs. Indeed, a number of immunosuppressive approaches which downregulate activated microglia have been shown to function both *in vitro* and in lesion models resulting in neuronal cell death *in vivo* (cited by Wood[102]). The S-phase-specific antimitotic, cytosine-arabinoside, inhibits a brainstem microglial proliferation response induced by hypoglossal nerve injury in rats.[105] The intercalating cytostatic agent, doxorubicin, blocks microglial proliferation in the rat brainstem induced by injections of toxins into the facial nerve.[105] The immunosuppressant, deoxyspergualin, which interferes with heat shock protein function,[113] suppresses microglial function. Also, the controversial effect of dexamethasone may be mediated by the partial inactivation of microglia at low doses[114] which induce nerve cell death at higher concentrations (Figure 7.3, C). Immunosuppressive approaches are neuroprotective in *in vivo* nonretinal ischaemia models and include:

1. A combination of chloroquine and colchicine, which suppresses the secretory and phagocytic activities of microglia, resulting in neuronal protection in spinal cord ischaemia induced by the temporary occlusion of the abdominal aorta in rabbits.[83]
2. The immunosuppressant cyctosporin A, which downregulates activated microglia[94] and protects against neuronal and muscarinic receptor losses in the gerbil hippocampus after transient global ischaemia.[115]
3. The IL-1 receptor antagonist protein, which antagonises the actions of microglia-derived IL-1α and IL-1β is neuroprotective in rat focal cerebral ischaemia[116] and against brain lesions induced by IL-1α or excitotoxins.[117]
4. Inhibition of protein synthesis with cyclohexamide is neuroprotective in rat focal cerebral ischaemia;[118] while inhibition of protein synthesis would occur in all cell types and presumably inhibits the synthesis of "cell death proteins,"[80] the role of microglial suppression via this approach remains undefined.

Leukocyte-derived cytokines like IL-4[119] have been found to protect the cocultured neurons from the neurotoxic activity of brain microglia. Although these experiments are performed with nonretinal microglia, they allow a certain degree of extrapolation as to their possible use as inhibiting cytokines for blocking retinal

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microglia. Indeed, cutting of the optic nerve and instillation of either human or rat IL-4 into the vitreous results in a remarkable delay of RGC death, similar to that seen with injection of MIF (Thanos, S. et al., in preparation). Simultaneous grafting of a piece of peripheral nerve to facilitate regrowth of axons was accompanied by vigorous growth of nearly half of the neurons axotomised at time of grafting. It now becomes apparent that cytokine-mediated communication with the circulating immune system plays a key role in both the mechanisms of microglial activation and in the mode of cell destruction.

As mentioned in the previous section, reactive oxygen intermediates (e.g., superoxide O_2) generated by microglia may play a role in host defense and injury within the CNS. Priming of microglial cell cultures with IFN-γ or TNF-α results in a dose- and time-dependent enhancement of O_2 production, mediated by a protein kinase C signal transduction pathway.[73,74] The priming effects of these two cytokines is additive, suggesting that they act by independent mechanisms.[120] Treatment of the microglia with TGF-β , IL-4, or IL-l0 suppresses this O_2 production.

7.7.3 Hormone-Derived Microglial Inhibitors

Neurohormones like ACTH and α-MSH have been located in the amacrine cells of the IPL.[121] The first 24 amino acids of ACTH are phylogenetically well preserved and responsible for its biological activity. ACTH plays a role in the restoration of neuronal function after CNS lesions[122] and has receptors widely distributed throughout the brain. Since it can inhibit IFN-γ production of macrophages and T-helper cells, it could possibly inhibit phagocytosis by microglia.

The RCS rat model of photoreceptor degeneration has been used[123] to test the effect of the ACTH fragments 4-10 and 4-9 and demonstrates a neuroprotective effect only with the fragment 4-9. This remarkable and significant difference between the two oligopeptides points to receptor-binding of the fragment 4-9, but not of the fragment 4-10. To examine whether this effect is microglia-mediated, microglia are labelled with internalised dyes and studied both morphologically and quantitatively. Although microglia remain morphologically intact, their numbers are significantly reduced nine days after transection of the optic nerve, indicating a blockade mechanism different from that described for MIF.[37] ACTH fragment 4-9 may act as an antagonist of the natural ACTH receptors and alter the mode and speed of microglial activation, thus providing the photoreceptor cells with a higher chance to remain unphagocytosed over longer periods of time (Figure 7.5). This mechanism remains undeciphered, although the number of photoreceptor cells which survive with ACTH analogue 4-9 treatment is similar to that obtained with MlF treatment (Figure 7.5).

Otto[124] found increased adenyl cyclase activity in RCS rats as compared to Sprague-Dawley rats, which increased with age. Alternatively, the fragment 4-9 may activate the adenyl cyclase pathway as was determined for dopaminergic neurons.[125] Finally, the fragment 4-9 may bind to its receptor and release cytokines of the autocrine type which inhibit the phagocytic activity of microglia, as was also postulated by Ulenkate et al.[126] based on their electron microscopic data. Retrograde labelling studies[123] confirmed that the ACTH fragment does not directly affect the phenotype of microglial. The reduction in cell number after intravitreal injection,

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indicates a transient, indirect inhibition of the phagocytic activity of microglia, as was observed after application in the hippocampal model.[126] This is possible if neurons form a receptor-ligand complex with the ACTH fragment and prevent the release of ATP, K^+ or Ca^{2+},[127,128] or cytokines (e.g.,TNF-α). Thus, a possible signal pathway used by microglia could be interrupted and suppressed. The destructive effect of either glutamate[129,130] or nitric oxide[87] could be prevented in this manner.

7.8 FUTURE PERSPECTIVES AND CONCLUSIONS

Traumatic neuronal cell death, and to some extent excessive hereditary cell death, requires the contribution of specific, concerted mechanisms. These include intrinsic cellular decisions at molecular and metabolic levels, and activation of an intraretinal surveillance cascade devoted to limiting the undesirable side effects of cell death. A key role in this biological process is ascribed to microglia residing within the tissue which are activated by numerous mechanisms and diseases, integrate the signals that activate the machinery of possible responses, and ultimately respond specifically. Partial abolition of TCD can be achieved with substances that block certain stages of microglial activation and neurotoxicity. Among these, PI, deactivating cytokines, and various antimetabolites constitute a spectrum of neuroprotective substances. Recent discoveries in the field of protease-mediated cell death, and the remarkable neuroprotective effects of simple PI with a wide range of actions, have contributed to the evolution of our understanding of the cell death cascades and offer exciting opportunities to improve curative interventions after trauma.

ACKNOWLEDGMENTS

The authors would like to acknowledge the contributions of M. Wild, C. Riedinger, W. Richter, and H. Siebert for technical advice with parts of the work. The work was supported by the DFG, BMBF, IRIP, and the EU in the frame of BARNEF. R.N. was a recipient of a stipend from the Friedrich Ebert Foundation and S.T. was a recipient of a Hermann and Lilly Schilling professorship.

GLOSSARY OF ABBREVIATIONS

4Di-10ASP4-[4-(Didecylamino)styryl]-*N*-methylpyridinium iodide
ACTHAdrenocorticotrophin hormone
ADAlzheimer's disease
AIDSAcquired immune deficiency virus
BBBBlood-brain barrier
BDNFBrain-derived neurotrophic factor
BRBBlood-retinal barrier
CBCathepsin B
CNSCentral nervous system
CR3Complement type 3 receptor
CSFCerebrospinal fluid

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DiI1, 1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyamine perchlorate
EAEExperimental allergic encephalomyelitis
ECDExcitatory cell death
EMElectron microscopic
GCLGanglion cell layer
GM CSFGranulocyte macrophage colony-stimulating factor
HIV-1Human immunodeficiency virus type-1
IFN-γInterferon gamma
ICEInterleukin-1-converting enzyme
ILInterleukin-l, -2, -3, -4, -6, -10
ILMInner limiting membrane
IPLInner plexiform layer
LCALeukocyte common antigen
LPSLipopolysaccharide
MCSFMacrophage colony-stimulating factor
MHC I, MHC IIMajor histocompatibility complex I, -II
MIFMacrophage inhibiting factor
MSMultiple sclerosis
MSHMelanocyte-stimulating hormone
NDPaseNucleoside-5′-diphosphatase
NFLNerve fibre layer
NGFNerve growth factor
NMDA*N*-Methyl-D-aspartate
NONitric oxide
OFLOptic fibre layer
ONLOuter nuclear layer
OPLOuter plexiform layer
PCDProgrammed cell death
PLPlexiform layers
PIProtease inhibitors
RCS ratRoyal College of Surgeons rat
RGCRetinal ganglion cell
RPERetinal pigment epithelium
SCSuperior colliculus
TCDTraumatic cell death
TNF-αTumour necrosis factor-alpha
TPPaseThiamine pyrophosphatase

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8 Transforming Growth Factor-β and CNS Scarring

Ann Logan and Martin Berry

CONTENTS

8.1 GLIAL SCAR FORMATION IN THE CNS

Penetrating injuries of the CNS initiate a complex cellular wounding response comprising sequential and overlapping events. Acute haemorrhage and inflammation is associated with neuron degeneration; this is followed by glial/collagen scar formation, which is accompanied by an abortive regeneration response by axotomised but still viable neurons.[1-3] The cellular events that culminate in glial scar formation are complex and are summarised in Figure 8.1. Whilst this figure illustrates the process of scar formation in the brain, the cellular events shown are representative of those that occur throughout the CNS.

Within a few hours of injury to the brain or spinal cord, the activated microglia and macrophages interact with intact astrocytes to initiate a reactive gliosis within the damaged tissue.[4] Reactive astrocytes have increased immunoreactivity for the intermediate filament glial fibrillar acidic protein (GFAP), which may reflect attempts either to preserve or enhance the structural integrity of the tissue. Between 5 and 7 days after injury, the cytoplasmic processes of the astrocytes become concentrated

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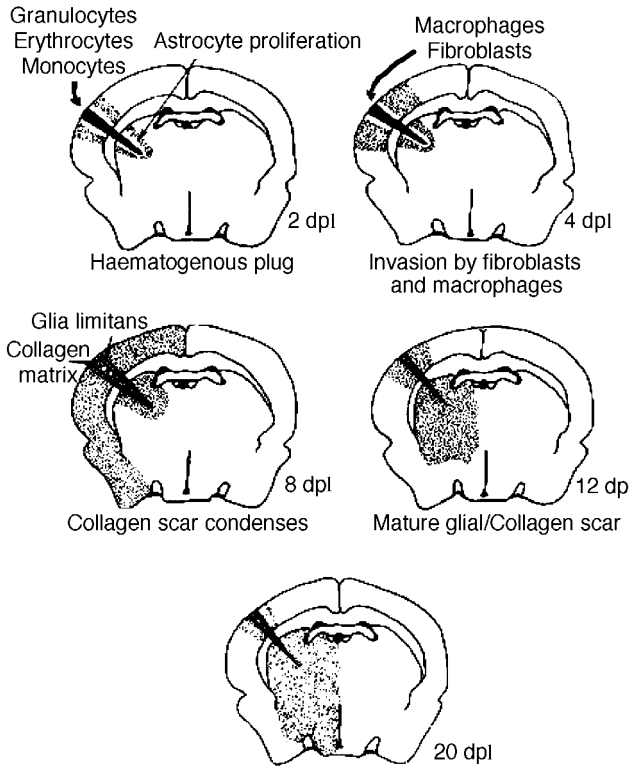

FIGURE 8.1 The spatial and temporal changes that occur after a penetrating injury to the cerebral cortex of the brain. During the acute phase (1 to 3 days postlesion (dpl)), a haemorrhagic period results in an influx of haematogenous cells, including macrophages, which help to clear away necrotic tissue, whilst a reactive gliosis is initiated in the surrounding neuropil. In the subacute phase (4 to 8 days postlesion) the clot contracts and is infiltrated by fibroblasts that start to produce fibrous collagens, laminin, and fibronectins in the core of the wound. The astrocytes begin to associate at the wound margins to form a glia limitans. Finally, during the consolidation phase (9 to 14 days postlesion) the scar contracts and matures. A declining reactive gliosis is seen at subsequent time points.

at the CNS/mesenchymal boundary, form a continuous multilayered sheet, and are locked together by tight junctions. Mesodermal cells, including fibroblasts, are chemoattracted into the core of the wound from the meninges, initiating deposition of matrix molecules such as collagens I, III, and IV, laminin, and fibronectins as they migrate inwards. Thus, the fibrous scar matures sequentially from pial surface to the depths of the lesion. During this time, compromised neurons that have survived the initial injury and the immediate neurotoxic events show outgrowth of new side branches and terminal growth cones, a process termed sprouting, and some may even make synapses with adjacent neurons.

Between the astrocytic processes and the mesenchymal core a basal lamina is deposited, probably by the astrocytes under the influence of fibroblasts, and a limiting

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membrane (glia limitans) starts to organise which eventually delineates the cut edges of neuropil. The glia limitans established in the wound is identical to, and continuous with, that of the external limiting glial membrane. A dense fibrous scar which is surrounded by a glial membrane is thereby constructed within the wound between 5 and 8 days. During this period an angiogenic response also occurs in the neuropil bordering the wound. By 8 to 14 days, the trilaminar scar is fully formed, the central mesenchymal fibrous core contracted, and the palisades of astrocyte processes compacted at the lesion margins (see Chapter 1). GFAP-activity in astrocytes thereafter declines, except at the wound margins, and a small number of macrophages remain in the core, but none are seen in the surrounding CNS tissue. By 14 days, all newly grown axonal processes have died back to their original parent axons, a large number of which remain *in situ* in perpetuity, making no further attempt to grow. This abortion of regeneration coincides with the maturation of the impenetrable fibrous glial scar and functional reconnection of severed neural pathways is therefore impossible.

The scarring response to injury described above is acquired neonatally in the rat, for example, between 8 and 12 days postpartum (dpp). The process of wound repair in the immature CNS is described in some detail in Chapter 1. Before 8 dpp, no mesenchymal elements accumulate in the wound, and although some astrocytes become reactive a glia limitans is not formed, compromised neurons show excellent growth capacity, and the neuropil grows together obliterating all signs of the original lesion. Typical scarring first appears at 8 dpp subpially and, over the next 4 days, invades the depths of the wound. Although the microglial response is qualitatively mature in the neonate, macrophage and fibroblast invasion from the pia into the wound is minimal. Curiously, however, the glia limitans externa is repaired after injury before 8 dpp. It has been suggested that understanding the biology of scar acquisition in the perinatal CNS could direct pharmacological strategies aimed at replicating the neonatal response to injury in the adult.

The observations of scar formation coinciding with axon growth arrest in the mature CNS provided an early explanation of the aborted regeneration. It was suggested that, in adults, axon growth arrest is scar related, since the cicatrix constitutes a physical barrier through which axons are unable to pass. But neuromata never form around a scar and, accordingly, this barrier hypothesis has received little support. Nevertheless, more recent analysis has demonstrated that reactive astrocytes and the glial scar they form do constitute a significant physical and biochemical hurdle for growing axons and have been targeted as one of the primary contributors to the environment of the injured CNS that fails to support neuronal regeneration.[1,5] The adult rat glial scar in culture inhibits neurite outgrowth[6] and the results of electron microscope studies suggest that growth of axons is arrested in the immediate vicinity of reactive astrocytes.[7] However, due to the presence of fibroblasts, meningeal cells, and macrophages in such preparations of scar tissue, it is difficult to assess the specific contribution of the astrocyte component to regenerative failure of neurons. Chondroitin-6-sulphate proteoglycan and cytotactin/tenascin are neurite growth-inhibitory factors present on astrocytes or their precursors during development.[8,9] Following injury to the mature rat CNS, these molecules are reexpressed and colocalise with reactive astrocytes adjacent to wounds.

