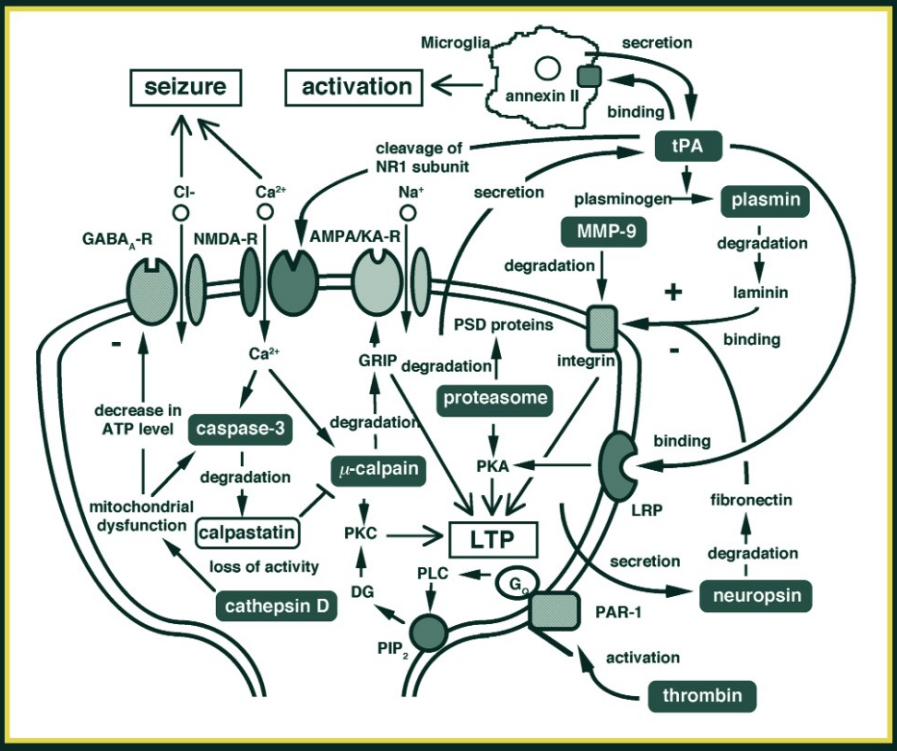
PROTEASES IN BIOLOGY AND DISEASE

PROTEASES IN THE BRAIN



Edited by

UWE LENDECKEL

AND

NIGEL M. HOOPER

PROTEASES IN THE BRAIN

PROTEASES IN BIOLOGY AND DISEASE

Volume 1
PROTEASES IN TISSUE REMODELLING OF LUNG AND HEART
Edited by Uwe Lendeckel and Nigel M. Hooper

Volume 2
AMINOPEPTIDASES IN BIOLOGY AND DISEASE
Edited by Nigel M. Hooper and Uwe Lendeckel

Volume 3
PROTEASES IN THE BRAIN
Edited by Uwe Lendeckel and Nigel M. Hooper

PROTEASES IN THE BRAIN

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Preface

This, the third volume in the *Proteases in Biology and Disease* series, focuses on Proteases in the Brain. In all organs of the body proteases have critical roles to play both in normal development and functioning and in disease states. The brain is no exception to this, with proteases having emerging roles in synaptic plasticity, memory, neurodegenerative disorders such as Alzheimer's, Parkinson's and prion diseases, ischemia and traumatic brain injury, inflammatory and infectious diseases, and tumour progression. This volume brings together a wide range of topics under this central theme and highlights the large number of proteases involved in these normal and disease processes.

The first chapter by Hans-Gert Bernstein reviews the current knowledge about the impact of proteolytic processes in the development and progression of Alzheimer's disease and whether proteases may be promising new therapeutic targets for the treatment of this fatal disease. In Chapter 2, Stephen Crocker, Patrice Smith and David Park review the current hypotheses about how dopamine neurons of the substantia nigra pars compacta degenerate in Parkinson's disease, focusing attention on the recent evidence supporting a central role for calcium-dependent calpains in this process. The prominent role of the cysteine proteases of the calpain and caspase families in the pathogenesis of brain ischemia are discussed in Chapter 3 by Swapan Ray. A description of the use of calpain and caspase inhibitors for neuroprotection in brain ischemia is also presented. Multiple proteases, including the calpains, caspases, cathepsins, serine proteases and matrix metalloproteases have all been implicated in the mechanisms underlying traumatic brain injury. In Chapter 4, Susan Knoblach and Alan Faden review the involvement of these proteases in traumatic brain injury and the potential that inhibition of them may have in its treatment. The next chapter by Bernd Kieseier and Fabian Bernal describes the role of caspases,

calpains, cathepsins, matrix metalloproteases and ADAM proteases in the pathogenesis of inflammatory and infectious diseases of the central nervous system, with particular focus on multiple sclerosis and bacterial meningitis. In Chapter 6, Stefan Brocke and colleagues describe how dipeptidyl peptidase IV regulates T cell function in CNS inflammation. Proteases are known to be critically involved in a number of steps in tumour progression, such as tumour growth, invasion, migration and metastasis. In Chapter 7, Sajana Lakka and Jasti Rao discuss the role and regulation of proteases, including cathepsins, plasminogen and matrix metalloproteases in human glioma.

The role of proteases in the metabolism of the prion protein is discussed in Chapter 8 by Antonieta Valenzuela and colleagues, while in Chapter 9, Mathias Hallberg, Pierre Le Grevès and Fred Nyberg cover the role of proteases in the processing, conversion and inactivation of neuropeptides. In the next chapter, John Wright and Joseph Harding describe the role of matrix metalloproteases and their inhibitors (TIMPs) in the degradation and preservation, respectively, of the extracellular matrix during neuronal plasticity. They also discuss the potential roles of calpains, tissue plasminogen activator and matrix metalloproteases in memory consolidation. The theme of proteases in neuronal plasticity is continued by Nobuko Mataga and Takao Hensch in Chapter 11, where the roles for the serine proteases plasminogen activators in development and plasticity in the normal mammalian brain is reviewed, with emphasis on the experience-dependent plasticity in the visual cortex. In Chapter 12, Hiroshi Nakanishi discusses the regulation of proteases in the context of modulating synaptic activity. The role of proteinase-activated receptors in brain function is the focus of Chapter 13 by Barry Festoff, with particular emphasis on thrombin and the protease-activated receptors (PARs) in nervous system function and dysfunction. In the final chapter, Corey Ford and Gary Rosenberg discuss the role of matrix metalloproteases in the neuroinflammatory damage evident in multiple sclerosis and the potential therapeutic strategies being explored to control their activities.

We hope that, like the first two volumes in the *Proteases in Biology and Diseases* series, this third volume will prove to be a timely and useful source of information both for those well versed in the role of proteases in the brain and for those who are beginning to realize the important role of this family of enzymes in brain function and dysfunction. Finally, we would like to thank all the authors for their scholarly and timely contributions and apologise to them for editorial changes in the interests of consistency and clarity.

N.M. Hooper and U. Lendeckel
July 2004

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Contents

Chapter 1 1
Proteases and Alzheimer Disease:
Present Knowledge and Emerging Concepts of Therapy
Hans-Cert Bernstein

Chapter 2 25
Calpain Proteolysis and the Etiology of Parkinson's Disease:
An Emerging Hypothesis
Stephen J. Crocker, Patrice D. Smith and David S. Park

Chapter 3 63
Cysteine Proteases in Neuronal Death in Brain Ischemia
Swapan K. Ray

Chapter 4 79
Proteases in Traumatic Brain Injury
Susan M. Knoblach and Alan I. Faden

Chapter 5 109
Proteases in CNS Infection and Inflammation
Bernd C. Kieseier and Fabian Bernal

Chapter 6 139
Dipeptidyl Peptidase IV Regulates T Cell Function in CNS Inflammation
Stefan Brocke, Andreas Steinbrecher, Aliza Biton, Maya Ratner, Vera Preller, Bettina Rotthauwe, Sabine Wrenger and Dirk Reinhold

Chapter 7 151
Role and Regulation of Proteases in Human Glioma
Sajani S. Lakka and Jasti S. Rao

Chapter 8 179
Proteases and Prion Diseases
M. Antonieta Valenzuela, Luis Cartier, Aron D. Mosnaim, Marion E. Wolf, Ana María Kettlun, Lucía Collados, Lorena García

Chapter 9 203
Neuropeptide Processing
Mathias Hallberg, Pierre Le Grevès and Fred Nyberg

Chapter 10 235
Proteases and Neuronal Plasticity
John W. Wright and Joseph W. Harding

Chapter 11 271
Serine Proteases and Neuronal Plasticity *in vivo*
Nobuko Mataga and Takao K. Hensch

Chapter 12 303
Proteases and Synaptic Activity
Hiroshi Nakanishi

Chapter 13 323
Role of Proteinase-Activated Receptors in Brain Function
Barry W. Festoff

Chapter 14 351
Matrixmetalloproteinases and Neuroinflammation in Multiple Sclerosis
Corey C. Ford and Gary W. Rosenberg

Index 373

Chapter 1

Proteases and Alzheimer's Disease: Present Knowledge and Emerging Concepts of Therapy

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1. INTRODUCTION

Nearly a century ago, Alois Alzheimer published a short note about the famous case of Auguste D., a 56 years old woman who had suffered from severe memory impairment and delusions (Alzheimer 1907). When morphologically analyzing her brain after death Alzheimer saw some of the characteristic structural alterations (compromised neurons, extracellular senile plaques, intraneuronal tangles), which now are commonly accepted as neuropathological hallmarks of the disease carrying his name. Alzheimer disease (AD) is currently the fourth leading cause of death and most common cause of dementia. Nosologically, AD is not a single disorder in spite of a common clinical phenotype. Etiologically, at least two different types exist. In a minority of 5% of the cases or even less; AD is due to mutations in certain genes, resulting in the permanent generation of Aβ fragments (see below). The majority of cases of AD are sporadic in origin, with old age as a main risk factor (Hoyer 2004). With age being an important risk factor for the development of AD, and with the population aging rapidly in developed countries, the number of persons suffering from the disease will dramatically grow during next decades. Suffering from AD clearly extends beyond the patient and immediate caregivers, challenging the society as a whole. Hence, many efforts have been directed towards revealing the cellular and molecular events behind the development of AD and to establish therapeutic strategies in order to cure the disease or, at least to decelerate its progression. Although the mechanisms leading to AD are

still far from being really understood, it became evident during the last years that proteases are prominently and in many ways involved in the pathobiology of the disease. The present article aims at reviewing current knowledge about the impact of proteolytic processes in the development and/or progression of AD and to ask, whether proteases may be part of an "avenue of hope" (Samuels and Grossman, 2003) taking us to promising new therapeutics of this treacherous, fatal disease.

2. PROTEASES AND NEUROPATHOLOGICAL HALLMARKS OF AD

AD is a progressive degenerative encephalopathy, which is clinically characterized by profound behavioural disorders, loss of memory and reasoning, and personality changes. Neuropathological hallmarks of AD are accelerated atrophy and loss of neurons from specific areas of the brain, reduction of synapses on surviving neurons, deposition of amyloid in neuritic plaques and within the walls of the cerebral microvasculature, and the increased appearance of neurofibrillary tangles (for overview, see Masliah and Terry, 1993; Selkoe, 1994; Bernstein *et al* 1996). Although some of these pathological changes may occasionally be observed in brains of aged patients without clinical signs of AD, there is no doubt that these hallmarks are highly indicative of AD, and that the degree of their expression correlates with the severity of the disease. A plethora of findings shows that protein-cleaving enzymes are active players in almost all of these processes, and one hardly can find a brain-associated protease which never has been suspected to be involved in AD. However, recent research has helped to draw a much clearer picture now, and I will try to relate specific proteases to specific AD-associated processes. Special emphasis will be given to the process of amyloidogenesis, which is regarded a core event in AD pathogenesis (Hardy and Selkoe, 2002).

2.1 Proteases and Amyloidogenesis in AD

A primary neuropathological hallmark of AD brains is the accumulation of β-amyloid peptide (Aβ) in amyloid (diffuse and later neuritic) plaques and, to a lesser extent, blood vessels. The neurotoxic Aβ peptides are derived by proteoloytic processing of the amyloid precursor protein (APP) by certain proteases called β- and γ- secretases, generating three forms of Aβ corresponding to Aβ (1-40), Aβ (1-42), and Aβ (1-43). A scheme showing APP cleavage by the secretases is shown in Fig. 1. The amyloid precursor protein (APP) gene is located on human chromosome 21 and

consists of 18 exons, which are alternatively spliced into several different transcripts (APP isoforms), named accordingly to their length in amino acids, APP_{695}, APP_{751}, and APP_{770} (Beyreuther and Masters, 1990). Two of them have an extra sequence homologous to the Kunitz-type trypsin inhibitor (Tanzi *et al* 1988, Selkoe, 1994). APPs are transmembrane type I proteins which are not only widely distributed within normal and AD brains, but also in non-neural tissues. Their physiological role in neural tissues is largely unknown but might be connected with nerve cell proliferation and survival as well as with synaptogenesis (for recent considerations, see Caille *et al* 2004).

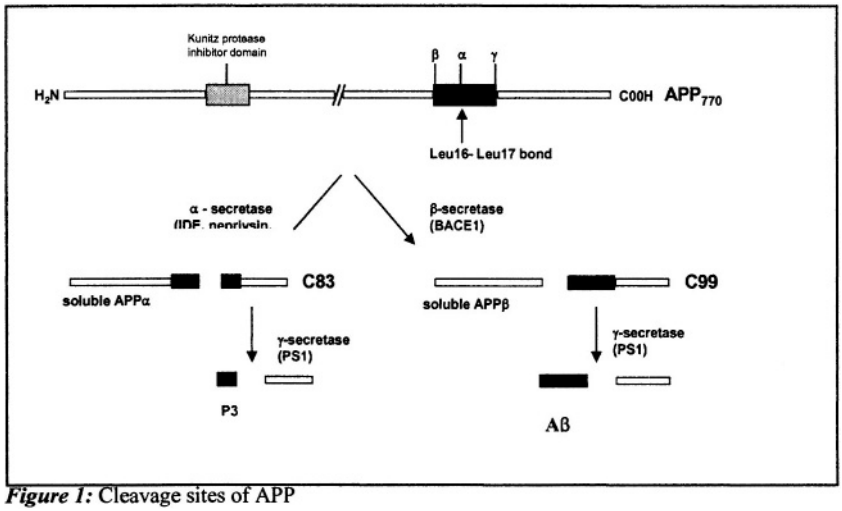

Figure 1: Cleavage sites of APP

2.1.1 Normal processing of APP by α-secretases: first cut is the deepest

In the normal brain, most APP is processed in a way that prevents the formation of potentially amyloidogenic fragments. This non-amyloidogenic secretory pathway is predominant over the amyloidogenic pathway and involves the obligate cleavage of APP by a so-called **α-secretase(s)** (Selkoe 1991; Asai *et al* 2003 and many others). By cleaving the APP molecule at the Leu16-Leu17 bond within the Aβ region of the APP molecule thereby producing a soluble α-APP fragment and an 83-residue COOH-terminal fragment, the formation of "dangerous" amyloidogenic fragments is precluded. Moreover, the large ectodomain released from the cell surface by the action of α-secretase has several neuroprotective properties (reviewed in Allinson *et al* 2003) Thus, the cut made by α-secretases determines whether or not amyloid formation may follow. During the past decade, several candidate

proteases have been identified, which are capable of acting as α-secretases. However, most data now favours one of the ADAMs (a Disintegrin and Metalloprotease) family of proteases, ADAM9 (EC 3.4.24.-), ADAM10 (Kuzbanian; EC 3.4.24.81) and ADAM17 (TNFα-converting enzyme, TACE; EC 3.4.24.86) as the physiological α-secretases.