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Further evidence suggests that the influence of reactive astrocytes need not necessarily be exclusively inhibitory to axonal regeneration. As well as providing inhibitory molecules, astrocytes may also elaborate appropriate extracellular substrates for neurite adhesion and extension. Glial tissue taken from neonatal rats, in which neural regeneration is relatively successful, but not from adults, supports neurite outgrowth *in vitro*.[10] Similarly, cultured astrocytes from neonatal rats actively facilitate neural outgrowth, whilst those isolated from injured adult brain demonstrate a dramatically reduced capability of promoting neurite outgrowth.[11] Astrocytic scar tissue taken from the optic nerve of adult rats several months after transection is also a good substrate for neurite growth.[12] Most conclusively, in the presence of nerve growth factor (NGF), reactive astrocytes are able to provide a substrate for the growth of sympathetic neurites.[13]

Laminin, which is secreted by astrocytes forming the glia limitans, has attracted much attention as an astrocyte-derived substrate for regenerating axons since, *in vitro*, neurite outgrowth of many neuronal cell types is greatly enhanced when they are grown on a substratum that contains the molecule.[14] However, laminin is not found in the healing CNS other than in basement membranes, and neither is it essential for the growth of adult mammalian ganglion cells *in vitro* or *in vivo*.[15] Of more relevence, a glycoprotein neurite outgrowth-promoting substrate molecule, designated as L1, has recently been described and located in astrocytes of the embryonic CNS, and shown to be downregulated in the neonate after major tracts are established. Expression of the L1 gene in adult transgenic mice significantly enhances neurite outgrowth of the optic nerve *in vitro*.[16]

It seems likely that effective neurotrophism by astrocytes is mediated by a combination of astrocyte-derived neurotrophic factors, neurite growth-inhibitory factors, cell-surface molecules/receptors, and extracellular matrix. It is clear that, as regeneration is now possible in the CNS, concomitant inhibition of scarring could augment the reconnection of functional pathways by increasing the numbers of axons regrowing through the lesion. It seems that transforming growth factor-βs (TGF-βs) are key factors responsible for organising astrocytes and initiating matrix deposition to form a glial scar. Recent work by ourselves and others has established that the expression of these fibrogenic cytokines is rapidly upregulated in neurons and glia of damaged CNS tissue,[17-22] so that between 1 and 7 days postlesion significantly elevated levels of these trophins are present within wounds to orchestrate glial/collagen scar formation.

8.2 BASIC BIOLOGY OF TRANSFORMING GROWTH FACTOR-β (TGF-β)

8.2.1 Molecular Structure of TGF-βs

The reader is directed to the recent excellent review of Cui and Akhurst[23] which gives a full description of the TGF-βs; the important biochemistry relating to TGFβ bioactivity is summarised herein. Three genetically distinct isoforms have been cloned and characterised in mammals, designated TGF-β1, -β2, and -β3, respectively. The three human TGF-β genes are situated on separate chromosomes: TGF-β1

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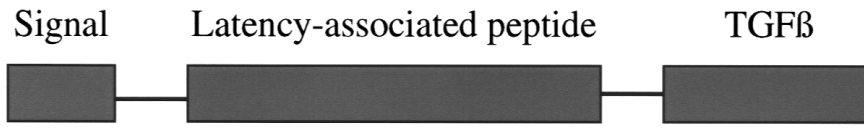

FIGURE 8.2 Pre-pro-TGF-β. The TGF-βs are synthesised as large precursor molecules (pre-pro-TGF-β) consisting an N-terminal signal peptide preceding the latency-associated protein (LAP), with the bioactive TGF-β forming the C-terminus.

on chromosome 19q13, TGF-β2 on 1q41, and TGF-β3 on 14q24. All three have a similar seven-exon gene structure and exhibit a high DNA sequence homology, suggesting a common ancesteral gene, but each have distinct 5′ regulatory regions indicating that their expression can be differentially regulated. The genes encode precursors of 390 (TGF-β1) and 412 (TGF-β2 and -β3) amino acids. Each protein is made in a precursor form that is proteolytically processed to generate the biologically active molecule. The large pre-pro-TGF-βs each comprise an N-terminal short (29-amino-acid) signal peptide for secretion, followed by a β-latency-associated peptide (LAP) of 249 amino acids and a C-terminal domain of 112 amino acids wherein lies the biological activity (Figure 8.2). Whilst the C-terminal bioactive peptide is highly conserved between isoforms (70 to 80% amino acid homology), the sequences of the β-LAPs are markedly dissimilar. The β-LAPs do, however, share some important structural features, such as three cysteine residues (involved in disulphide bonding of the β-LAP dimer) and several isoform-specific N-linked glycosylation sites, where mannose-6-phosphate residues are added posttranslationally. The β-LAP region is probably important for protein folding, secretion, and activation.

The mature proteins exist as 25-kDa homodimers held together by disulphide bonds which are generated by proteolytic cleavage of the C-terminus of the pro-TGF-β molecules. The cleaved homodimers remain noncovalently associated with the two β-LAPs from which they were cleaved, and hence are kept in an inactive state (Figure 8.3). Since all three TGF-βs are synthesised and secreted as biologically inactive homodimer complexes which are unable to bind to their cell surface receptors, activation of latent TGF-β by dissociation from the β-LAPs is a possible control point for regulation of bioactivity. *In vivo*, TGF-β is secreted as a large, latent complex which includes an additional protein component, the "latent TGF-β-binding protein" (LTBP, a glycoprotein of 125 to 160 kDa), which is covalently linked to β-LAP (Figure 8.3). The function of LTBP is not defined but it certainly plays a role in conferring latency and may be important for efficient activation of latent TGF-β at the cell surface.

The latent TGF-β complexes can be activated *in vitro* by either acidification, alkalinisation, heating, or the use of chaotropic agents such as SDS and urea, although the mechanism of activation by *in vivo* targets remains to be elucidated. However, controlled proteolysis may be involved and urokinase-type plasminogen activator and transglutaminase type II have been implicated in the process. Interaction of the mannose-6-phosphate residues on the LAP with cell-surface mannose-6-phosphate/insulin-like growth factor receptors may also play a role, but this interaction

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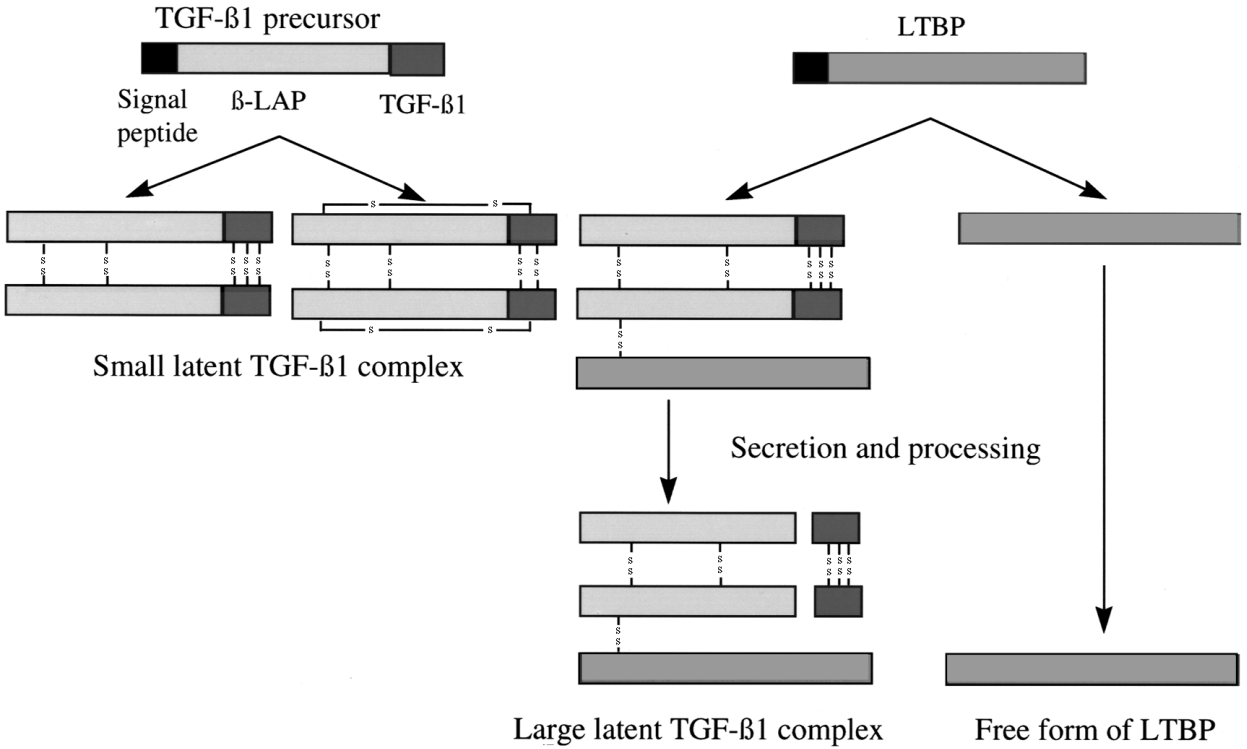

FIGURE 8.3 Molecular structure and processing of TGF-β. After synthesis, the N-terminal signal peptide is cleaved from the pre-pro-TGF-β to yield an inactive pro-TGF-β. The so-called "small latent complex" comprises two LAPs and a TGF-β dimer. Cell-secreted "large latent complex" latent TGF-β comprises a complex between the "small latent complex" and one molecule of latent TGF-β-binding protein (LTBP), which is covalently bound to LAP. The TGF-β dimer becomes activated by dissociation from LAP.

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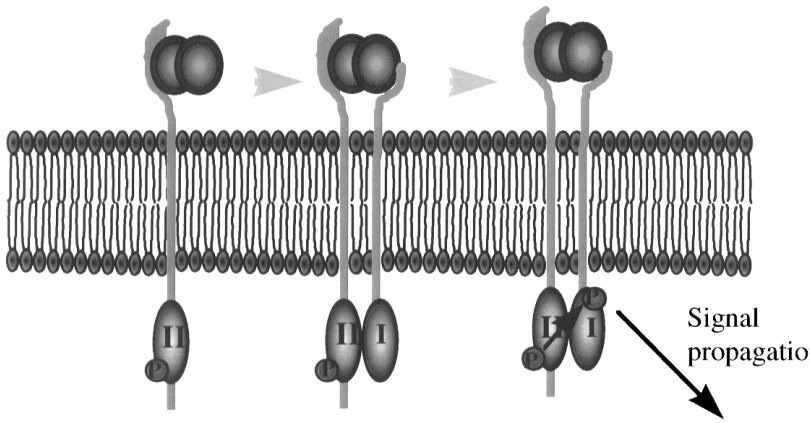

FIGURE 8.4 A model of TGF-β receptor activation. The type II receptor kinase is constitutively active, even in the absence of ligand. It binds the ligand dimer and then recruits type I receptor to form a heteromeric complex. In this complex, the type I receptor is phosphorylated by the type II receptor kinase. The now activated type I receptor then propagates the signal to downstream effectors via its kinase domain.

is not essential.[24] By whatever mechanism, release of the C-terminal 25-kDa TGF-β homodimer from its β-LAP confers bioactivity to the peptide.

8.2.2 TGF-β Receptors

Currently, type I, type II, and type III TGF-β receptors (TGF-βR) have been cloned and biochemically characterised.[25] Types I and II TGF-β R are glycoproteins of 55 to 60 and 70 to 95 kDa, respectively, and both belong to the transmembrane serine-threonine kinase family. Both have an N-terminus containing a signal sequence preceding a hydrophilic cysteine-rich extracellular domain. This ligand-binding domain is followed by a hydrophobic transmembrane region and an intracellular serine-threonine kinase signalling domain, which is terminated by a small C-terminal tail. Despite the organisational similarities between these two receptors, the ligand-binding domains are only 17% homologous. The distinctive type III receptor, also known as betaglycan, is a 300-kDa proteoglycan capable of binding TGF-βs, but it has no enzymatic potential.

All three receptors cooperate in the ligand-dependent signalling process[26] (Figure 8.4). Though the type II receptor can bind ligand directly it cannot signal, and the type I receptor is unable to interact with TGF-β in the absence of a functional type II receptor. Type II receptors exist on the cell surface as homo-oligomers which are constitutively phosphorylated. By contrast, type I receptors are not phosphorylated in the absence of ligand. Binding of TGF-β to the extracellular domain of the type II receptor results in the recruitment of type I receptor into the complex, resulting in a ternary receptor-ligand complex. Consequently, the type I receptor becomes phosphorylated by the type II recepor and this activates the type I receptor kinase.

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The activated receptor I kinase can subsequently phosphorylate or interact with specific substrates/effectors, sending signals to the downstream pathways.

The type III receptor (betaglycan) is a large (280- to 300-kDa) proteoglycan comprising a core protein covalently linked to heparan sulphate and chondroitin sulphate glycosaminoglycans. These carbohydrate moieties are not required for ligand binding. This receptor has no cytoplasmic signalling motif and is not directly involved in signal transduction, but it is important for facilitating ligand-receptor interaction. It acts to present ligand to the signalling receptors; on binding TGF-β it forms a transient complex with the type II receptor which allows high-affinity bonding between the type II receptor and the ligand. The type III receptor dissociates after bonding, allowing the type II receptor to recruit a type I receptor, thereby forming an active signalling complex. In addition to its role in presenting ligands to the signalling receptor, the type III receptor may also act to antagonise the effects of the TGF-βs. Its extracellular domain may be proteolytically released from cell membranes to form a soluble molecule or binding protein which sequesters TGF-βs, thereby rendering TGF-βs biologically inactive.

8.2.3 Biological Activities of TGF-βs

8.2.3.1 Cell Growth and Differentiation

The biological effects of TGF-βs *in vitro* are remarkably diverse. They can stimulate or inhibit cell proliferation, down-regulate or up-regulate cell differentiation, or alter cellular phenotype. Their precise action depends on the cell type, the proliferative or differentiation state of the cell, and the culture conditions. The predominant growth-related effect of all three isoforms is inhibitory, with growth-stimulatory effects being rare and usually indirect, mediated by other growth factors or the extracellular matrix.