ADAMs are membrane proteins containing a disintegrin and metalloprotease domain, which are widely distributed and play important roles in a plethora of cellular events (Wolfsberg *et al* 1995). At least 17 individual members of the ADAM family are expressed in the brain (Karkainen *et al* 2000). By the work of several groups ADAMs 9, 10, and 17 have been shown to be putative α-secretases (Koike *et al* 1999; Lammich *et al* 1999; Skovronsky *et al* 2000; Marcinkiewicz and Seidah, 2000; Lopez-Perez *et al* 2001; Hooper and Turner, 2002; Allinson *et al* 2003; Asai *et al* 2003). There is some recent evidence that ADAMs 9, 10, and 17 together might represent the "true" α-secretase (Asai *et al* 2003). Of these enzymes, ADAM 10 is currently the best studied one with regard to AD. ADAM 10 was found to be located in neurons and senile plaques of AD brains and brains of patients with Down syndrome (Fig. 2; Bernstein *et al* 2003). Together with ADAM 17, ADAM 10 is implicated in Notch signaling, which is disturbed in AD (reviewed in Hartmann *et al* 2001) and was shown to compete with β-secretase for cleavage of APP (Marcinkiewicz and Seidah, 2000). Alterations in ADAM 10 activity is a very early event in the progression of AD (Colciaghi *et al* 2004). Unfortunately, there are as yet no genetic studies linking mutations of ADAM enzymes with AD.

2.1.2 Processing of APP by β-secretase: opening the route to amyloid formation

Beta-secretase generates the NH_2-terminus of Aβ. By splitting APP it produces a soluble fragment of APP (β-APP_S) and a 99-residue COOH-terminal molecule (C99), which remains membrane-bound. Nowadays there is little doubt that the authentic β-secretase is a type 1 transmembrane protein belonging to the pepsin family, which is called **BACE-1** (for β-site APP-cleaving enzyme, EC 3.4.23.46) (Vassar 2001, 2004; Haass 2004; Hook and Reisine, 2003). It contains aspartyl protease activity. Its physiological function is yet poorly understood. There exists a close homologue to BACE-1, called **BACE-2,** which does not contribute to the amyloidogenic processing of APP (Haass 2004). Instead, BACE-2 seems to have α-secretase-like properties (Farzan *et al* 2000). Under normal conditions there is a competition between α-and β-secretases for the substrate APP (Asai *et al* 2003; Neve 2003). The gene coding for BACE-1 is located on chromosome 11, but no AD-causing mutation in this gene has been identified so far. However, polymorphisms in the BACE gene seem to

influence the risk for AD (Kirschling *et al* 2003). There is evidence that a high molecular complex variant of BACE has a higher β-secretase activity than the monomer (Marlow *et al* 2002).

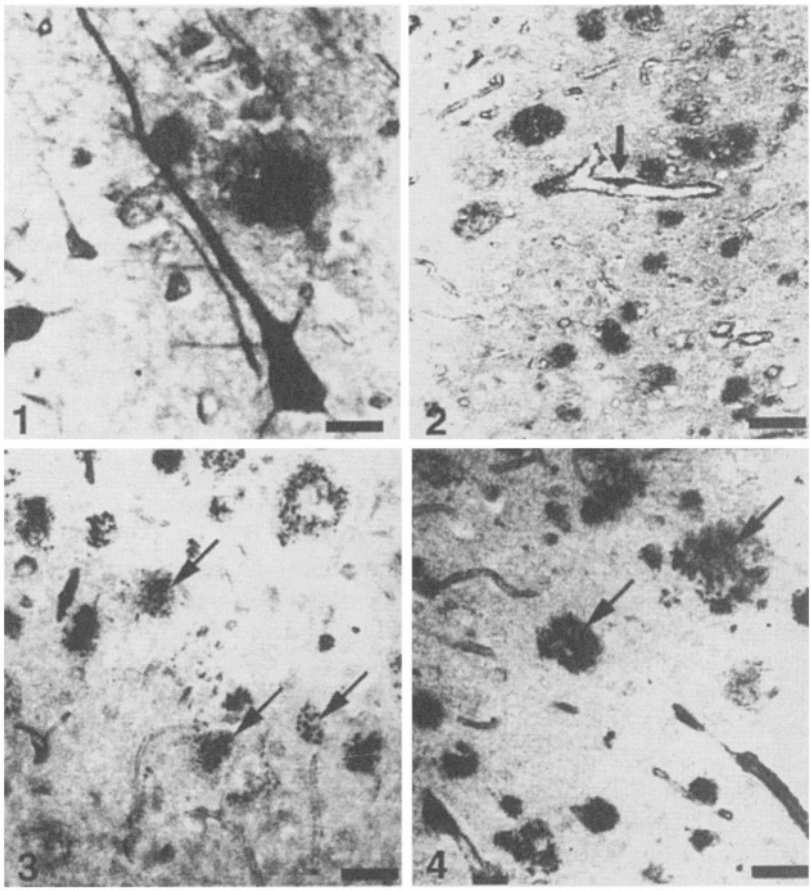

Figure 2: Immunohistochemical localization of proteases in AD brains. **1** and **2:** IDE-immunoreactivity in neurons and senile plaques in the neocortex of an AD patient (from Bernstein *et al* 1999 with kind permission of Elsevier). **3:** ADAM 10 immunoreactive material decorating multiple plaques in an AD brain. **4:** ADAM 10 immunoreactivity associated with plaques in an adult Downs patient (3 and 4 from Bernstein *et al* 2003 with kind permission of Kluwer Academic Publishers).

Interestingly, the BACE-2 homologue maps to chromosome 21, raising the possibility of an involvement of this protease in Down syndrome, but due to its minute brain concentrations and its α-secretase properties this possibility may be ruled out (Esler and Wolfe, 2001; Citron 2002). Beta-secretase mRNA is highly expressed in the brain as well as in other tissues. Intracellularly,

the enzyme is located in the Golgi apparatus, the endosomes, and at the cell membrane (for review see: Esler and Wolfe, 2001). BACE-1 appears to be co-localized with its cleavage product, β-APP_S and the putative α-secretase ADAM 10 in human cortical neurons (Marcinkiewicz and Seidah, 2000; Sennvik *et al* 2004). Strong support for the pivotal (and exclusive) role of BACE-1 as β-secretase comes from experiments with BACE-1-deficient mice: absolutely no Aβ is found in their brains (Cai *et al* 2001).

2.1.3 The γ-secretases: presenilins and beyond

Both the C83 (generated by α-secretase) and C99 (produced by β-secretase) APP fragments are substrates to γ-secretase, which performs a rather unusual proteolysis in the middle of the transmembrane domain to liberate the 4-kD Aβ from C99 and a 3-kD peptide (P3) from C83 (Esler and Wolfe, 2001; Haass 2004). Thus, only proteolysis of the C99 molecule yields amyloidogenic peptides. Hence, β-secretase initiated Aβ-generation is a prerequisite for "amyloidogenic" γ-secretase activity. A recently discovered ε-cleavage site within the APP intracellular domain does not influence the generation of Aβ by γ-secretase activity (Bergman *et al* 2003). The nature of the γ-secretase(s) has long been elusive. Now there is consensus that γ-secretase activity is largely due to the action of **presenilins** (EC 3.4.23.-) which are membrane-bound aspartyl proteases. There are two homologous presenilins, **PS1** and **PS2.** Clearly, PS1 and 2 have important biological functions in the brain other than to produce Aβ peptide (which was called a "rather unfortunate by-product" of PS action by Haass 2004). Among their natural substrates one can find cadherins, nectins and neuregulin receptors, erbBs 2 and 4 (reviewed in Bergman et *et al* 2003; Haass 2004).

Initially, presenilins were linked to familial (early-onset) AD (Sherrington *et al* 1995). Common to all known familial cases of AD are point mutations in the APP gene, which all seem to influence γ-secretase activity and bring about a shift in the ratio of γ-secretase generated Aβ fragments towards an increase of the highly amyloidogenic Aβ-42. This process is thought to significantly contribute to the aggressive progression of early-onset AD (Hardy and Selkoe, 2002; Haass 2004). Moreover, mutations in the presenilin molecule itself play a pivotal role in early-onset AD (Kim and Tanzi, 1997). Their role in sporadic AD is less clear. Good evidence for the dominant role of PS1 and PS2 in general amyloid formation comes from observations in PS1 knockout mice, which have drastically reduced Aβ generation (De Strooper *et al* 1998). Moreover, PS1/PS2 double knockout mice show no Aβ generation at all (Herreman *et al* 2000). It is now widely accepted that PS1 and PS2 alone do not explain γ-secretase activity. In search for additional players in the amyloid game new factors were identified: **nicastrin** (Yu *et al* 2000), **APH-1** (anterior pharynx-

defective phenotype) and **PEN-2** (PS-enhancer) (for recent considerations see De Strooper *et al* 2003). It is thought that these factors form a complex with the presenilins and are able to limit PS activity (Haass 2004).

2.1.4 Aβ degrading proteases

The following proteases have been shown, at least in vitro, to degrade the Aβ peptide:

- Insulin-degrading enzyme, IDE (insulinase, insulysin, insulin-glucagon protease; EC 3.4.24.11)
- Neprilysin (neutral endopeptidase, enkephalinase, acute lymphoblastic leukaemia antigen; EC 3.4.24.11)
- Plasmin(EC3.4.21.7)
- Thimet oligopeptidase (EC 3.4.24.15)
- Angiotensin-converting enzyme (ACE; EC 3.4.15.1)

Insulin-degrading enzyme (IDE) is an intracellular protein with important binding, regulatory and adaptive functions (Hamel *et al* 1998). Since IDE is capable of splitting insulin with high specificity the enzyme is regarded a reliable marker for insulin catabolism in various tissues including the brain (Dorn *et al* 1983, 1986; Reiser *et al* 1987). Besides insulin, IDE degrades other peptides, too (glucagon, β- and γ-endorphins and others, Safavi *et al* 1996). Work from several laboratories has demonstrated the Aβ degrading properties of the enzyme (Kurochkin and Goto 1994; Kurochkin 1998; McDermott and Gibson 1997; Qui *et al* 1998; Vekrellis *et al* 2000). Insulin-degrading enzyme has immunohistochemically been demonstrated to be present in normal and AD brains (Fig. 2). At the cellular level the enzyme was found in neurons and senile plaques (Bernstein *et al* 1999; Cook *et al* 2003). Although there are hints for an increased expression of IDE in neurons adjacent to senile plaques (Bernstein *et al* 1999), the overall Aβ-degrading capacity in AD brains is only about 50% of that of control brains (Perez *et al* 2000; Hoyer 2004). Moreover, mice lacking the IDE gene show a considerable accumulation of brain amyloid due to reduced degradation of Aβ (Craft and Watson, 2004), whereas transgenic animals overexpressing the enzyme have significantly reduced Aβ levels (Leissring *et al* 2004). On the other hand, rats carrying partial loss-of-function mutations of IDE show impaired degradation of Aβ (Farris *et al* 2004). Furthermore, IDE activity may depend on the intracellular metabolic state and the apolipoprotein-E genotype of the individual, which in itself may be a risk factor for AD (Cook *et al* 2003). In addition to that, genetic variants in the haplotype of IDE gene have been shown to be significantly associated with plasma levels of Aβ and risk for AD (Ertekin-Taner *et al* 2004). Interestingly enough, the main substrate of IDE, insulin, as well as brain

insulin receptors, have repeatedly been shown to be prominently involved in the pathophysiology of AD (Hoyer 1998, 2004; Frolich *et al* 1998; Craft and Watson, 2004 and many others). Hoyer (1998) was the first to come up with the idea that sporadic AD might be the brain type of non-insulin dependent diabetes mellitus.

And perhaps, brain insulin (either transported to brain tissue through the blood-brain barrier or locally synthesized) competes with Aβ for degradation by IDE. Consequently, permanent excess of one substrate, insulin (resulting from hyperinsulinaemia as observed in type II diabetes) would reduce degradation of the other, Aβ (Craft and Watson, 2004). Indeed, diabetes mellitus was found to double the risk for AD, with patients treated with insulin having the highest risk for dementia (Ott *et al* 1999).

Neprilysin is a zinc metalloendopeptidase which metabolizes substance P, somatostatin and other neuropeptides. Originally, the enzyme came into focus of AD research because of altered neuropeptide levels and degradation rates in AD brains (Weber *et al* 1992; Waters and David, 1995). Experimental work of Iwata and colleagues has clearly shown that neprilysin is an Aβ degrading enzyme, which significantly contributes to the metabolic regulation of brain Aβ (Iwata *et al* 2001; Carson and Turner, 2002). Neuropathologically, neprilysin is associated with neuritic plaques (Sato *et al* 1991). Moreover, there is evidence that formation of amyloid plaque is prevented by increased brain levels of neprilysin (Mohajeri *et al* 2002, 2004; Leissring *et al* 2004). A reduction of neprilysin levels has the opposite effect. Interestingly, region-specific reductions of enzyme levels seem to be part of the aging process of the brain – at least in the mouse (Iwata *et al* 2002). Finally, a recent genetic study showed that a newly discovered bi-allelic polymorphism of the neprilysin gene is associated with an increased risk for AD in an age-dependent manner (Clarimon *et al* 2003).

In some reports **plasmin**, **thimet oligopeptidase** and **ACE** have also been implicated in AD (for review see: Carson and Turner, 2002). Despite their property to cleave Aβ in vitro, comparatively little is known about the actual role of these enzymes in Aβ degradation in vivo. Here, further work is needed to better understand their impact.

Among the intrinsic factors modulating amyloidogenesis, the age-dependent activation of the endosomal-lysosomal system is the best studied one (Nixon 2001; Nakanishi 2003). During normal aging, and very prominently in AD brain, certain enzymes such as **calpains** (EC 3.4.22.-) and especially lysosomal cathepsins become dramatically upregulated in expression and activity. Years ago it has been shown that changes of the lysosomal cathepsins (cathepsin D and others) is an early and reliable cellular marker for AD (Bernstein *et al* 1989, 1990; Cataldo and Nixon, 1990). **Cathepsin D** (EC 3.4.23.5) has repeatedly been shown to contribute to the intracellular clearance of Aβ peptides and to be responsible for

degradation of Aβ which accumulates in neuronal lysosomes or is phagocytosed by microglia (for review see Nakanishi 2003). Thus, cathepsin D seems to play important roles as an enzyme that precludes amyloid formation by digestion of Aβ (Bendiske and Bahr, 2003). Curiously, cathepsin D possesses β-and γ-secretase activities as well, and its actual role in amyloid formation is far from being understood (for review see: Nixon 2002). However, a recent meta-analysis of the association of cathepsin D gene polymorphism with the risk of AD came to the conclusion that there is yet little support for the idea that cathepsin D is a major risk factor for the disease (Ntais *et al* 2004). It should be emphasized that the activation of the endosomal-lysosomal system does not only activate cathepsins, but may also lead to enhanced γ-secretase activity of the presenilin complex, as recent findings demonstrate the localization of nicastrin and PS at membranes of lysosomes (Pasternak *et al* 2004).