8.2.3.2 Formation of Extracellular Matrix

One of the most striking bioactivities of TGF-βs is the modulation of both extracellular matrix (ECM) deposition and composition and also cell-substratum interactions (see Figure 8.5). The ECM is not only a support for tissue structure, but more importantly it is a complex and dynamic molecular substratum which can modify a wide range of cellular activities. The ECM can affect cell proliferation, differentiation, morphology, adhesion, migration, and interaction between cells. Thus, TGF-β may affect cell activity indirectly via the ECM. In general, TGF-β promotes ECM formation and can enhance the response of cells to ECM via alterations in integrin expression. TGF-β stimulates the deposition of several ECM components, including fibronectins, interstitial collagens (I, III, IV, and V), thrombospondin, tenascin, laminin, and chondroitin/dermatan sulphate proteoglycans.[27] The enhancement of ECM protein synthesis results from the increased transcription of the corresponding genes and, in some cases, stabilisation of their mRNAs. In addition, TGF-β potentiates ECM production by inhibiting matrix protein degradation, which is achieved through its action on genes encoding proteases and protease inhibitors. TGF-β decreases synthesis and secretion of several proteases, including tissue plasminogen

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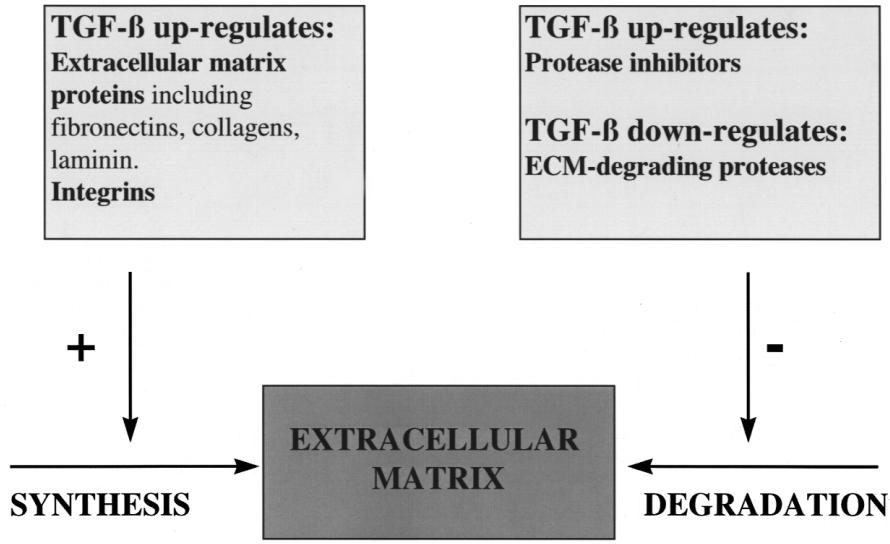

FIGURE 8.5 The four cumulative effects of the TGF-βs on ECM synthesis/maintenance.

activator, urokinase-type plasminogen activators, thiol protease, collagenase, and transin, but increases expression of protease inhibitors such as plasminogen activator inhibitor type 1 (PAI-1), urokinase, and the tissue inhibitor of metalloproteinases (TIMP).[27] The net effect of these activities is to promote matrix deposition.

8.2.3.3 Cell Migration

TGF-β also regulates cell-substratum interactions by a selectively increasing expression of integrins, a major class of cell-adhesion receptors. Integrins bind fibronectin, collagens, and other ECM proteins[28,29] and thus modulate the adhesion cascade, which modulates cell migration.

8.2.3.4 Inflammation

TGF-βs affect the immune system by usually acting as immunosuppressors.[30] During inflammation these cytokines have multiple effects. Initially, they act positively to stimulate monocyte migration and macrophage production, by chemoattraction. After the initiation of the inflammatory response, TGF-βs become antiinflammatory by (1) preventing the adhesion of neutrophils and T-lymphocytes to endothelium,[31,32] (2) downregulating macrophages, and (3) blocking cytokine actions.[33]

8.2.3.5 Angiogenesis

There is now considerable evidence to suggest a key role for TGF-βs both in vasculogenesis and angiogenesis.[34] *In vivo,* TGF-β1 and -β2 may promote stabilisation of the vessel wall through direct interaction with endothelial cells as well as by promoting differentiation and recruitment of other vessel wall cells. They may also

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have indirect angiogenic effects by inducing chemotaxis of inflammatory, epithelial, and connective tissue cells, which are subsequently activated to produce direct-acting positive endothelial regulators such as vascular endothelial growth factor (VEGF).

8.3 TGF-β AND WOUND HEALING

The observations that platelets are the most concentrated source of TGF-βs[35] and that TGF-βs can stimulate ECM synthesis, activate macrophages/monocytes, and suppress immune cell responses, implies important roles for TGF-βs as regulators of wound healing. Indeed, these factors have been shown to be involved in a variety of peripheral tissue repair processes such as the promotion of bone fracture healing,[27] closure of defective skull bones,[36] and production of retinal defects.[37] The fibrogenic activity of TGF-βs have been most studied in the cutaneous healing process. Following haemorrhage in dermal wounds, large amounts of TGF-βs are released by degranulation of platelet α granules;[35] both the newly delivered cytokines and the TGF-βs bound locally to the ECM become activated at the site of injury. Immunohistochemical studies of skin injuries have shown that, within hours of trauma, levels of TGF-β1 increase at the wound site.[38,39] Keratinocytes at the wound margins form the leading edge of a migrating epithelial sheet and coordinately increase TGF-β1 expression. The resulting induction of fibronectin and integrin synthesis could modulate tissue integrity and cell migration.[38] TGF-βs exert a twofold effect on fibrosis: they are strongly chemotactic for fibroblasts,[40] and increase the deposition of ECM by the fibroblasts. Injection of TGF-β1 into dermal wounds results in rapid induction of collagen synthesis and accelerated healing.[36,41-44] A very similar induction of expression[39] and fibrogenic activity[45] of TGF-β2 isoform has also been demonstrated in dermal wounds. In addition, TGF-βs have other important functions in dermal healing and tissue repair. They promote the development of new blood vessels and they chemoattract and activate monocytes and neutrophils, thereby modulating the inflammatory response. Taken together, the evidence from peripheral tissues suggests that TGF-βs have important homeostatic actions in the process of injury repair, increasing repair efficiency.

8.4 TGF-β EXPRESSION IN CNS WOUNDS

There is little or no expression of TGF-β1 in the adult CNS neural parenchyma. The protein is exclusively restricted to the mesenchymal support structures such as the choroid plexus and meninges. By contrast, TGF-β2 and TGF-β3 are both widely, coincidentally, and constitutively expressed by neurons and glia as well as by the CNS mesenchymal support structures.[46] The precise functions of TGF-βs in the intact and injured CNS remain to be defined, but *in vitro* studies have indicated potential gliogenic, neurotrophic, angiogenic, and fibrogenic activities.[47-53]

After a CNS injury that breaches the blood-brain barrier, TGF-β1 and TGF-β2 are released from platelets and secreted from cells of the monocyte/macrophage lineage at the earliest stages of the injury response. In a number of *in vivo* experimental CNS injury paradigms, the local expression of TGF-β1 increases.[17-22] For

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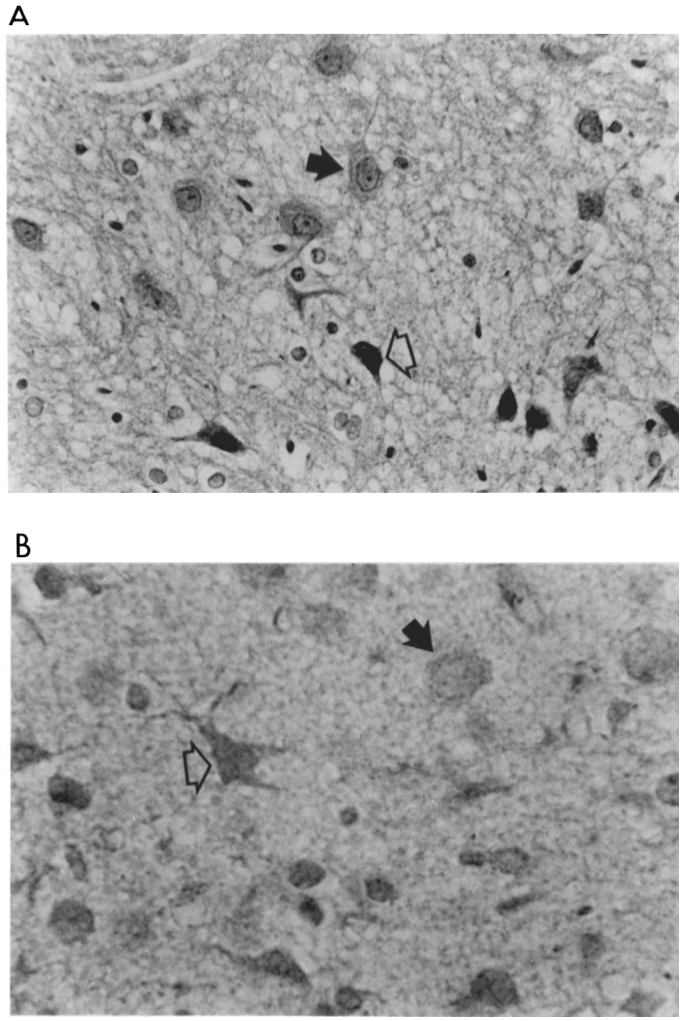

FIGURE 8.6 A/B Localisation of immunoreactive **A:** TGF-β1 and **B:** TGF-β2 in injured cerebral cortex parenchyma. Five days postlesion significant levels of immunoreactive TGF-β1 and TGF-β2 are seen in neurons (closed arrows) and astrocytes (open arrows) of the neuropil bordering the wound.

example, within traumatic wounds of the rat cerebral cortex and spinal cord, the influx of haematogenous TGF-β1 and TGF-β2 is rapidly followed by a transient upregulation of expression of both peptides by injury-responsive cells;[18,21] so that after injury, TGF-β1 and TGF-β2 proteins are localised to astrocytes, microglia, neurons, vascular endothelial cells, and macrophages (Figure 8.6). Further, the localisation of TGF-β1 and TGF-β2 mRNA to choroid plexus epithelial cells and the measurement of significantly increased levels of both proteins in the cerebrospinal fluid (Logan, A. and Berry, M., unpublished observations) indicate a supply of factors

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to the damaged neuropil posthaemorrhage, both via the CSF as well as by local synthesis within the wound. In accordance with the observations in rodent experimental models, TGF-β1 levels have also been investigated in a preliminary study of the cerebrospinal fluid of humans with severe traumatic brain injury;[54] levels are dramatically raised, with the highest values observed in the first few days postinjury. TGF-β1 levels also rise in human brain tissue damaged by hypoxic/ischaemic insults,[55] suggesting a neuroprotective role as well as stimulating angiogenesis and scarring.

In vivo studies are now establishing a major role for TGF-βs in the repair of the injured brain. In addition to inducing fibrosis and reactive gliosis,[21] TGF-βs probably have other homeostatic functions. They may play an important role in CNS microvasculature angiogenesis,[56] which is needed for the neovascularisation of the scar tissue. The TGF-βs may probably also act as inflammatory mediators by recruiting, priming, and activating cells of the immune system, including microglia.[57,58] Nerve growth factor (NGF) synthesis is also induced by TGF-β1,[59] probably contributing to the reported neurotrophic activity of the cytokine, since NGF not only has a trophic effect on CNS neurons, but also stimulates astrocytes to become reactive.[53] The ability of TGF-βs to upregulate their own synthesis as well as that of other cytokines suggests a mechanism for the initiation and amplification of the trophic regulatory cascade that occurs in wounds.[60]

Since the foetal and neonatal CNS heals without scarring, one might predict that TGF-βs would be absent from such wounds. This is not the case. The intact immature CNS expresses all three isoforms of TGF-β at significant levels and a role for these factors in regulation of neuronal migration and differentiation as well as glial proliferation is suggested.[48-53] Furthermore, there is a rapid though transient increase in TGF-β1 expression within damaged perinatal CNS tissue (Logan, A. and Berry, M., unpublished observations). The question of why the immature CNS does not scar despite the presence of potent fibrogenic factors remains to be fully answered. However, it may relate to the developmental competence of target cells to respond appropriately to the cytokines and the presence or absence of other trophins. It is clear that individual growth factors do not act in isolation and that cellular responses are dependent on the context in which trophins are presented, and this is particularly true of the TGF-βs.

8.5 EXPERIMENTAL MODULATION OF TGF-β ACTIVITY IN CNS WOUNDS

We have shown that the increased levels of TGF-β1 and TGF-β2 within CNS wounds are important determinants of scar production. Intraventricular infusion of recombinant TGF-βs markedly enhances matrix deposition in penetrating CNS wounds.[21,22] Conversely, local immunoneutralisation of TGF-β1 and TGF-β2 activity via intraventricular injections of isoform or pan-specific antibodies into such CNS wounds inhibits mesodermal scarring[21] (Figure 8.7).

We have also achieved suppression of scarring in the CNS using decorin, a small dermatan sulphate proteoglycan, which is a more universal antagonist of the TGF-β

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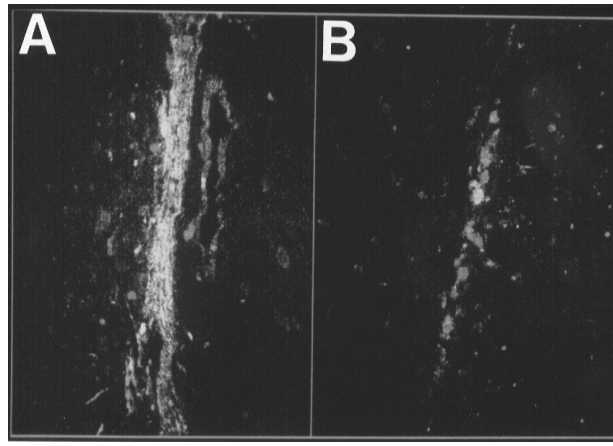

FIGURE 8.7 A/B Attenuation of scarring in TGF-β2 immunoneutralised wounds. At 14 days postlesion, specific fibronectin immunoreactivity is visualised in the core an incisional cerebral cortex wound of an untreated animal **(A)**. When neutralising TGF-β2 antibodies are administered to wounds paraventricularly, a significant reduction in matrix deposition in the wound is evident **(B)**.

family.[61] All three treatment strategies significantly attenuate the deposition of collagenous and matrix elements (including collagens I, III, and IV, fibronectins, tenascin, and chondroitin sulphate proteoglycan) of the scar, together with its astroglial endowment and organised basal lamina which normally reconstitute the glial limiting membrane (despite the presence of a normal reactive astrogliosis response). However, some isoform-specific differences in the cellular responses are revealed by the various treatments. For example, when TGF-β1-specific antibodies are used, the effects on macrophages and microglia are disparate. Macrophage invasion is reduced whilst the microglial response is significantly enhanced.[21] By contrast, if TGF-β2-specific antibodies, pan-specific antibodies, or decorin are applied, both macrophage and microglia responses are reduced.[21,61] Together, these observations suggest that these factors conspire to exert multiple effects in the injured tissue, leading to a diverse range of cellular effects which all culminate in scar formation. To this end, TGF-β1 and TGF-β2 may act (1) as chemoattractants for blood/meningeal-derived cells (macrophages and fibroblasts), (2) as activators of astrocytes and microglia, and (3) also as potent desmoplastic agents promoting matrix deposition by invading meningeal fibroblasts.

However, but perhaps not surprisingly, in such scar-inhibited wounds no evidence of enhancement of nerve regeneration is observed. Whether antagonism of any residual neurotrophic activity of TGF-βs contributes to the continued neuronal refractoriness in TGF-β-neutralised wounds remains to be established. Presumably, antifibrotic treatments must be used as adjuncts to concurrent neurotrophic pharmacological strategies in order to mobilise the inherent growth response of CNS neurons (see Chapter 1). A multiplicity of trophic and tropic (mechanochemical) factors may determine the final regenerative response of damaged CNS neurons. Neurotrophic

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factors, proteolytic enzymes, glial inhibitory factors, the mechanochemical substrata, and scar deposition are all cited as examples of elements which interact to determine the axonal response to injury; it may be that all aspects must be addressed by any neurotrophic strategy.