2.2 Proteases and Formation of Neurofibrillary Tangles in AD

Another core pathological feature of AD is the presence of intracellular neurofibrillary tangles (NFT), which are composed of microtubule-binding protein tau assembled into paired helical and straight filaments. It has been disputed for a long period of time, whether amyloid or tangle formation represents the primary pathological event in AD. Now there is some evidence that these pathological entities may be functionally linked (for review see Gamblin *et al* 2003). In AD brains, the tau present in NFTs is aberrantly (hyper)phosphorylated and often proteolytically truncated at the carboxy-terminus (Grundke-Iqbal *et al* 1986). Histological labeling of tau pathology has been introduced to trace and grade the progression of the disease (using the so-called Braak staging, Harding *et al* 2000). Truncation seems to be crucial for enhanced tau filament assembly in vitro and, possibly, under vivo conditions of AD (Abraha *et al* 2000). Recently it was found that **caspase-3** (EC 3.4.22.-; a member of the caspase family of cysteine proteases) is responsible for removal of amino acid residues from the C-terminus (Gamblin *et al* 2003). Interestingly, upregulation of caspase-3 and other members of the caspase family is observed in AD brains and plays a crucial role in Aβ-induced neuronal death (see below). Meanwhile, additional caspases (6, 7, 8 and 9) have been identified which also cleave tau in neurons undergoing Aβ-induced apoptosis (Gamblin *et al* 2003). The importance of this mechanism is underlined by the finding that tau-depleted neurons in knockout mice are resistant to Aβ-induced neurotoxicity (Rapoport *et al* 2002). Normally, tau protein is turned over by **calpains.** Hyperphosphorylated tau, however, is highly resistant against cleavage by calpain (Nixon 2002). The neuronal calpain system becomes activated early

in AD. Activated μ-calpain is detectable in neurites before any tau pathology is visible. At later stages of AD the enzyme is found in close association with tau-containing granules (the assumed precursor of neurofibrillary tangles in AD) as well as in neuropil threads with tangles (Grynspan *et al* 1997). Calpains are known to modulate phosphorylation and proteolysis of tau and might thus contribute to the formation of paired helical filaments in AD (Litersky and Johnson, 1995; Grynspan *et al* 1997). The described alterations in calpain activities might be the result of a profoundly disturbed neuronal calcium homeostasis, which is a key phenomenon of aging and AD (Braunewell *et al* 2001). Another typical manifestation of tauopathy-related events in AD is the accumulation of mutant ubiquitin as a morphological marker of proteasomal dysfunction (for review see Fischer *et al* 2003). However, the deposition of ubiquitin is not AD-specific. It can be also observed in other neurogenerative diseases (Fischer *et al* 2003). Interestingly, ubiquitin interacts with the Aβ degrading enzyme, IDE (Saric *et al* 2003), which might have consequences for the clearance of Aβ. In patients with sporadic AD or with Down syndrome dinucleotide deletions within the ubiquitin B gene can be found (Van Leeuwen *et al* 1998). The disturbed function of the **proteasome** leading to ubiquitin accumulation in AD may have fatal consequences for the neurons, since the proteasome is a major factor for protein degradation of the cell (Ding and Keller, 2003; Fischer *et al* 2003). It has been proposed that a chronic low-level inhibition of the proteasome complex is the functional basis for some of the observed neuropathological changes (Ding and Keller, 2003). Paired helical tau filament itself further contributes to proteasome inhibition in AD (Keck *et al* 2003). Finally, a certain contribution of cathepsins to tangle pathology cannot be ruled out, because cathepsins B and D are capable of splitting neurofilament proteins (reviewed in Bernstein *et al* 1996).

2.3 Proteases in Neuronal Loss and Synaptic Pathology of AD

Neuronal loss distinguishes AD from normal aging and correlates best with cognitive decline in AD individuals (LeBlanc *et al* 1999). The extent of nerve cell death in AD is high. So, in mild cases of AD, there is already a 50% loss of neurons in the entorhinal cortex, a region which is very early affected in the development of AD (Braak and Braak, 1996; Hyman and Gomez-Isla, 1996). The progressive nature of neuronal cell dysfunction and death in AD patients points to an apoptotic mechanism of neuronal cell death. Some researchers believe that the initiation of the apoptotic program in AD is prior to amyloid and tangle formation (Su *et al* 2002). Undoubtedly, both processes influence one another. Neuronal cell death leads to an increase of Aβ generation by activation of proteolytic enzymes.

Aβ in turn is neurotoxic and induces apoptosis (Dickson 2004). Thus, apoptosis-mediated increase in Aβ may be part of a cascade of events that further enhances neuronal cell death (for review see LeBlanc 1997). The main actors in apoptosis are the **caspases**. Caspases normally exist in the cytosolic fraction of the cell as inactive precursors that become activated by enzymatic cleavage during apoptosis. Several studies have shown that caspase-3 activation is both necessary and sufficient to trigger apoptosis (Mehmet 2000; Takuma *et al* 2004). Morphologically, elevated levels of activated caspase-3 immunoreactivity is found in neurons, astrocytes and blood vessels of AD patients (Su *et al* 2002). Moreover, these authors could show that the activation of apoptotic mechanisms takes place in selective compartments containing granules of granulovascuolar degeneration. These granules are rare in normal brain, but appear markedly increased in number in AD patients (Su *et al* 2002). They are suggested to arise from microautophagy and may be formed through lysosomal autophagy of intraneuronal substances (Okomato *et al* 1991). If this interpretation is correct, the caspase-mediated apoptotic pathway of neuronal (and glial?) (Takuma *et al* 2004) death would be linked to activation of the endosomal-lysosomal system (Nixon 2000; Nakanishi 2003), bringing the cathepsins into play again. Exciting new findings show that unsuccessful attempts to re-enter the cell cycle always precede nerve cell death in AD (Arendt 2003; Yang *et al* 2003; Reiser and Bernstein, 2004). A role of proteases in this process remains to be defined yet. Data from rat models of AD show that there is a calpain- and caspase-induced disruption of neurogenesis and perturbed neural progenitor cell homeostasis in these animals (Haughey *et al* 2002). Such pathomechanisms might play a role in humans, too. Another defining feature of AD is synaptic loss, whereby the hippocampus is the most affected brain region (for recent work see Honer 2003). As most other neuropathological hallmarks, synaptic injury is found very early in AD evolution. Loss of terminals is accompanied by the reduction of specific presynaptic proteins such as GAP-43, SNAP-25, synapsin (Honer 2003). Synaptic changes in AD are thought to mainly result from the neurotoxic attack of Aβ peptides (Lue *et al* 1996) or disturbed glutamate metabolism and/or transport (Masliah *et al* 1996; Lue *et al* 1999). There is evidence from aged mouse brain that neprilysin is presynaptically localized and protects the synapse from Aβ neurotoxicity by effectively clearing Aβ (Iwata *et al* 2004). It would be of interest to know, whether or not neprilysin is a component of all synapses. A similar synapse-specific role of the enzyme in normal and AD human brain has not been demonstrated yet. The immune processes which are implicated in destruction of cells and synapses in AD, will be regarded below.

3. PROTEASES AND INFLAMMATION IN AD

Neuroinflammation is a central feature in AD (McGeer and McGeer, 2001). The hypothesis that inflammatory processes might occur in AD brains emerged when activated microglia, expressing immunocompetent protein HLA-DR were detected in association with typical AD lesions (McGeer *et al* 1987). Although activation of microglia has long been considered as a secondary event following neuronal damage, there is increasing evidence for an independent role of microglia-related immune processes in AD (Nakanishi 2003). Various molecules acting as key mediators in peripheral immune reactions, are present at high concentrations in AD brains (Rogers *et al* 1988; McGeer and McGeer, 1995; Nakanishi 2003). The main result of neuroinflammation with regard to AD is a self attack by host defence mechanisms (autotoxicity), which finally leads to cell death. Proteases are believed to play significant roles in the inflammatory processes, since proteolytic mechanisms are involved in clearance of abnormal proteins, reformulation of extracellular matrix, facilitation of pathways for cell chemotaxis and other immune processes (for overview, see McGeer and McGeer, 2002). Amazingly little is known about the authentic proteases in neuroinflammation, however. Polymorphisms of two protease inhibitors, **α-2-macroglobulin** and **α-1-antichymotrypsin** seem to increase the risk for AD (Blacker *et al* 1998). Several observations suggest that activated microglia express and secrete lysosomal cysteine proteases, the **cathepsins S**, **L** and **B** (EC 3.4.22.27, EC 3.4.22.15 and EC 3.4.22.1; Banati *et al* 1993; Bernstein *et al* 1996; Petanceska *et al* 1996; Kingham and Pocock, 2001). Cathepsin S is cabable of degrading extracellular matrix proteins at neutral pH, which might play a certain role in neurodegeneration. Cathepsin B seems to be even more important in this context, because it has been identified to be the major causative factor of microglia-mediated neuronal apoptosis (Nakanishi 2003). The essential role of cathepsin B as a mediator of neuronal death induced by Aβ-activated microglial cells has recently been confirmed and extended by biochemical and functional genomics studies (Bohne 2004; Gan *et al* 2004). Nishioku and co-workers (2002) have demonstrated that that another cathepsin, the non-lysosomal aspartic protease **cathepsin E** (EC 3.4.23.37) is linked to the processing of exogenous antigens and MHC class molecules through the microglial endosomal-lysosomal system. This is of special interest, since cathepsin E immunoreactivity, which is fairly low in normal brain tissue (Nakanishi *et al* 1994), becomes sharply upregulated in AD brains (Bernstein and Wiederanders, 1994). In brains of AD patients this enzyme was seen in multiple microglial cells and "dying" neurons of Nuc. basalis of Meynert (Bernstein and Wiederanders, 1994). Additional support for a prominent impact of several cathepsins in microglia-mediated inflammatory processes

is lent by convincing findings in cathepsin B, L, and D single or double knockout mice (summarized in Nakanishi 2003). How far endogenous brain-associated inhibitors of cysteine proteases (cystatins A, B, and C) play a significant role in cathepsin-mediated inflammatory processes in AD remains to be established (Bernstein *et al* 1994; Nixon 2002; Nakanishi 2003). Thus, both lysosomal and non-lysosomal cathepsins appear to have central pathophysiological functions in neuroinflammation.

4. PROTEASE-BASED THERAPEUTIC CONCEPTS IN AD

Nobody will deny that the search for successful treatment strategies for AD is one of the most challenging tasks for contemporary medicine. Endeavours to cure AD certainly imply conceptual considerations, where proteases play an outstanding role. Unfortunately, most of the proteases which are implicated in AD pathobiology have also eminent functions in normal cell proteolysis. Hence, any effort to manipulate ("normalize") the expression and/or activity of these enzymes in AD will have considerable side effects. Thus, some of these manipulations might appear to be jumps out of the frying pan into the fire.

If assumed that AD represents the effects of a chronic imbalance between Aβ production and Aβ clearance, several therapeutic strategies may be proposed (Hardy and Selkoe 2002; Neve 2003; Ferrer *et al* 2004; Zlokovic 2004):

- Partial inhibition of either of the two Aβ-generating proteases, β-and γ-secretase(s)
- Partial enhancement of α-secretase(s) activity
- Prevention of the oligomerization of Aβ or enhancement of its clearance
- Depression of the Aβ-induced inflammatory response
- Anti-amyloid vaccination
- Modulation of the cholesterol homeostasis
- Chelation of Cu^{2+} and Zn^{2+} ions which contribute to Aβ deposition
- Prevention of synaptotoxic and neurodegenerative effects putatively triggered by Aβ accumulation
- Enhancement of clearance of amyloid through the blood-brain barrier

Of these approaches, the first two directly concern brain proteases. In the case of the β-secretase (BACE1) there is ongoing research for reliable small-molecule inhibitors that can fit into the large active site of this

aspartyl protease and still penetrate the blood-brain barrier (Citron 2002; Hardy and Selkoe, 2002; Vassar 2002, 2004). An important step towards reaching this goal was the development of the first BACE inhibitor which is based on the sequence around the β-secretase cleavage site of APP, where the Leu-Asp amide bond of EVNL/DAEF was replaced by a hydroxyethylene transition state analogue isostere (Roggo 2002). Another potent β-secretase inhibitor (hispidin) is a natural product. It was isolated from mycelial cultures of *Phellinus linteus.* Unfortunately, this inhibitor affects α-secretase activity as well (Park *et al* 2004). It has been suggested that an inhibitor against the BACE polymer would be even more effective in depressing intraneuronal β-secretase activity (Marlow *et al* 2002). In the case of γ-secretases, potent inhibitors already exist which work well in cellular model systems and mice. However, experience with AD patients is almost completely lacking. The first compounds applied in humans were described by Dovey *et al* (2000). Concerns with these inhibitors regard possible interference with the important Notch signaling system and other cell surface receptors (Selkoe 2001; Hardy and Selkoe 2002; Sisodia *et al* 2002; Lewis *et al* 2003). Inhibition of Notch processing seems to be responsible for some of the undesirable biological effects which occur after chronic treatment with γ-secretase inhibitors (for example, altered lymphopoiesis and intestinal cell differentiation, Wong *et al* 2004). Since the development of β-secretase inhibitors is not so easy, a promising way to reduce amyloid formation was the stimulation of α-secretases in order to facilitate the non-amyloidogenic APP pathway. Very recently a new signaling pathway was found in AD - sumoylation (Li *et al* 2003, Neve 2003). SUMOs (**s**mall **u**biquitin-like **mo**difiers) 1- and –2 are intraneuronal proteins which occur at higher concentrations in AD and Down brain. They are capable of mediating α-secretase, in preference to β-secretase-mediated cleavage of APP (Li *et al* 2003). ADAMs-related α-secretase activity may be upregulated by muscarinic agonists, steroid hormones, cholesterol-lowering drugs and certain metal ions (summarized by Hooper and Turner, 2002; Allinson *et al* 2003). Insulin-degrading enzyme's main substrate is insulin. Perhaps, attempts to metabolically lower "intracerebral hyperinsulinaemia" would help to increase the Aβ-degrading activity of IDE (for review see Hoyer 2004). Interestingly, light chains of certain anti-Aβ-antibodies were revealed that have Aβ-degrading activity. Application of these light chains might have an amyloid-clearing effect (Rangan *et al* 2003). Of note, partial blocking or activation of other putative APP-cleaving enzymes (cathepsins, calpain, caspases) might also have beneficial influence on the prevention of amyloid formation and, possibly, on other neuropathological signs of the disease as well. Cathepsins of the cysteine proteases type (i.e. B, H, L, and S) have endogenous inhibitors which are widely distributed in neural tissue, the cystatins. These cystatins become

strongly upregulated in AD and are associated with typical hallmarks of the disease (Bernstein *et al* 1994). It would possibly be ingenious to include such natural factors in the search for protease inhibition. Development of protease-based treatment of AD is still at the very beginning. Most of our current experience was collected in rodents, not in humans. So, the future will eventually see both groundbreaking successes and bitter defeats in curing AD by use of protease inhibitors and activators.

5. CONCLUSIONS

Proteases play an eminent role in the pathophysiology of Alzheimer's disease (AD). A core feature of AD is amyloid formation, which results from the aggregation of Aβ fragments (1-40, 1-42, 1-43) of the amyloid precursor protein, APP. Aβ is liberated from APP by cleavage by so-called β-and γ-secretases. Most likely, BACE1 and the presenilin-1 are the authentic β-and γ-secretases. APP is cleaved also by α-secretases, most probably members of the ADAMs family, to yield non-amyloidogenic fragments. Several proteases have been implicated in the degradation of Aβ, with IDE and neprilysin being the two key candidates. Several other proteases (calpains, caspases, cathepsins) are involved in other AD-related processes, such as tangle formation, neuronal cell death and neuroinflammation. Currently, many attempts are made to develop strategies to cure AD by applying brain protease inhibitors and activators. Unfortunately, most of these approaches have undesirable side-effects.