Since it is established that adult CNS neurons can regrow if provided with a compatible trophic and mechanochemical environment, then it would seem reasonable that a strategy of acute-phase multifactoral trophic and tropic manipulation of CNS wounds might establish a neuronally supportive environment in situations of severe trauma with massive necrosis and tissue damage. The first step in developing such a strategy to promote regeneration and reconnection in such severely damaged neural pathways must involve removal of a developing mechanochemical barrier as a glial/mesenchymal scar becomes organised. We suggest that this may be achieved pharmacologically in the acute phase of the injury response by antagonising TGF-β activity.

8.6 CONCLUSIONS

This article summarises the current knowledge concerning functions of TGF-β1 and TGF-β2 in the injured CNS. Many of their actions in this tissue are reminiscent of those in peripheral injuries. The known activities of TGF-βs suggest diverse roles in wounds, including modulation of certain aspects of inflammation, angiogenesis, glial scarring, and nerve regeneration. It seems clear that inhibition of TGF-β activity in CNS wounds might help provide an environment that facilitates successful nerve regeneration after trauma. A range of pan-specific and isoform-specific TGF-β antagonists are now available, including proteoglycans such as decorin and isoform-specific recombinant human neutralising antibodies. Some of these antagonists are currently undergoing clinical trials for application in a wide variety of peripheral tissue fibrotic diseases. In addition, the soluble receptor III (betaglycan) and engineered soluble subunits of the other TGF-β receptors may be used to bind soluble TGF-βs and render them inactive. The latency-associated peptide may prove particularly useful. Alternative antifibrotic strategies are to inhibit the activation of latent TGF-βs in CNS wounds when the precise mechanism of activation is elucidated, or to inhibit the trophic/signalling events downstream of the TGF-β receptor. Sugars such as mannose-6-phosphate might prove useful in this regard by competing with the LAP mannose-6-phosphate residues for binding mannose-6-phosphate receptors on target cells and thereby blocking TGF-β activation. Precise definition of the mechanism of action of TGF-βs in the injured CNS will herald the identification of much more specific and selective means of manipulating the CNS wounding response, and those TGF-β antagonists most appropriate to the CNS will become obvious. The potential for pharmacological treatment of fibrotic CNS disease by antagonising TGF-β activity is therefore explicit and will be the focus of much future attention.

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9 Neurotrophic Factors

Theo Hagg

CONTENTS

9.1 INTRODUCTION

Many types of central nervous system (CNS) neurons undergo degeneration in response to injury, and when severely damaged, undergo cell death. Degenerative changes include loss of transmitter-related function, retrograde degeneration of the lesioned axons, and atrophy of the cell body. Treatment strategies for CNS injury should ideally be aimed at preventing these degenerative events and rescuing cells

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from injury-induced death. However, patients often present with progressing injuries years after an acute injury has occurred and in such cases axonal contacts may have been interrupted and many neurons may be atrophic or have been lost. Therefore, development of treatment strategies for CNS injury should also be aimed at reversing degenerative changes, and should include (1) the promotion of neurite (axons and dendrites) regeneration, and (2) replacement of the lost populations of neurons and other cells.

This review will deal with the rapidly evolving field of neurotrophic factors and will focus on animal studies that have revealed the therapeutic potential of these proteins for CNS injury and chronic degenerative disorders. This review will discuss a selected number of factors to illustrate several concepts related to neurotrophic factor treatment strategies for CNS injury and will focus on the effects of neurotrophic factors on neurons. Although some neurotrophic factors also appear to have protective and recovery-inducing effects on oligodendrocytes whose degeneration is an important component of CNS injury, this topic is beyond the scope of the present review. Research findings during the past decade have led to the conclusion that neurotrophic factors are able to promote the several processes that are crucial for functional recovery, e.g., cell survival, axonal regeneration across bridging materials, regrowth into the original innervation territory, synaptogenesis, and transmitter-related functions (Figure 9.1). The challenge now is to discover how the different properties of various neurotrophic factors can be harnessed to affect selectively these individual repair processes and how they are to be applied to CNS trauma and neurodegenerative disorders.

9.2 NEUROTROPHIC FACTORS AND RECEPTORS

9.2.1 General Aspects of Neurotrophic Factors

Developing neurons become dependent for their survival on neurotrophic factors, typically ~10- to 30-kDa proteins which originate from their neural surroundings and/or innervation territory.[1,2] The prototypical nerve growth factor (NGF) was recognized for its ability to promote survival, neurite outgrowth, and expression of transmitter-synthesizing enzymes of developing peripheral neurons.[1] Neurotrophic factors, which typically promote the growth (cell size or neurite length) of neurons, have been distinguished from growth factors, i.e., factors that promote growth or proliferation of a cell population. This distinction is not always clear since many growth factors also have survival-promoting activity for neurons. Moreover, neurotrophic factors are not exclusively directed towards the nervous system since they have actions for nonneural cells. Nor are they exclusively "trophic", since they have other types of activities including the induction of proliferation of certain cells.[3,4] Neurotrophic factors generally have many types of actions on several types of neurons which may limit their ability to affect only injured neurons or certain repair processes.

In the CNS, the different neurotrophic factors are synthesized by neurons and glia and can reach the neurons via autocrine, paracrine, or telecrine release mechanisms.[5] The normal synthesis of neurotrophic factors in neurons appears to be

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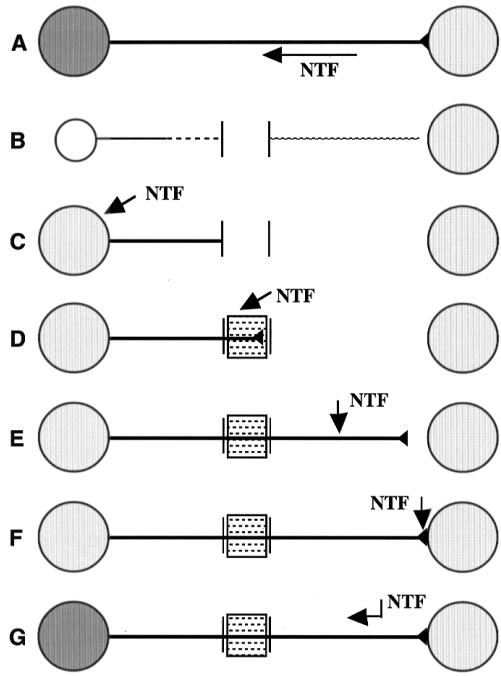

FIGURE 9.1 Potential sites for neurotrophic factor applications in the treatment of CNS injury. A: In the normal adult mammalian CNS, target-derived neurotrophic factors (NTF) are essential for the maintenance of normal neuronal function. B: After injury, neurons may lose the ability to synthesize transmitters and degenerate, in part because of the relative (increased requirement) or absolute deficiency in neurotrophic factors. C: Treatment with neurotrophic factors could enhance survival. D: Sprouting into synthetic or living bridging materials. E: Regeneration into the original innervation territory. F: Synaptic reconnection. G: Transmitter-related functions.

regulated, at least in part, by neuronal activity and transmitter release.[6,7] Other regulators likely include hormones such as thyroid hormone and glucocorticoids that can stimulate NGF synthesis in CNS neurons.[6] After traumatic CNS injury in adult rats, the synthesis of several neurotrophic factors such as NGF, ciliary neurotrophic factor (CNTF), and transforming growth factor-β is greatly increased.[8-11] Such a response to injury may be facilitated by cytokines such as interleukin-1 (IL-1) that are released through inflammatory mechanisms. For instance, after CNS trauma increased NGF synthesis by reactive astrocytes appears to be stimulated by release of IL-1 by microglia and immune cells.[11-13] However, the increase in neurotrophic factors is apparently not sufficient in the injured mammalian CNS to completely prevent neuronal death and promote regeneration (see Sections 9.3 and 9.4). The fact that neurotrophic factors are synthesized in the CNS raises the possibility that stimulation of their synthesis can be enhanced by small molecules,[14] and such agents might therefore be useful in the treatment of CNS injury.

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After binding to their receptors on neuronal processes, most neurotrophic factors are retrogradely transported to the cell body[15-17] although some have been shown to be anterogradely transported for axodendritic release.[18] It has not yet been resolved whether the neurotrophic factors act in the cell body after retrograde transport or whether receptor binding at the axonal terminal activates a second messenger that activates downstream signaling elements in the cell body.[19] Whether receptors that are found on the cell bodies can be activated by neurotrophic factors is difficult to establish *in vivo,* but is likely. Understanding where neurotrophic factors act has important implications for choosing the administration site of a neurotrophic factor treatment.

9.2.2 Neurotrophins

Neurotrophic factors with structural and functional homology to NGF have been found and together form the neurotrophin family that consists of NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3, -4/5, and -6 (NT-3, NT-4, NT-6).[20,21] Neurotrophins are homodimers of approximately 120 amino acid protein strands (~13 kDa), and any two neurotrophins are 50 to 60% identical. Disulfide bonds in the monomers provide a three-dimensional structure with several loops that are important for specific receptor binding. The neurotrophins activate high-affinity transmembrane tyrosine kinase (Trk) receptors that activate various intracellular signaling pathways.[21-24] NGF binds specifically to and activates TrkA, BDNF and NT-4 preferentially activate TrkB, and NT-3 primarily activates TrkC and in some cell types TrkB. TrkB and TrkC also exist in truncated forms without an intracellular domain that can bind the neurotrophins, but do not transduce intracellular signals. The function of such binding proteins is still unclear. Through targeted mutations of the amino acid composition of the neurotrophins and the determination of the crystal structure of NGF, various binding domains of the neurotrophins have been identified.[25] Such information may help in the rational design of neurotrophin mimics or their antagonists.

The low-affinity p75 NGF receptor ($p75^{NGFR}$)[21-24] is a product of a different gene family and is a member of the tumor necrosis factor receptor superfamily. In contrast to the above-mentioned Trk receptor specificities, $p75^{NGFR}$ can bind all the neurotrophins. The $p75^{NGFR}$ provides ligand-binding specificity for NGF to TrkA, can enhance Trk phosphorylation in the presence of ligand, but can decrease TrkA autophosphorylation in the absence of NGF. In the peripheral nervous system $p75^{NGFR}$ facilitates retrograde transport of selected neurotrophins.[26] The $p75^{NGFR}$ also has TrkA-independent signaling abilities through activation of sphingomyelin hydrolysis, which produces the lipid second messenger ceramide.[27,28] Ceramide can induce apoptosis[29,30] and recent *in vitro* and *in vivo* findings have revealed that $p75^{NGFR}$ can mediate neuronal apoptosis.[31-33] Interestingly, activation of $p75^{NGFR}$ by all neurotrophins can produce ceramide, but in Schwann cells (which lack Trks), NGF, but not BDNF or NT-3, also causes activation of the transcription factor NFkB.[34] This suggests that different ligands can activate different intracellular pathways through

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$p75^{NGFR}$. The selective activation by different ligands of different signaling pathways that may result in cell death or survival through the same receptor may constitute a powerful therapeutic strategy.

9.2.3 Ciliary Neurotrophic Factor

Based on its predicted structure and receptor complex, CNTF can be viewed as a member of the hematopoietic cytokine family, that includes leukemia-inhibitory factor (LIF) and interleukin-6 (IL-6).[35-39] CNTF has ~200 amino acids, migrates in western blots at ~22 kDa, and under physiological conditions acts as a single-stranded protein. In contrast to other hematopoietic cytokines, CNTF and its receptor are expressed almost exclusively in the nervous system. CNTF can affect various cell types, including neurons, oligodendrocytes, astrocytes, microglia, and muscle cells.[36,40-43] Synthesis of CNTF by astrocytes is increased after CNS injury[9] and astrocytes become reactive in response to CNTF,[42,43] suggesting a lesion-related autocrine function of CNTF.

CNTF binds specifically to the CNTF α receptor which lacks a transmembrane domain and cannot directly activate intracellular signaling pathways.[38,44] Upon binding CNTF, this receptor associates in a tripartite receptor complex with the transmembrane LIF-β and gp130 receptors, which autophosphorylate and transduce the CNTF-CNTF α receptor signal. LIF can directly activate the dimer of the LIF-β receptor and gp130, which is the basis for findings that CNTF and LIF have almost identical biological activities. Under physiological and pathophysiological conditions the CNTF α receptor is soluble and can associate with the LIF-β-gp130 receptor dimer of other cells.[45] Such soluble receptors might be utilized therapeutically to influence cells that are not normally responsive to CNTF (provided those cells contain the LIF-β and gp130 receptor).[46]

9.2.4 Glial Cell Line-Derived Neurotrophic Factor

Glial cell line-derived neurotrophic factor (GDNF) is a distant member of the transforming growth factor-β superfamily,[47] but apparently has a narrower range of target cells and activities. Because of its initially perceived relative specificity and its relatively high potency, GDNF has received much attention in the neurotrophic factor field. GDNF is a ~134-amino, ~15- to 20-kDa protein that acts as a homodimer. GDNF synthesis is not unique to the nervous system, in fact, its expression levels are much higher in many peripheral organs during development, and in lung, liver, and ovary during adulthood.[48-50] Whether GDNF only affects neurons in the CNS has not yet been resolved.

A specific receptor for GDNF has been identified.[51,52] GDNF binds to a non-transducing GDNF α receptor, which in turn associates with and activates the transmembrane transducing Ret receptor, perhaps as a tripartite receptor complex containing two Ret receptor monomers. GDNF receptors are expressed in CNS and peripheral nervous system (PNS) neurons and also in the liver and kidney.[51,52]

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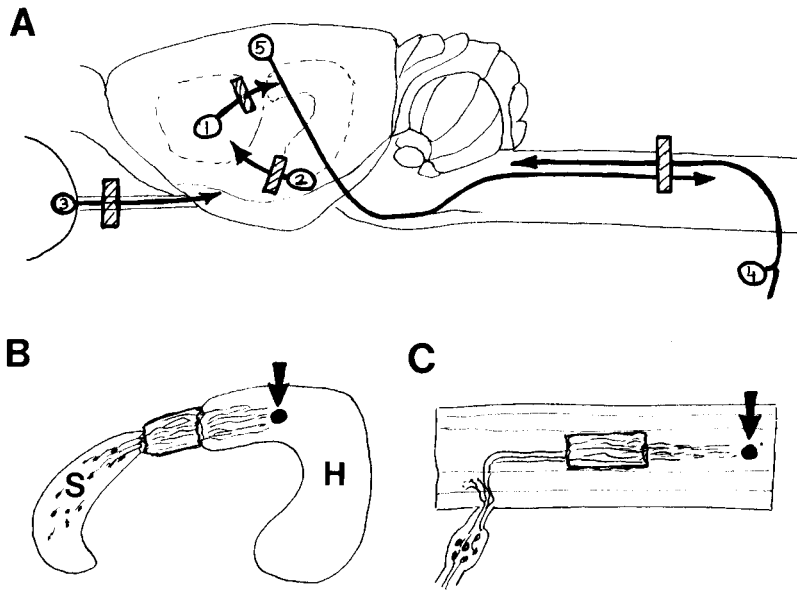

FIGURE 9.2 Axotomy models for investigating the therapeutic potential of neurotrophic factors for CNS injury. **A:** Different neurons can be axotomized by transection (hatched boxes) of their projections; (1) cholinergic medial septum neurons project through the fimbria-fornix to the hippocampal formation; (2) dopaminergic neurons of the substantia nigra project through the nigrostriatal pathway to the neostriatum; (3) retinal ganglion cells project through the optic nerve and central tracts to the optic tectum (not indicated here); (4) central processes of a portion of peripheral sensory neurons ascend through the dorsal funiculus to the cuneatus and gracilis nuclei; (5) corticospinal motor neurons descend through the dorsal corticospinal tract in the spinal cord to innervate lower motor neurons. **B:** NGF infusion (arrow) into the hippocampal formation can promote reentry of cholinergic septohippocampal axons that have grown across peripheral nerve bridges, S = septum, H = hippocampal formation. **C:** NGF infusion (arrow) into the dorsal funiculus of the spinal cord can promote reentry of ascending sensory axons that have grown across peripheral nerve bridges.