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Chapter 2

Calpain Proteolysis and the Etiology of Parkinson's Disease: An Emerging Hypothesis

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1. INTRODUCTION

In his 1817 monograph entitled "An Essay on the Shaking Palsy", Dr. James Parkinson provided the first description of a collection of clinical signs that distinguished what has come to be known as *Parkinson's Disease* (PD) from other forms of palsy or chorea. Although Parkinson speculated that the nature of this condition likely resided within the nervous system, little was known of brain function or neurodegenerative disease at this time and it wasn't until 1862 that French Neurologist Jean Martin Charcot determined upon postmortem analysis that there was a "softening" of the substantia nigra pars compacta (SNpc) in the brains of Parkinson's disease cases. Just over 50 years later, in 1919, Tretiakoff identified two hallmarks of this disease; degeneration of pigmented neurons and the presence of eosinophilic intracellular inclusions, called Lewy bodies, within the surviving pigmented neurons (Figure 1). However, at the time of these initial pathological findings little was known of the biochemistry of the brain and it wasn't until shortly after the identification of dopamine as a neurotransmitter by Montagu in 1957 (Montagu 1957), that PD was also identified as a condition of dopamine deficiency (Carlsson 1959). Subsequently, it was determined that nigral neurons innervate the striatum where dopamine modulates the control of movement and the loss of SNpc neurons in PD causes a depletion of dopamine levels in the striatum. It is estimated that once striatal dopamine levels are reduced by greater than

80% and SNpc neuron loss exceeds 60% that the clinical syndrome described by James Parkinson starts to emerge (Bernheimer *et al* 1973). From the first hundred years of clinical and neurological investigation, the foundations that now define the criteria for Parkinson's disease were established.

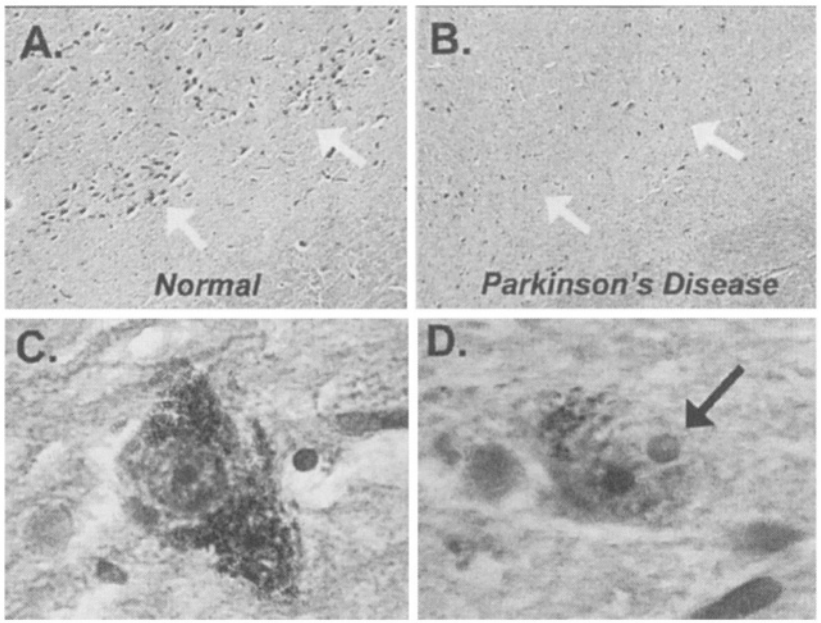

Figure 1: Parkinson's disease is associated with a selective loss of pigmented neurons in the Substantia Nigra pars compacta (SNpc). (A,B) Sections from the human brainstem stained with hematoxylin and eosin reveal the profound loss of pigmented neurons in the SNpc of an individual with Parkinson's disease when compared to an individual without documented neurological disease (Crocker *et al* 2003). At higher magnification, the characteristic dense and darkly pigmented neuromelanin (black substance in latin is substantia nigra) fills the cytoplasmic compartment of dopamine neurons in healthy SNpc neurons (C), whereas surviving nigral neurons from individuals with PD exhibit a less robust phenotype (D) that may also include the presence of Lewy Bodies (D, arrow).

Epidemiological evidence suggests that the incidence of Parkinson's disease ranges from 18-234/100,000 people over the age of 55, with the number of PD cases increasing with age. PD occurs throughout the world with a slightly greater incidence in North America and Europe than in Africa or Asia (Tanner and Goldman, 1996). Although the primary risk factor for developing PD is age, there are several instances where incidence may be increased by environmental factors of disease. The 1917-1928 pandemic of encephalitis lethargica *(sleeping sickness),* for example,

produced a cohort of patients that developed a post-encephalitic Parkinsonian condition (an event popularized by the book "Awakenings" by Dr. Oliver Sacks). In a broader population, however, there have been correlative studies that suggest strong associations with environmental or genetic factors. Within Canada, for instance, the prevalence of PD increases from East to West, where the western provinces there is a larger proportion of the population living in an agricultural environment (Svenson 1991). Additionally, several candidate genes for certain subtypes of PD [i.e. familial (inherited) or early-onset (juvenile PD)] have been identified (Polymeropoulos *et al* 1997; Kitada *et al* 1998), though present studies have not established a causal relationship between the presence of gene mutations and the prevalence of the sporadic (idiopathic) form of this disease. It is thought, however, that by elucidating how PD is caused in certain inherited forms of PD, for example, a generalizible hypothesis applicable to all forms of PD may be determined. The finding that genetic or environmental factors, either individually or collaboratively, are potential causes of PD has lead to the development of models for the neuroetiology of PD. We will discuss the topic of experimental models of PD in greater detail in below (Section 2.1: Models of PD).

The diagnosis of Parkinson's disease is initially based upon clinical presentation. The clinical signs of PD are categorized into primary and secondary symptoms. The cardinal signs of a resting tremor, muscular rigidity, a slowness (bradykinesia) or absence of movement (akinesia) and postural instability are considered primary symptoms because they represent the most readily identifiable signs of the disease. Secondary symptoms of PD can be subdivided into negative and positive signs where there is a loss of normal function and/or development of symptoms not observed in unaffected individuals. Examples of secondary symptoms in PD include cognitive impairment (dementia) and autonomic dysfunction (excessive sweating). Because the diverse collection of symptoms associated with PD, this disease can be difficult to accurately diagnose. Hence, a standardized protocol for the clinical evaluation of PD has been developed. Called the Unified Parkinson's Disease Rating Index (Fahn and Elton, 1987), this evaluation scheme surveys mental, motor and social criteria for the diagnosis of Parkinsonism and incorporates the Hoehn and Yahr Staging Scale to determine disease severity. When used in conjunction with brain imaging (cranial CT scan) a diagnosis of PD can be made, although without neuropathology diagnoses are often indeterminate (Lang and Lozano, 1998).

The marked reduction in dopamine associated with Parkinson's disease first described by Carlsson and colleagues lead to several notions about the changes in brain chemistry in PD. It was conceived and then demonstrated that administration of L-DOPA, the metabolic precursor in the synthesis of dopamine, could provide an efficacious method for dopamine replacement

in PD. However, disability in PD was also proposed to stem from an imbalance between levels of other neurotransmitters, such as acetylcholine, and the very low levels of dopamine in PD (Spehlmann and Stahl, 1976), under the assumption that restoring the balance between dopamine and acetylcholine (albeit at dramatically reduced concentrations of each) would be beneficial for PD patients. Hence, anticholinergics are sometime prescribed for treatment in early PD. Indeed, in early PD the ability of the brain to function in spite of profound decreases in striatal dopamine (i.e. compensation) is not attributable to compensation by surviving dopamine neurons (Bezard *et al* 2003), indicating that systems outside the basal ganglia may contribute to functional disability in PD (Bezard *et al* 2001). Nevertheless, L-DOPA remains the mainstay of current pharamacotherapy for PD. L-DOPA it not without its limitations as it does not abate the continued degeneration SNpc neurons or accompanying secondary changes in the PD brain that can stem from the ongoing disease process - therein mitigating its long-term utility. Since it is known that by the time a patient clinically presents with PD they have already lost greater than 80% of their nigral dopamine neurons (Riederer and Wuketich, 1976; McGeer *et al* 1977), current research efforts are focusing upon neuroprotective and/or neurorestorative strategies. With studies also indicating that the loss of SNpc neurons begins decades prior to obvious clinical disability, advances in technologies for the early and timely diagnosis PD may help to realize the potential for neuroprotection as the next generation treatment for PD.