9.3 NEUROTROPHIC FACTORS AND NEURONAL SURVIVAL

9.3.1 Cholinergic Basal Forebrain Neurons

9.3.1.1 Neurotrophins

In the septohippocampal system, cholinergic neurons of the medial septum and vertical limb of the nucleus of the diagonal band of Broca project their axons predominantly to the ipsilateral dorsal hippocampal formation through the fimbria-fornix and supracallosal stria (Figure 9.2, A).[53] In the 1970s and 1980s, evidence from animal studies established that NGF has a physiological role for the cholinergic neurons of the basal forebrain. The septal cholinergic neurons have Trk and $p75^{NGFR}$

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receptors.[54-57] NGF is produced in the hippocampal formation and in the basal forebrain[58-61] and the cholinergic neurons retrogradely transport NGF.[15] Exogenous NGF causes an increase in levels of the acetylcholine-synthesizing enzyme choline acetyltransferase (ChAT) *in vivo*.[62,63] Evidence for an endogenous role of NGF was also provided by the finding that NGF-antibodies decrease levels of ChAT *in vivo*.[64,65] Nonetheless, the neurons appear not to be dependent for their survival on NGF or other neurotrophic factors from the hippocampus, because hippocampal removal leads to their atrophy but not to their death.[66] Subsequent axotomy of such cholinergic neurons (with a removed hippocampal formation) does induce their apparent loss, suggesting that other endogenous sources of neurotrophic factors (e.g., basal forebrain) cannot protect the cells against the more severe insult.

The extensive use of the septohippocampal axotomy model, at a time when NGF was the only neurotrophic factor available in sufficiently large quantities for experimentation in adult animals, has enabled the formulation of many fundamental concepts about neurotrophic factors. Advances in the understanding about degeneration and regeneration of the cholinergic basal forebrain neurons have been reviewed extensively elsewhere.[67] Over a 2-week period following transection of the fimbria-fornix, which interrupts the septohippocampal axons, ~70% of the axotomized cholinergic neurons disappear. Over this period, these neurons atrophy and lose their markers ChAT, TrkA, and $p75^{NGFR}$ by which they can be identified among other more numerous septohippocampal and septal neurons.[55,68-70] As is the case with other types of neurons, axotomy closer to the cell body causes greater apparent cell loss.[71] Whether this is due to an increased severity of the lesion or to decreased support by cells around the removed portions of the axons remains to be resolved.

In adult rats, intracerebral injection or chronic infusion of exogenous NGF close to the septal cholinergic neurons over the 2-week period after the fimbria-fornix transection can completely prevent the axotomy-induced degeneration of these cholinergic neurons (Figure 9.3, A and B).[55,69,72,73] NGF prevents the reduction in the expression of ChAT and of $p75^{NGFR}$. Moreover, the rescued neurons have a normal size and do not show the axotomy-induced degeneration of the proximal axonal stumps normally seen after axotomy.[74] In fact, NGF induces sprouting of the lesioned cholinergic axons (Section 9.4.1.1).[55,69,75,76] NGF also protects the cholinergic neurons of the nucleus basalis of Meynert from atrophy after removal of the cortex that they innervate.[77,78] Consistent with the exclusive expression in the basal forebrain of TrkA on cholinergic neurons, NGF has no effect on the GABAergic septohippocampal neurons which also disappear after axotomy.[69,79] Thus, in the basal forebrain NGF is a specific factor for cholinergic neurons.

To what extent the axotomized cholinergic neurons undergo cell death has not been completely resolved. Several authors have provided evidence that some death occurs between 2 and 4 weeks after axotomy,[80-82] whereas others have provided evidence to the contrary.[83] We have found that the cell body atrophy and ChAT and $p75^{NGFR}$ expression can be reversed within 3 to 7 days in up to 60% of the axotomized neurons by NGF infusions started up to 15 weeks after the lesion.[55] This suggests that many of these injured neurons may not die, but apparently become “dormant”, i.e., atrophic and dysfunctional. Most importantly, they remain amenable to NGF treatments — a finding that may have relevance for patients with CNS injuries that

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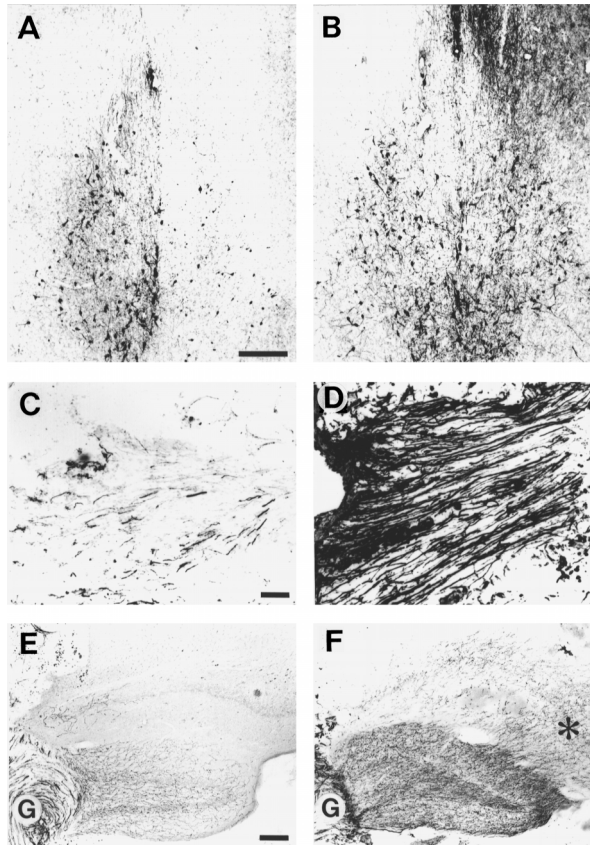

FIGURE 9.3 A-F NGF prevents degeneration and promotes regeneration of cholinergic basal forebrain neurons. **A:** After a unilateral transection of the fimbria-fornix in adult rats, axotomized cholinergic neurons in the ipsilateral medial septum (right side) lose their markers and degenerate over a 2-week period. Bar = 250 µm for A and B. **B:** NGF infusion into the lateral ventricle prevents this degeneration and induces sprouting of cholinergic axons toward the site of administration and even into regions previously not occupied by the cholinergic axons, such as the dorsolateral septum (right top). **C:** Peripheral nerve grafts devoid of cells and debris and consisting of basement membrane tubes support growth of only a few cholinergic septohippocampal axons even after one month of implantation. Bar = 100 µm for C and D. **D:** Such acellular nerve grafts, when soaked in NGF before implantation, promote ingrowth of cholinergic axons almost as well as living peripheral nerve grafts. **E:** Only a limited number of cholinergic axons reenter the hippocampal formation from cell-containing peripheral nerves placed as bridges between the septum and dorsal hippocampal formation for one month. Bar = 250 µm for E and F. **G:** Indicates the end of a peripheral nerve graft. **F:** Infusion of NGF into the hippocampal formation over the same time promotes ingrowth of cholinergic axons, leading to an almost normal fiber density in the dorsal hippocampal formation.

have occurred a long time before treatment would be applied. The axotomized cholinergic neurons appear to be dependent on support by exogenous NGF until they can reestablish contact with the hippocampal formation through regenerative

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events, i.e., NGF injections had to be given for 22 weeks before the neurons remained detectable after withdrawal of the treatment.[84] This finding is consistent with the idea that the expression of the markers by which these neurons are detected is dependent on synaptic contact, e.g., the induction of transmitter function-related proteins such as ChAT is dependent on stable synaptic contact.

NGF can diffuse readily from the lateral ventricles through the brain tissue to reach neurons in the medial septum or the neostriatum.[85,86] Although endogenous NGF is normally retrogradely transported from the hippocampal formation to the basal forebrain cell bodies,[15] it is still unclear whether the effect of the diffused exogenous NGF on lesioned cholinergic neurons is mediated through receptors on the axonal endings, the dendrites, or the cell body. This is an important issue when considering neurotrophic factor application in humans where distances from a potential administration site are much greater than in the rat. Encouragingly, in monkeys, where such distances are also greater, NGF infusion into the lateral ventricle is also very effective in protecting axotomized cholinergic basal forebrain neurons.[87,88] NGF can protect the cholinergic neurons when delivered through alternative methods, such as through transfected BHK cells or fibroblasts that overexpress NGF and that are implanted close to the septum.[89-92] Others have shown such protective effects by NGF released from implanted polymer rods.[93]

The other neurotrophins appear to have much less effect on the cholinergic basal forebrain neurons than NGF. When injected into the hippocampal formation, much less ^{125}I-labeled BDNF, NT-3 and NT-4 is retrogradely transported to the septal cell bodies than ^{125}I-NGF (Kromer, L. F., Van der Zee, C. E., and Hagg, T., unpublished).[15] After a fimbria-fornix transection, BDNF[79,94-96] but not NT-3[79] has been shown to protect septal cholinergic neurons. However, BDNF is less effective and at least ten times less potent than NGF.[97] When injected into the lateral ventricles BDNF does not diffuse well into the brain parenchyma, apparently because of its sequestration by truncated TrkB receptors in the ventricle lining.[85,86] When injected into the septum BDNF can diffuse much better, but still much less widespread than NGF, and is more effective in protecting the lesioned septal cholinergic neurons than after ventricular injection.[95] Thus, neurotrophins have different diffusion characteristics and degrees of effectiveness in protecting the cholinergic neurons, suggesting that neurons may be responsive to a variety of neurotrophic factors but are optimally responsive to a more limited number of factors. Like NGF, the other neurotrophins appear to be specific for the cholinergic neurons and do not rescue axotomized GABAergic septohippocampal neurons.[79]

9.3.1.2 CNTF and GDNF

CNTF was the second neurotrophic factor after NGF that became available in sufficiently large quantities for *in vivo* testing in adult animals. In the adult rat, infusions of CNTF for 2 weeks after a fimbria-fornix transection can completely prevent the axotomy-induced degeneration of the cholinergic basal forebrain neurons.[69] In contrast to the neurotrophins, CNTF rescued both the cholinergic and noncholinergic septohippocampal neurons including GABAergic ones. The cell body size of these protected cholinergic and other neurons was normal. Although CNTF

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TABLE 9.1
Neurotrophic Factors Have Different Effects on Axotomized Neurons in the Adult CNS

	Septal Cholinergic Neurons			Nigral Dopaminergic Neurons		
	Survival[a]	Outgrowth	Function[b]	Survival	Outgrowth	Function[b]
NGF	+++	+++	+++	0	0	0
BDNF	+/++	+	+	++	ND[c]	–
NT-4	ND	ND	ND	++/+++	ND[c]	–
NT-3	0	0	0	+	ND[c]	+++[d]
CNTF	++/+++	0	0	+++	0	0/–
GDNF	++	++	+/++	+++	+++	–[e]

Note: Listed are the relative potency of the neurotrophic factors, where: – = inhibitory; 0 = no effect; + = little; ++ = moderate; +++ = high; ND = not determined.

[a] As discussed in the text, a substantial portion of the cholinergic neurons do not die.
[b] Function as measured by levels of transmitter or transmitter enzymes.
[c] Neurons had normal morphology, including processes, suggesting protection of axons and dendrites.
[d] At high doses.
[e] With continuous infusion.

maintained the expression of $p75^{NGFR}$ in the lesioned cholinergic neurons, it did not prevent the axotomy-induced loss of ChAT.[69] CNTF infusion also induced expression of $p75^{NGFR}$ in the cholinergic interneurons of the neostriatum.[69] In unpublished studies, we have determined that CNTF does not induce sprouting or prevent retrograde degeneration of the proximal axons of axotomized septal cholinergic neurons. This lack of action in the CNS is in contrast to findings in the peripheral nervous system, where motor axons sprout in response to systemic injections of CNTF.[98] CNTF can also activate CNS microglia and induce their expression of $p75^{NGFR}$.[40] Since antigen presentation is one of the proposed functions of microglia, this finding suggests that CNTF has an immune-mediator role in the injured CNS. In addition, exogenous CNTF can cause astrocytes to become reactive,[42,43] suggesting another injury-related role for CNTF. Thus, compared to NGF, CNTF affects more types of neurons and cell types in the CNS, but appears to lack some of the biological activities of NGF (Table 9.1). Such differences between neurotrophic factors in their effects and specificity may constitute useful therapeutic tools.

Recently, GDNF was shown to protect the expression of ChAT- and $p75^{NGFR}$-staining in axotomized cholinergic septal neurons but was ten times less potent than NGF.[97] GDNF prevented the loss of $p75^{NGFR}$-staining but had less effect on the reduction in ChAT-staining of the cholinergic cell bodies. GDNF induced sprouting of the axotomized neurons into the lateral septum, a finding that may account for the increased level of GDNF-induced ChAT activity above normal despite the modest effect in maintaining the number of ChAT-positive cell bodies.

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The question can be asked whether factors such as CNTF and GDNF (and see BDNF, Section 9.3.2.1) would be less useful as therapeutic agents because of their presumed inhibitory effect on transmitter synthesis. However, injured neurons are known to "switch" from synthesizing function-related proteins, particularly neurotransmitter enzymes, to those involved in repair and regeneration,[99] thereby possibly conserving cellular energies. In fact, BDNF can induce regeneration-associated genes in other axotomized CNS neurons[100] and therapeutic induction of a "switch" by such factors could be beneficial for neuronal recovery.

9.3.2 Dopaminergic Substantia Nigra Neurons

9.3.2.1 Neurotrophins

The dopaminergic nigrostriatal system consists of dopaminergic neurons of the substantia nigra pars compacta that project their axons to the neostriatum (caudate-putamen) (Figure 9.2, A).[101] This is an excellent *in vivo* model and has the advantage that 90% of the neurons in the compacta region are dopaminergic, i.e., it is a highly homogeneous population. Since in the compacta region only the dopaminergic neurons project to the striatum, they can be readily identified through retrograde labeling. In the adult rat, these neurons express TrkB and TrkC receptors.[102,103] Low levels of BDNF and NT-3 are produced in the striatum[104-106] and retrogradely transported to the nigrostriatal neurons.[107,108] BDNF and NT-3 are also produced by a portion of the nigral neurons,[109] suggesting that these factors can also play an autocrine or paracrine role. In normal nigral neurons, BDNF increases DA turnover[110,111] and enhances fast electrical activation.[112] On the other hand, BDNF does not increase striatal dopamine or tyrosine hydroxylase (TH) levels.[110,113] In fact, others have shown that BDNF can induce a decrease in TH mRNA in the substantia nigra and cause a decrease in striatal dopamine levels.[114] The fact that BDNF reduces transmitter-related enzyme levels, as also discussed below, distinguishes it from the effects of NGF on cholinergic basal forebrain neurons.

After a nigrostriatal pathway transection in adult rats, axotomized nigrostriatal neurons die over a 2- to 4-week period and only ~20% of these neurons survive as confirmed by retrograde labeling with fluorescent markers.[115] In contrast, we have shown that a 2-week infusion of BDNF in adult rats close to the rostral pole of the nigral complex almost completely prevented the axotomy-induced death of the nigrostriatal neurons in the rostral half of the nigra (Hagg, T., unpublished observations).[116] BDNF was less effective in protecting neurons further away from the rostral infusion site, consistent with the poor diffusion characteristics of BDNF. In contrast, the effects of NT-3, which diffuses better, were similar throughout the nigral nucleus. The rescued neurons had an apparently normal size and morphology. Dose-response curves comparing all neurotrophins revealed that NT-4 was more potent than BDNF, which was more potent than NT-3, and as expected by the lack of TrkA receptors, NGF had no effect. Others had reported that injected BDNF was unable to prevent loss of axotomized nigral neurons,[94,114] but given the ED_{50} of BDNF (10 to 30 µg/d), the tissue concentrations achieved with the intermittent injection protocols in those studies were likely insufficient. This indicates that injections of neurotrophic factors,

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although perhaps more ideal in some clinical settings, are much less effective than continuous infusions. It also suggests that the neurons are dependent for their survival on higher tissue concentrations or continuous presence of the neurotrophic factors. Consistent with this notion, others have successfully employed an alternative mode of delivery by implanting BDNF-producing fibroblasts in adult rats to prevent MPP+-induced loss of substantia nigra neurons.[117,118]

After the nigrostriatal transection, the optical density of TH-immunostaining in the nigral neurons is reduced by ~40% per surviving neuron. BDNF and NT-4 did not prevent this axotomy-induced loss of TH (Hagg, T., unpublished observations).[116] In fact, infusion of BDNF close to the substantia nigra in normal nontransected animals resulted in a ~25% reduction of neuronal TH staining intensity. As mentioned above, BDNF causes a decrease in TH mRNA in the substantia nigra and a decrease in dopamine levels in the striatum.[114] This suggests that BDNF and NT-4 actively down-regulate TH expression, which results in a reduced synthesis of dopamine. NT-3, in sharp contrast to BDNF and NT-4, maintained TH-immunoreactivity in the axotomized nigrostriatal neurons, but was much less effective in preventing neuronal death. Thus, after neuronal injury, and potentially also in the normal CNS, some neurotrophic factors may be more effective in the regulation of survival and some in the regulation of function (Table 9.1). A better understanding and utilization of this difference may prove to be a powerful therapeutic strategy for various types or stages of neurological disorders. For instance, in some diseases where endogenous regulation mechanisms of transmitter functions might be intact, treatments may only have to facilitate neuronal survival. In other cases, regulation of enzyme levels and transmitter synthesis without affecting neuronal survival or axonal outgrowth may be more appropriate.

9.3.2.2 CNTF and GDNF

Low levels of CNTF α receptor mRNA has been detected in normal substantia nigra pars compacta neurons[119] but the protein is apparently not expressed in detectable levels.[120] CNTF can prevent the degeneration of axotomized substantia nigra neurons in adult rats.[115] The level of protection provided by CNTF was similar throughout the nigra, and was higher than with the neurotrophins, i.e., CNTF was more effective. The ED_{50} of CNTF was much lower compared to that of BDNF and comparable to that of NT-4. The cell bodies of these CNTF-rescued neurons had a normal size but appeared rounder than normal, perhaps reflecting the inability of CNTF to prevent axotomy-induced retrograde degeneration of the proximal processes (see Section 9.3.1.2). As was seen in the septum, CNTF did not prevent the axotomy-induced loss of the transmitter synthesizing enzyme (tyrosine hydroxylase).

GDNF was discovered to be a neurotrophic factor for cultured developing dopaminergic mesencephalic neurons.[47] GDNF can promote survival of several other types of neurons *in vitro* and *in vivo*, including injured lower motor neurons.[121,122] Nigrostriatal neurons can retrogradely transport GDNF from the striatum[123] and express the GDNF-specific alpha receptors.[52] Others have reported that GDNF injections into the adult nigra can increase dopamine turnover, induce sprouting of lesioned nigral axons in the striatum and prevent axotomy-,[124] 6-OHDA- or MPP^+-induced

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death of nigrostriatal neurons.[125-127] We have found that continuously infused GDNF is more potent (a much lower ED_{50}) and more effective (more surviving neurons) in promoting survival than BDNF. GDNF infusion also results in higher survival levels than previously reported in other studies, all of which used injection techniques (Lu, X. and Hagg, T., unpublished observations).[128] When started one week after the transection, GDNF can maintain the survival of those neurons that have not yet died. This may be relevant for chronic degenerative diseases where a diagnosis often is made at a time when neurons are undergoing degeneration and a remnant of neurons is still alive. However, infusion of GDNF did not prevent reductions of TH-immunostaining in lesioned nigral neurons and induced reductions of TH-staining in normal noninjured ones (Lu, X. and Hagg, T., unpublished observations).[128] Thus, when comparing the effects of neurotrophic factors in the nigrostriatal system, each of the factors appears to have a slightly different set of activities (Table 9.1). On the one hand CNTF predominantly promotes survival, GDNF promotes survival and neurite outgrowth, and NT-3 predominantly regulates expression of neurotransmitter-related enzyme. It is possible that these neurotrophic factors or their derivatives can be combined in a treatment cocktail to enhance some neuronal repair processes but not affect other processes that would lead to negative side effects.

9.3.3 Retinal Ganglion Cells

With regard to CNS trauma, the visual system is clinically more relevant than the central cholinergic and dopaminergic systems. The well-defined properties of the adult rat visual system, including the relatively homogeneous population of retinal ganglion cells (RGCs) projecting through the optic nerve to the optic tectum, have made it a convenient model for regeneration research (Figure 9.2, A).[129] Almost all adult rat retinal ganglion cells that project to the tectum, degenerate and die over the first 2 weeks after an intraorbital lesion of the optic nerve close to the eye. A more central axotomy further away from the eye does not result in such a rapid and extensive neuronal loss. A limited number of neurotrophic factors have been investigated for their ability to prevent this neuronal loss and their ability to promote regeneration. RGCs are immunoreactive for $p75^{NGFR}$ and express mRNA for $p75^{NGFR}$,[130] and have TrkB receptors.[131] Therefore, it is not surprising that BDNF and NT-4 can rescue injured RGCs when injected into the eye immediately after optic nerve injury in adult[132-134] or ablation of the tectum in neonate rats.[135] BDNF and NT-4 also rescue RGCs in cultured explants of adult rat retina.[136] CNTF can also prevent the loss of axotomized retinal ganglion cells *in vivo*.[132] On the other hand, single injections of NGF and NT-3 into the eye have no or very little survival-promoting effect.[134] In contrast, repeated injections of high doses of NGF can prevent ~20% of the otherwise occurring neuronal loss.[137] Thus, as is the case in the cholinergic or dopaminergic systems, continuous infusions are expected to be more effective and represent an approach that may reveal the responsiveness of retinal ganglion cells to yet other factors. As is the case for other neuronal systems, it remains to be tested whether the prevention of neuronal loss will translate into an enhanced regeneration. A first indication that this is not straightforward is provided

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by the finding that BDNF and NT-4 injections into the eye induce sprouting of the lesioned axons, but that these axons turn back at the optic nerve exit and do not enter otherwise regeneration-promoting peripheral nerve grafts.[138]

9.4 NEUROTROPHIC FACTORS AND AXONAL REGENERATION

9.4.1 Septohippocampal Pathway

9.4.1.1 Sprouting and Neurotropism

One of the earliest observations on NGF was the discovery of its effect in promoting neurite and axonal outgrowth.[1] NGF has tropic (directional guidance) activities for developing peripheral neurons, i.e., their neurites sprout and grow toward the source of NGF.[1,139,140] In the adult basal forebrain, NGF induces sprouting of lesioned cholinergic fibers toward the infusion site and can induce axons to grow into brain regions that they did not occupy before (Figure 9.3, B).[76,89,91,141] Compared to NGF, BDNF has very little enhancing effects on the sprouting of the cholinergic axons,[79,95-97] whereas GDNF has moderate effects.[97] In an attempt to combine the survival-promoting with the regeneration-promoting effects of NGF, we infused NGF into the lateral ventricle for 2 weeks in adult rats implanted with a septohippocampal peripheral nerve graft into which many of the cholinergic fibers would normally grow (see Section 9.4.1.2).[141] However, such fibers remained in the region of the NGF infusion (close to the lateral ventricle, septum, or fornix) and did not enter the septohippocampal grafts. Thus, NGF infusions close to the cell body seem incompetent to induce neurite outgrowth into an environment with lower levels of NGF such as the graft, in agreement with findings *in vitro*.[142] This indicates that neurite-promoting molecules like NGF have to be administered in the tissue region into which regenerating fibers have to grow. Others have shown that intraventricular NGF infusion during the first 2 weeks after implantation of a fetal hippocampal tissue graft, enhances the longer-term preservation of the cholinergic cell bodies and the regeneration of cholinergic fibers into the graft and hippocampal formation.[143,144] This suggests that an early and temporary protection of the cholinergic neurons allows more of them to participate in the regeneration process.

Of interest is our observation that exogenous NGF infusions close to the septal region did not induce obvious sprouting in the septum of nonlesioned cholinergic neurons.[141] On the other hand, endogenous NGF enhances collateral sprouting of nonlesioned septohippocampal cholinergic axons that occurs in denervated hippocampal regions vacated by removal of entorhinal cortex projections.[145] This apparent discrepancy may be due to the possibility that collateral sprouting of these axons only occurs close to the axonal terminals and not in the septum. We have also observed that lesioned cholinergic axons do not grow into nonlesioned septal terrain,[141] suggesting that nonlesioned tissue is nonpermissive for entry of regenerating axons. These concepts are of importance when considering treatment strategies for the injured CNS where most tissues and neuronal systems may be intact. Thus, it is possible that NGF treatments would not affect sprouting in nonlesioned tissues or of nonlesioned neurons.

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9.4.1.2 Bridges

Peripheral nerve and other types of grafts have been used to promote regeneration of the cholinergic septohippocampal neurons[146-148] in the knowledge that they can promote regeneration from a variety of CNS regions.[129] In general, septal cholinergic axons enter fetal or nerve grafts after 3 to 7 days, with a substantial number of axons reaching the end of such bridges between 2 and 4 weeks.[148] The success of these grafts to recruit regenerating axons may reflect the ability of the living graft cells to produce and release neurotrophic factors[149,150] as it is known that Schwann cells and fibroblasts in living peripheral nerve grafts produce increased amounts of NGF and BDNF.[151-153] Conversely, the failure of freeze-thawed nerves to do so may result from a lack of or reduction in viable cells.[154]

To investigate the potential trophic role of these cells we prepared "acellular" nerves[155] which were completely devoid of myelin, axons, and intact cells and consisted of longitudinally arranged basal lamina tubes essentially free of debris. Such preparations are rich in laminin and collagen that are known for their *in vitro* neurite-promoting activities.[156] These acellular grafts did not support cholinergic axonal regeneration. However, when soaked in purified NGF before implantation they became as supportive as normal cell-containing nerve grafts (Figure 9.3, C and D).[155] Thus, exogenous NGF can replace the putative endogenous neurotrophic factors of the graft, suggesting that neurotrophic factors can facilitate regeneration into mechanochemical scaffolds with appropriate substrates. Another interesting laminin-rich substrate for regeneration was revealed by the finding that cholinergic fibers grow into bridges of human amnion membrane.[157] Fibroblasts that are transfected to overproduce NGF constitute another very good cellular bridging material.[90] The possibility that cellular or artificial materials, when combined with neuron-selective neurotrophic factors could recruit and promote the regeneration of specific sets of CNS neurons, is a potentially powerful tool in the treatment of CNS trauma.

9.4.1.3 CNS Terrain and Reinnervation

CNS axons can grow very rapidly across essentially all suitable grafts, sometimes over distances which are longer than their original projections, indicating the growth potential of CNS neurons. However, entry and further elongation into the CNS, for example, the septal cholinergic neurons into the dorsal hippocampal formation, is much less "vigorous" and less rapid than in the grafts.[129,148] Limitations or restrictions on regeneration in the CNS may be caused by the presence of neurite-outgrowth inhibitors.[158-162] In fact, neutralization of the myelin-associated inhibitor NI35 leads to enhanced regeneration of cholinergic axons into the hippocampal formation of adult rats.[163] Regeneration in the CNS may also fail because of insufficient levels of growth-promoting substances such as NGF in the hippocampal terrain. We have shown that infusion with NGF for 1 month directly into the dorsal hippocampal formation 2 mm from an implanted nerve graft dramatically enhanced the number of cholinergic axons that had regenerated into the hippocampal tissue (Figures 9.2, B; 9.3, E and F).[164] This enhanced presence of fibers represented better penetration into the hippocampal tissue by the axons and did not result from recruitment

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of more cholinergic neurons or an increased availability of fibers at the nerve end. Others have reported similar promoting effects of NGF on cholinergic axonal regeneration into denervated CNS terrain of adult rats.[143,165-167] Thus, the naturally nonpermissive CNS environment can be modified by administration of neurotrophic factors. The relative selectivity of such factors for a limited number of axonal types in a given CNS region could be utilized to enhance regeneration of selected neuronal populations.

NGF infusions into the ventral hippocampus promoted axonal regeneration from the septum into the dorsal hippocampal formation, but did not induce sprouting of the nonlesioned cholinergic pathway that projects to the ventral hippocampal formation.[164] Thus, as was also mentioned in Section 9.4.1.1, it is possible that NGF or other neurotrophic factors only or predominantly promote regeneration of lesioned axons in the adult brain. If so, the side effects of such treatments may not include or have limited effect on aberrant collateral sprouting of noninjured axons. One important question, which has not been resolved, is whether or how the neurotropic and guidance effects of NGF can be harnessed or even circumvented to allow a normal pattern of reinnervation and the formation of appropriate synaptic reconnections.

9.4.2 Spinal Cord

9.4.2.1 Central Sensory Axons

Recent advances in research on spinal cord degeneration and regeneration have been extensively reviewed elsewhere.[168] The primary peripheral sensory neurons project one of their fibers peripherally through the nerve and one through the dorsal root to the cord, where they terminate and/or form the ascending sensory tract of the dorsal column system (Figure 9.2, A). This tract lies in the dorsal funiculus making it very accessible for experimental manipulations. After injury of either or both of their processes, adult sensory neurons do not die and their axons can regenerate very well in the peripheral nerve and the dorsal root and to a lesser extent into peripheral nerve and fetal grafts placed into the lesioned spinal cord. However, such adult regenerating fibers fail to reenter the cord more than ~1 mm, again emphasizing the nonpermissive nature of the CNS.[168-170] Of particular interest to sensory regeneration (as well as for other neurons) is the finding that regeneration of the central projecting axon into peripheral nerve grafts is greatly enhanced by or even dependent on a previous conditioning lesion of the peripheral sensory projections.[171-173] The mechanism of this so-called “conditioning lesion” apparently involves the inflammatory response and satellite cell proliferation in the ganglion[174-176] and could very well be initiated by cytokines released from the injured neurons.[177] The understanding of how this conditioning can be achieved in the CNS will be a major advance toward resolving the problem of CNS regeneration.

Adult rat sensory neurons express the three Trk receptors in partially overlapping but largely distinct subpopulations of neurons.[178-182] The sensory neurons can retrogradely transport neurotrophins.[15,26,183] NGF can affect a ~40 to 50% population of DRG neurons that express TrkA and contain substance-P and CGRP.[178] In the adult rat these neuropeptides are lost as a result of injury, but intrathecal infusion of NGF

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can counteract the injury-induced loss[184,185] and loss of NGF receptors.[179] Thus, under normal conditions these neurons probably utilize peripheral as well as spinal cord-derived NGF. Most of the large caliber ascending sensory tract axons originate from neurons that express TrkC,[181,182] suggesting that such fibers are responsive to NT-3.

Because adult sensory neurons respond to NGF with enhanced neurite outgrowth in culture[186] and regeneration of their peripheral projections *in vivo*,[187,188] the effect of NGF on sensory regeneration in the adult rat spinal cord has been investigated. Intrathecal infusion of NGF can enhance regeneration of sensory fibers from the spinal cord into nerve grafts.[189] NGF-coated nitrocellulose bridges can guide and promote ingrowth of reimplanted dorsal root fibers into fetal spinal cord transplants and into the host spinal cord tissue itself over very short distances.[190-192] Intraspinal grafts of NGF-producing[193,194] or NT-3-producing fibroblasts[194,195] cause a robust ingrowth of sensory axons into the grafts. The limited extent of ingrowth back into the spinal cord in such studies is most likely caused by insufficient levels of neurotrophin in the spinal cord and/or the tropic properties of NGF that may have impeded outgrowth away from the grafts. In the septohippocampal system, NGF treatment was required in the terrain into which the regenerating axons were to grow (Section 9.4.1.3). To test this local requirement for neurotrophic factors, adult rats with a conditioning lesion of the sciatic nerve received a nerve graft across a lesion gap through the thoracic dorsal column. NGF was infused for 2 weeks directly into the denervated dorsal funiculus 3 to 5 mm rostral to the graft-host interface (Figure 9.2, C).[196] NGF promoted the reentry into the dorsal funiculus white matter of approximately 50% of the fibers that had crossed the nerve bridge (>80% of the total number of "sciatic" fibers in the ascending sensory tract, anterogradely labeled). The number of fibers diminished with distance away from the graft (~20% at the infusion site) and fibers did not grow beyond the infusion site, probably due to the neurotropic effects of NGF. The percentages of fibers that had grown 2 to 3 mm into the spinal cord from the graft were greater than those we had observed in the septohippocampal model with cholinergic fibers. This is surprising because the cholinergic system has been known as among the most readily regenerating ones in the CNS. Since most of the ascending sensory axons apparently express TrkC, it remains to be resolved how NGF stimulates their regeneration. It is possible that the 50% population that had reentered the cord from the nerve grafts represents divergent sprouts of a smaller population of TrkA-expressing ascending fibers.

Our preliminary studies show that NT-3 and BDNF are equally potent in promoting the reentry of the sensory axons from peripheral nerve grafts back into the spinal cord white matter. Sensory axons from crushed dorsal roots in adult rats regenerate vigorously but cannot cross the root-cord transition into the cord.[170,196] Similar to the results seen with the nerve graft model, NGF infusion into the lumbar spinal cord promoted the reentry into the spinal cord of ~20% of the sensory axons in crushed L4 dorsal roots.[196] Thus, neurotrophins can modify the otherwise non-permissive CNS environment to facilitate regeneration. It is, however, clear that much more progress is needed when one considers the relatively low number of axons that had reached 3 mm into the cord and the total length of the spinal cord through which such fibers need to regenerate.

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Because of the possibility that problems of limited regeneration cannot be resolved we have taken another approach to the problem of spinal cord injury by investigating the application of neurotrophic factors in preventing axonal degeneration. One of the earliest events after spinal cord injury is the degeneration of the terminal portions of the proximal axonal stumps and the formation of terminal clubs.[197,198] Rupture of these swellings may contribute to the secondary damage that is characteristic of spinal cord injury. We have now established in a transection model that the neurotrophins can prevent the formation of these terminal clubs in the ascending sensory tracts.[199] Similarly, others have shown in the lesioned optic nerve of neonate rats that BDNF can prevent axonal dieback or degeneration.[200] Immediately after contusion of the spinal cord, which is the more common type of injury in humans, most spinal axons are still intact and degenerative processes ensue over the next hours to days. It is likely that after spinal cord contusion the neurotrophins can prevent the axonal disintegration and permanent dysfunction and potentially also the extent of secondary spinal damage. If so, neurotrophic factors will have gained yet another potential for therapeutic strategies for CNS trauma.

9.4.2.2 Corticospinal Motor Axons

Corticospinal upper motor neurons in the motor cortex project to the lower motor neurons in the brainstem and spinal cord and are critically involved in voluntary movement (Figure 9.2, A). When transected at the spinal cord or brainstem level, these neurons do not die but also do not or rarely regenerate into peripheral nerve grafts. Attempts at inducing their regeneration have until recently failed and this failure may be related to the lack of induction of proteins associated with regeneration.[100] It is not known if these neurons can be "conditioned", a requirement that was clearly shown for sensory axons to enter nerve grafts (Section 9.4.2.1). However, in a recent study where acidic fibroblast growth factor was used in combination with multiple nerve grafts, corticospinal axons crossed the grafts and grew back into the spinal gray matter.[201] The first clear-cut success in inducing corticospinal regeneration in mammals *in vivo* was achieved in neonate rats where neutralization of the neurite-outgrowth inhibitor NI35 resulted in the regrowth of a small proportion of corticospinal axons over very long distances.[202] Corticospinal motor neurons express TrkC receptors and NT-3 can rescue these neurons from death induced by axotomy close to the cell body.[100] Single injections of NT-3, but not BDNF, at the spinal cord lesion site in adult rats promotes the regenerative sprouting of the corticospinal fibers.[203] Moreover, lengthy regrowth of some corticospinal axons was achieved when NT-3 injections were combined with antibodies against myelin-associated inhibitor NI35.[203] It remains to be resolved whether chronic infusions of NT-3 (or BDNF) at the lesion site or more distal from it would promote regeneration of more axons over longer distances. It is likely that major advances will be made in promoting corticospinal regeneration if the successful approaches discussed above for other CNS systems and the ascending sensory tract are fully exploited for corticospinal systems.

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9.5 STRATEGIES FOR NEUROTROPHIC FACTOR DELIVERY

Neurotrophic factors are moderately large proteins which cannot cross the blood-brain barrier and systemic administration does not result in significant concentrations in the CNS. Another consideration of neurotrophic factor treatments is related to the size of the affected brain or spinal cord region because, ideally, an administered neurotrophic factor should only reach injured structures. There also exists a need for control of the neurotrophic factor dose, time-course of delivery, and the possibility of interrupting the treatment. Several strategies are currently being developed, each with their specific advantages and drawbacks. First, direct administration into the CNS allows for local effects and precise regulation of the timing of the treatment. However, this approach requires (multiple) surgical intervention and if a large area is affected multiple injection sites may be needed. Second, since neurotrophic factors cannot cross the blood-brain barrier therapeutic approaches could include the temporary interruption of this barrier, which enables a systemic delivery that results in a global treatment of all the regions of the CNS. The drawback is the interruption of the protective barrier and potential effects on nontraumatized CNS regions. A solution to this problem may be the recent development of methods involving carrier molecules such as transferrin which can deliver neurotrophic factors across the barrier without interrupting the barrier. A third delivery approach may be achieved by viral transfection of local CNS cells with neurotrophin genes for continuous and novel delivery by endogenous cells. This approach will ensure a desired chronic and local delivery but may have undesirable effects on the normal functions of the host cells and the difficulty in regulating neurotrophic factor doses and time-course of delivery. A fourth delivery strategy consists of the implantation of neurotrophin-producing cells such as transfected fibroblasts. These cells can have a very local and chronic effect, which may be an effective approach. On the other hand, such cells also produce other products perhaps not common to the CNS and the nature of these cells may constitute a risk of neoplastic transformation.

Given the fact that essentially all neurotrophic factors are produced in the CNS, efforts are underway to develop agents that can induce the local or global production of endogenous neurotrophic factors. If these agents are small they could be given systemically, and their doses readily regulated. Such agents obviously also have effects on nondiseased tissue, including nonnervous tissues where neurotrophic factors have a normal physiological role. A variation of this approach would be the use of inflammatory cytokines, or induction of inflammation itself, to induce increased neurotrophic factor synthesis and release. Yet another approach is the design of small agonist or antagonist molecules which mimic the binding sites of neurotrophins to their receptors. They have similar advantages and disadvantages as small molecules. As with all pharmacological agents the systemic side effects might be severe, as exemplified by the recent clinical trials with CNTF as a treatment for amyotrophic lateral sclerosis.[204] In the CNS, side effects of neurotrophic factors could potentially include the induction of neuronal[33] and oligodendrocyte[205] death, aberrant sprouting, and functional consequences for otherwise normal neural systems.

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9.6 CONCLUSIONS

This review has provided an overview of a selection of studies that have revealed the survival- and regeneration-promoting effects of neurotrophic factors. Such findings have created a great deal of excitement in the basic and clinical neuroscience community and some neurotrophic factors have been or are being tested in clinical trials for chronic degenerative disorders such as amyotrophic lateral sclerosis and peripheral neuropathies. Investigations into the effects of neurotrophic factors have revealed that they are very potent and can be used to promote neuronal survival, axonal outgrowth, and improved function in animals. The exciting possibility that these properties can be selectively utilized in treatment strategies has also been recognized. On the other hand, it is clear that the regeneration and recovery process is very complex and that this may limit the success of current possible restorative treatments in humans. Thus, a better understanding of the early events that follow CNS injury and the potential therapeutic and preventive role of neurotrophic factors should provide a new and possibly very successful approach to CNS injury. One excellent target for treatment after injury is the spinal cord, where neurotrophic factors may not only prevent axonal disintegration but may also play a role in the protection of oligodendrocytes and stimulation of remyelination. As with all novel pharmaceuticals, the challenge will be to understand the biology of these factors, develop more effective and specific derivatives of these potent neurotrophic factors, and solve problems of their delivery.

ACKNOWLEDGMENTS

The author's studies reviewed here were supported by grants from the International Research Institute for Paraplegia, United Parkinson Foundation U.S.A., Parkinson Foundation Canada, Canadian Network of Centres of Excellence, and a Scholarship from MRC Canada. I greatly appreciate the very helpful input on the manuscript by Dr. David Hopkins, Department of Anatomy and Neurobiology, Dalhousie University.

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10 Fibroblast Growth Factors

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CONTENTS

10.1 THE FIBROBLAST GROWTH FACTOR (FGF) FAMILY

Fibroblast growth factors (FGFs) are heparin-binding proteins that were initially characterized as mitogenic and angiogenic factors. FGFs were found to be involved in various cellular processes such as cell growth and differentiation, angiogenesis, cell maintenance, chemotaxis, and repair.[1] FGFs have become a focus of interest with regard to the treatment of central nervous system (CNS) injury and disease for several reasons: (1) FGFs exert multiple trophic actions on neuronal and glial cells both *in vitro* and *in vivo*; (2) FGFs are expressed in the intact nervous system; and (3) the expression of FGFs is altered during injury and disease. The purpose of this chapter is to summarize recent data within this scope and to discuss both the therapeutic value of the FGFs as well as strategies for their application.

The FGF family currently comprises ten members, including acidic FGF (aFGF, FGF-1) and basic FGF (bFGF, FGF-2), the best-studied members.[2-4] Analysis of the gene structures of FGF-1, FGF-2, and FGF-9 displayed the lack of a classical signal sequence which is considered as a prerequisite for processing via the ER-Golgi secretory pathway. For FGF-2 a species- and tissue-specific expression of isoforms has been reported (see below). Recent studies suggest that FGF-1, FGF-2, FGF-4, and FGF-8 are involved in embryonic developmental events like mesoderm induction, limb bud formation, and regeneration.[5-11]

With the exception of FGF-4, FGF-6, and FGF-10, all FGFs that have been characterized so far can be found in the CNS. Most of the data available for the

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CNS, however, emerged from studies with FGF-1, FGF-2, and FGF-5. Several groups have shown that FGFs are not only expressed in the CNS but also have multiple effects on developing, adult, and injured neuronal as well as glial cells.[12-14]

The extracellular FGF signal is known to be mediated by four different FGF receptors (Rs). These receptors are of the transmembrane tyrosine kinase type and occur as several isoforms, which are produced by alternative splicing.[15,16] Because of ten FGFs plus isoforms and four FGFR types with even more isoforms, the question arises as to which receptor variant is capable of binding to which ligand. It has been shown that the FGFRs are promiscous with regard to binding to different FGFs. Whereas FGFR1, for example, binds FGF-1 and FGF-2 with similar activity, FGFR3 displays a 20-fold tighter binding to FGF-1 than to FGF-2.[17] Very recently, it has been shown that the affinity of a FGF ligand is not only dependent on the FGFR type but also on its isoform. Ornitz et al.[18] reported on different binding capacities of FGFR1-3 isoforms for FGF-2.

In the developing and adult central nervous system FGFR1, 2, and 3 display a broad distribution in a distinct pattern.[19-29] High levels of FGFR1 are localized to astrocytes, low levels to oligodendrocytes.[30] Selected neuronal populations (e.g., amygdala and substantia nigra) also express FGFR1.[30] In addition, heparan sulfate proteoglycans, such as syndecan, seem to function as low-affinity FGF binding sites.[31,32] In neural tissue, the binding specificity of heparan sulfate proteoglycan for FGFs undergoes changes during development.[33,34]

Since FGF-2 and FGFR1 accumulate in the cell nucleus, an intracrine mechanism of growth factor action in the nervous system is discussed. FGFR1 was detected in the nucleus of astrocytes,[35] chromaffin cells,[36] and 3T3 fibroblasts.[37] This nuclear FGFR1 possesses kinase activity and undergoes autophosphorylation.[36] It is suggested that FGFR1 could translocate with the ligand to the nucleus and may play a role in regulating gene activity.[36,37]

10.2 DISTRIBUTION OF FGFs IN THE INTACT CNS

Most of the FGFs that have been characterized so far can be found in the CNS. With the exception of FGF-4, FGF-10 (not detectable), and FGF-6 (weakly detectable), all FGFs have been shown by RT-PCR or Northern blotting to be expressed in the developing and adult mouse brain.[4,38]

Although there is agreement that FGF-1 and FGF-2 are found in the CNS, controversy still exists with regard to the cellular distribution. FGF-1 and FGF-2 immunoreactivity has been localized to neurons and glial cells.[12-14] Depending on the certain primary antibody as well as on the tissue-processing procedure, FGF-2 was reported to be localized to the cytoplasm or to the nucleus. In a recent detailed study, Gonzalez et al.[30] reported the specific distribution of FGF-2 and its mRNA in neurons and glial cells of distinct brain areas. In accordance with other investigators,[28,39-46] Gonzalez et al.[30] found FGF-2-expressing neurons, for example, in the hippocampal CA2 region and in subpopulations of several motor and sensory nuclei of the brainstem.

Disparate findings concerning the subcellular distribution of FGF-2 may also be due to a differential binding of anti-FGF-2 antibodies to different FGF-2 isoforms.

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These isoforms originate from alternative translation initiation within a single mRNA species.[47,48] The 18-kDa FGF-2 initiates at an internal AUG codon, the higher molecular weight forms initiate at CUG codons located 5′ to the AUG. Whereas the 18-kDa form is predominantly found in the cytoplasm, the higher molecular weight forms are mainly localized to the nucleus.[49,50] The FGF-2 isoforms are known to display a tissue- and species-specific expression in the cytoplasm and nucleus.[50-61] A differential expression of FGF-2 isoforms has also been found in the brain of various species and during development.[30,51,52,54,58,62,63] Whereas the adult rat brain contains FGF-2 isoforms of 18, 21, and 23 kDa,[30] the embryonic and neonatal rat brain only shows expression of the 18- and 21-kDa isoforms. During postnatal development a 22/23 kDa appears, with maximal expression in the adult.[58] In the rat spinal cord, FGF-2 isoforms of 18, 21, and 23 kDa[64] and of 18, 22, and 24 kDa[65] were reported. Although there is no direct evidence so far, it may be speculated that differential expression of FGF-2 isoforms might be correlated with discrete functions. This suggestion is underscored by *in vitro* studies demonstrating that overexpression of the low molecular weight (18 kDa) or high molecular weight FGF-2 isoforms (21 to 24 kDa) induces differences in cell growth and cell phenotype.[66-70] Since the 18-kDa FGF-2 is the only isoform that has been tested *in vivo* and *in vitro* so far, it seems interesting to analyze the effects of the higher molecular weight forms on various neuronal parameters. Schwann cells overexpressing specific FGF-2 isoforms, show altered cell growth and morphology (Grothe, C. et al., unpublished observation).

Several recent studies have demonstrated the presence of a FGF-2 antisense transcript in addition to the sense transcript. FGF-2 antisense was found in the embryonic and adult rat, human, and *Xenopus* CNS.[71-76] Developmental analysis revealed that while the sense transcript level increased into adulthood, the antisense mRNA level decreased.[74,75] Regulation of the stability of the FGF-2 mRNA and, indirectly, of the FGF-2 protein level could be a physiological relevance of the antisense message. The presence of FGF-2 mRNA/antisense hybrids also offers the possibility to selectively destroy or modify the transcript.[77] In the rat brain a 28-kDa MutT-related protein which is translated from the antisense transcript was identified.[76] MutT gene products are 8-oxo-dGTPases which remove oxidatively damaged guanosine triphosphates from the intracellular pool (antimutator protein[78]). Since FGF-2 antisense mRNA was also found in nonneuronal tissues, its functional relevance is apparently not specifically related to neural functions.

High levels of FGF-3 mRNA in rhombomeres 4/5/6 of the embryonic mouse hindbrain[79] indicate that FGF-3 together with FGF-8[80] may be signalling molecules involved in normal hindbrain development. Northern blot analysis and *in situ* hybridization have shown that the FGF-5 transcript is mainly confined to neurons in limbic structures, and to the spinal cord of the adult mouse and rat CNS.[81,82] Mice with a null mutation of the FGF-5 gene showed normal CNS development.[83] FGF-7 mRNA seemed to be restrictedly expressed in the mouse brain at E14.5.[84] The FGF-8 transcript was found in the embryonic and adult rat brain in a distinct pattern.[85,86] Although FGF-9 was originally isolated from human glioma cells, *in situ* hybridization revealed a preferential neuronal expression in several regions of the rat brain.[87]

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Very recently, four new members of the FGF family referred to as FGF homologous factors (FHFs[88]) have been identified. All FHFs lack a classical signal sequence and contain clusters of basic residues that are known to act as nuclear translocation signals.[88] All FHFs are expressed in the developing and adult mouse nervous system.[88] The specific activities of these molecules are not yet known.

10.3 EXPRESSION OF FGFs IN THE INJURED AND DISEASED CNS

FGFs and FGFRs, as pointed out earlier, display a widespread distribution in the brain and spinal cord under normal conditions. One strategy for getting new insights into FGF biology and the future use of these molecules as therapeutics has been the study of FGF and FGFR expression under pathological conditions, such as experimental injury or disease.

There are many reports on the regulation of FGFs and their mRNAs in the injured spinal cord and in the pathologic brain. Experimentally induced demyelination of the spinal cord leads to an increase in FGF-1 which correlates with the beginning of remyelination.[89] Spinal cord injury was also found to increase the expression of FGF-1 and FGF-2 in a differential spatiotemporal pattern.[64,65] Elevated levels of the FGF-2 protein correlate with an increase of cellular (glial) and interstitial FGF-2 immunoreactivity.[65] FGF-1 immunoreactivity present in ventral motoneurons is amplified after destruction of the spinal cord.[64] These results imply that FGF-1 and FGF-2 subserve distinct roles following spinal cord lesion.

Focal brain wounds are accompanied by an increase of the FGF-2 immunoreactivity at the lesion site.[90] After entorhinal cortex lesion, the number and intensity of FGF-2 immunoreactive astrocytes in the outer molecular layer of the ipsilateral dentate gyrus is elevated.[43] Several studies demonstrated intensified immunoreactivity for FGF-2 and an increased FGF-2 transcript level following transient forebrain ischemia.[91-94] *In situ* hybridization revealed that the FGF-2 transcript is strongly induced in the granule cell layer of the dentate gyrus and in the pyramidal cell layer of the CA1 region within some hours after ischemia, and most likely is localized to neurons.[95] FGF-2 mRNA expression decreases in the pyramidal cell layer as neuronal death progresses.[95] The upregulation of the transcript is not followed by an upregulation of the FGF-2 immunoreactivity.[95] In addition to the neuronal induction of FGF-2, ischemia also causes a strong increase of FGF-2 in reactive astrocytes.[92,93,95] Furthermore, expression of FGF-1 in neurons and macrophages following cerebral infarction was found.[96] These results suggest that FGFs may be part of a coordinated repair process following ischemic injury.

Neuronal death and astrocytosis in the striatum are characteristic of Huntington's disease. In brains from Huntington's disease patients, FGF-1 immunoreactivity is strongly increased in astrocytes and the remaining neurons; FGF-2 immunoreactivity is only slightly enhanced in astrocytes.[97] Since the FGFR3 gene is located in the Huntington's disease region on chromosome 4,[98,99] FGF-FGFR3 interactions might be involved in Huntington's disease pathology.

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In Parkinson's disease, there is a preferential loss of dopaminergic neurons in the substantia nigra. Immunocytochemical studies of human Parkinsonian brains revealed a loss of FGF-2 in substantia nigra neurons.[100,101] Treatment with the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) as a mouse model for this disease induces lesions of dopaminergic neurons and elevates transcript levels of FGF-1 and FGF-2 in the striatum but not in the substantia nigra.[102] Furthermore, iodinated FGF-2 was shown to be anterogradely transported from the substantia nigra to the striatum.[103] FGF seems to be involved in other neurological disorders, too. Brains from Alzheimer patients, for example, display substantially enhanced specific FGF-2 staining of neurons and astrocytes.[104] In Alzheimer's disease, neurofibrillary tangles and neuritic plaques are formed in the cortex and hippocampus.[105] The neuritic plaques are reported to be dense accumulations of amyloid material derived from the amyloid precursor protein (APP), a membrane-associated glycoprotein.[106] Immunocytochemical studies revealed a frequent colocalization and focal concentration of FGF-1 and FGF-2 with APP within neuritic plaques,[104,107,108] suggesting a functional relationship of APP and FGFs. Since FGF-2 is associated with the neuritic plaques, locally available FGF-2 might be reduced, leading to neuronal degeneration.[109]

10.4 EFFECTS OF FGFs *IN VIVO* AND *IN VITRO*

During recent years several studies have shown that FGFs exert multiple trophic actions on cells of the nervous system. In this chapter, however, the focus will be exclusively on those effects of FGFs which might be relevant for the development of therapeutic strategies of nerve injury or disorder. The observation that FGFs display neurotrophic potential[12-14] and are mitogenic for neuronal and glial precursor cells has raised high hopes to rescue neurons from cell death after injury and during disease, or to replace them by differentiating neuronal progenitors, thereby restoring brain function.[110] Several studies have shown that FGF-2 together with epidermal growth factor (EGF) is a key molecule regulating the differentiation of neural precursors *in vitro*. FGF-2 stimulates both the proliferation and survival of embryonic cells from the striatum,[111] and in addition, increases proliferation in cultures of rat embryonic hemispheres, hippocampus, and spinal cord.[112-116] The majority of the generated cells display a neuronal phenotype. Comparison between the effects of EGF and FGF-2 on developing CNS precursors expanded by each factor revealed the ability of FGF-2 to stimulate proliferation of a multipotential precursor, giving rise to neurons and astrocytes as well as a committed glial precursor, whereas EGF only stimulates the glial precursor.[117] A recent report suggests that FGF-2 could also promote division of cortical glutaminergic precursor cells, but not of GABAergic precursors, which would allow independent regulation of these two major classes of cortical cells.[118] Division and delay of differentiation of dopamine precursor cells is also influenced by FGF-2.[119]

The olfactory bulb and the hippocampal dentate gyrus are the only structures that display generation of neurons well into adult life. It is, however, possible to isolate neural precursor cells from the adult brain using *in vitro* culture systems and

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the appropriate factors.[120-123] FGF-2 stimulates proliferation of precursor cells from the adult mouse brain.[124-126] The majority of the progeny of these cells were of an astrocytic phenotype,[121] whereas mitogenic action of FGF-2 on precursor cells from the adult hippocampus produced both neurons and astrocytes under the culture conditions used.[127]

Proliferation and differentiation of glial cells is also influenced by FGF-2. It is a mitogen for cultured oligodendrocytes[128] and modulates the platelet-derived, growth factor-driven pathway of oligodendrocyte development.[129]

It has also been shown that FGF-2 is able to rescue cells from cell death and stimulates transmitter metabolism of dopaminergic neurons,[130-133] cholinergic septal neurons,[134] and motoneurons.[135-140] Although it has been shown that FGF-2 can act directly on neurons[141] the neurotrophic effect of FGF-2 is indirect and is most likely mediated by glial cells.[131] Nevertheless, FGF-2 has been applied successfully in a variety of different lesion models. Whereas the first of these studies directly applied FGF (injection, gel foams), recent studies have used the transplantation of genetically modified cells for trophic factor supply. It was demonstrated that exogenously applied FGF-2 undergoes receptor-mediated retrograde transport in various CNS neurons.[138,142] Following fimbria-fornix transection, cholinergic septal neurons are rescued by FGF-2 which was injected or applied in a gel foam.[143-146] In combination with the ganglioside GM1, FGF-2 can improve spatial memory deficits after partial fimbria transection.[147]

Degeneration of the dopaminergic nigrostriatal system, such as the reduction of the striatal dopamine and tyrosine hydroxylase immunoreactivity induced by MPTP, can be opposed by exogenously administered FGF-2.[148-150] It was also reported that FGF-2, continuously infused into the lateral ventricle, can rescue hippocampal CA1 neurons from ischemic-induced death.[151] In a rat neonatal model of hypoxia ischemia, intraperitoneal administration of FGF-2 exerts dose-dependent neuroprotective effects.[152] Grafting of genetically engineered fibroblasts producing FGF-2 into the lesion cavity prevents death of entorhinal layer II glutamatergic neurons following lesion of the perforant pathway in rats.[153] After ablation of the somatosensory cortex, implantation of a gelfoam soaked with FGF-2 into the lesion cavity prolongs the lesion-induced increase of the *c-fos*-mRNA expression in nonneuronal cells of the cortex, suggesting that FGF-2 could enhance the contribution of *c-fos* to neuronal plasticity.[154]

Preganglionic sympathetic neurons of the intermediolateral column of the spinal cord (Th7-10) give rise to the peripheral splanchnic nerves that innervate the chromaffin cells of the adrenal medulla. Target ablation by adrenomedullectomy leads to a lesion-induced death of the preganglionic sympathetic neurons, which is prevented by the substitution of the ablated adrenal medulla with FGF-2.[155] Since iodinated FGF-2 injected into the adrenal medulla is specifically retrogradely transported by preganglionic sympathetic neurons,[29] and FGF-2 is synthesized in the adrenal medulla,[60] this rescue effect might be of physiological relevance.

In addition to FGF-2, two other members of the FGF family, FGF-1 and FGF-4, have been shown to display regenerative potential *in vivo*. FGF-1 was used to partially restore hind limb functions in adult paraplegic rats.[156] Transplantation of FGF-4-secreting Schwannoma cells as a bridge graft stimulated axonal regrowth in the mechanically lesioned adult rat nigrostriatal pathway.[157]

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10.5 FGFs AND THE TREATMENT OF CNS INJURIES

The data presented so far show that members of the FGF family are promising candidates for the treatment of nerve injury or disorder. Different ways of using FGFs as therapeutics can be suggested. With regard to the application mode, recent studies suggest to use genetically modified cells for FGF supply rather than to inject or infuse FGF directly.

An approach which might be convenient for a wide spectrum of nerve injuries is the so-called cell replacement or cell recruitment strategy. The recent demonstration of plasticity in neural progenitor cells when transplanted to different regions raises the hope that immature neurons generated from cultured stem cells could replace a variety of neuronal subtypes.[158-160] However, currently only a few studies have examined the fate and the effects of FGF-2- or EGF-expanded progenitor cells after transplantation in the CNS.[126] Another promising attempt with regard to the expansion of neuronal progenitor cells has been demonstrated using EGF. *In vivo* infusion of EGF into the adult mouse forebrain which contains both neuronal stem and progenitor cells, results in a dramatic increase in the proliferation and the total number of subependymal cells and induced their migration away from the lateral ventricle into adjacent parenchyma.[161]

Another possible way to take advantage of the neurotrophic capacities of FGF-2 is in the context of neural transplantation. The clinical use of fetal nigral and adrenal medullary grafts as an intracerebral source of dopamine in patients with Parkinson's disease is limited, at least in part, because of the poor survival of the donor tissue.[162,163] Enhancement of the graft survival can be achieved by co-grafting of fetal dopaminergic neurons with a FGF-2 source like, for example, genetically modified cells which produce FGF-2.[164] In addition, *in vitro* propagation of fetal precursor cells before transplantation leads to a long-term survival of the graft.[165] Alternative gene delivery techniques include the use of viral vectors and the *ex vivo* technology.[166] The *ex vivo* approach allows manipulation of the cells in culture for reimplantation after isolation from the patient.

FGF-2, the most promising candidate of the FGF family, occurs in different isoforms. However, all of the effects of FGF-2 reported so far have been achieved by application of the 18-kDa isoform. It is therefore not clear whether all isoforms display similar activities or whether they differentially regulate cell metabolism. A correlation between expression of a certain isoform and cell morphology has been demonstrated, for example, in cardiac myocytes.[69] In the peripheral nervous system, FGF-2 isoforms were found to be differentially regulated in spinal ganglia and at the lesion site after sciatic nerve injury.[167] This differential regulation might be evidence for an isoform-specific function. Analysis of the FGF-2 isoform effects may therefore lead to therapeutic tools with increased efficiency and specificity and, possibly, less side effects. The same is true for the recently identified FHFs, which are also present in the nervous system.

In addition to the neurotrophic activities of FGFs which could be exploited for treatment strategies of the injured CNS, the involvement of FGFs in tumor growth might be also relevant. FGF-2 seems to be a key molecule in glial tumorigenesis.[168]

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In vitro studies have shown that glioma cell growth is inhibited after application of FGF-2 antisense oligonucleotides.[168-171] Although, the therapeutic application of antisense oligonucleotides is still in an experimental stage,[172] the *in vivo* administration of FGF-2 antisense probes could be a promising strategy in CNS tumor treatment.[173]

In conclusion, treatment of neuronal precursor cells with FGF-2 and co-grafting of a FGF-2 source with specific embryonic neuronal subpopulations seem to be promising therapeutical strategies for the injured CNS. Analysis of the neurotrophic capacities of the higher molecular weight FGF-2 isoforms and of the FHFs could provide a more effective FGF molecule for injured central neurons.

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