In this chapter we will review current hypotheses about how dopamine neurons of the SNpc degenerate in PD and focus our attention on the recent evidence supporting a central role for calcium-dependent proteases, calpains. Accordingly, we shall also discuss evidence to support alternate modes of preventing motor deficits in PD through protection of the SNpc and restoring motor control by functional reorganization of the basal ganglia.

2. THE ETIOLOGY OF PARKINSON'S DISEASE

2.1 Heterogeneity of PD and Models for Its Study

Because little is known of the direct cause(s) of PD, aside from those instances where specific events can be determined (e.g. *encephalitis lethargica,* or genetic determinants of familial PD), an increasing number of models for the study of dopamine neuron loss have been discovered and developed. From this discussion it will become apparent that SNpc neurons can be induced to degenerate by an increasing variety of neurotoxins and forms of injury. This observation may also depict the nature of this disease,

since there is the distinct possibility that PD too may be induced by a variety of possible modes. Hence, what we currently know as PD may in fact represent a broad classification for an amalgam of individual conditions of heterogeneous origins. In this regard, select cases of PD are genetically linked *(familial PD)* to autosomal dominant genes and others to recessive alleles (discussed below in section 2.1.7), while the vast majority of cases worldwide, however, do not (yet) have any definable gene linkage or cause *(sporadic PD).* In the following sections we will briefly describe and outline models currently used for the study of PD which may reflect this heterogeneity.

2.1.1 6-hydroxydopamine (6-OHDA)

6-hydroxydopamine (6-OHDA) is a neurotoxic derivative of dopamine formed by the autooxidation of dopamine in the presence of reactive oxygen species (ROS). The discovery that intranigral injection of 6-OHDA could be injected into the SNpc and induce the selective destruction of nigral dopamine neurons in rats became the first animal model of PD (Ungerstedt, 1968). Unilateral injection of 6-OHDA into the nigra produces a hemi-parkinsonian condition that can be used to evaluate the efficacy of dopamine mimetics by measuring stimulated circling behaviour. Biochemically, 6-OHDA is recognized by the dopamine transporter where it is taken-up by dopamine neurons and interferes with complex I of the mitochondrial respiration chain resulting in potentiation of ROS formation by nonenzymatic oxidation (Heikkila and Cohen, 1971; Glinka and Youdim, 1995; Glinka *et al* 1997). A modification of the Ungerstedt model was more recently developed by Sauer and Oertel where direct injection of 6-OHDA into the striatum produces a more delayed and progressive loss of nigral neurons (Sauer and Oertel, 1994). The advantage of this intrastriatal 6-OHDA model is that the loss of neurons occurs over the course of weeks rather than days as with intranigral delivery. The protracted time course of neuronal loss affords investigators the opportunity to study gene expression and strategies to attenuate dopamine neuron loss and motor function (Crocker *et al* 2001b). Moreover, 6-OHDA may have unique relevance to the human PD condition since it has been suggested that 6-OHDA can be endogenously produced during dopamine metabolism in the SNpc that is facilitated by the high iron content of these neurons (Jellinger *et al* 1995). Hence, the experimental actions of 6-OHDA injected into the brains of rodents may reflect an unfortunate autotoxicity inherent to the dopamine neurons of the substantia nigra. Indeed, it is known that there is a approximately 0.7% loss of SNpc neurons per annum (McGeer *et al* 1988), thus predicting that at an age exceeding current life expectancies (i.e. very late into senescence) everyone would develop PD.

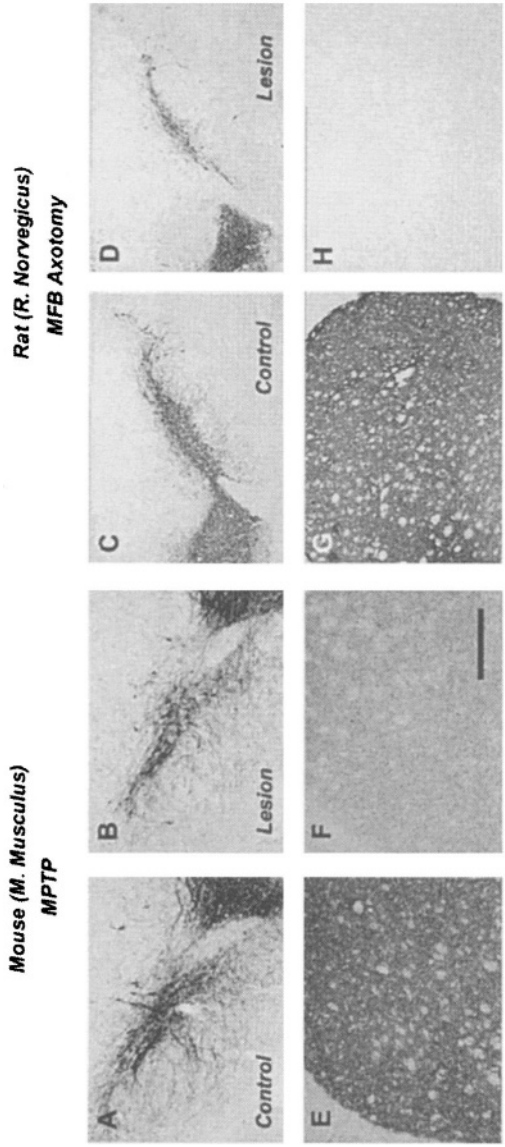

Figure 2: The selective loss of nigral dopamine neurons and striatal dopaminergic fibers can be modeled in two different experimental models of Parkinson's disease in rodents: MPTP in mice (A,B,E,F) and, MFB axotomy model in rats (C,D,G,H). Photomicrographs reveal dopamine neurons of the SNpc (A-D) and dopaminergic fibres of the striatum (E-H) using immunohistochemical detection of the dopaminergic phenotype marker, tyrosine hydroxylase (TH) – the rate-limiting enzyme in the synthesis of dopamine. The extent of dopaminergic lesions can be variable and incomplete in the MPTP model (depending upon dosing regime and mouse strain) whereas mechanical lesions produce a profound loss of SNpc neurons and almost complete loss of striatal dopamine fibres.

2.1.2 N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine(MPTP)

Interestingly, the observation that 6-OHDA interrupts mitochondrial respiration and leads to the loss of nigral dopamine neurons has lead to the hypothesis that loss or impairment of mitochondrial function may underlie the etiology of PD (Dawson and Dawson, 2003). This notion was furthered by the discovery of another dopaminergic neurotoxin that also blocks mitochondrial activity, N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP was discovered serendipitously in the 1980's by Dr. William Langston and colleagues when they identified MPTP as a byproduct of improper meperidine synthesis that induces a PD-like condition in humans (Langston *et al* 1983). When administered to non-human primates or vulnerable strains of rodents, MPTP produces a syndrome reminiscent of PD that also recapitulates the selective destruction of the SNpc (Burns *et al* 1983; Sedelis *et al* 2000). MPTP is a pro-toxin that requires metabolism by monoamine oxidases into N-methyl-pyridium ion (MPP+) to elicit neurotoxicity (Langston *et al* 1984a, b; Markey *et al* 1984). The recent use of MPTP in transgenic mice has advanced our understanding of the molecular pathology of PD and may to determine key factors involved in the cellular processes governing dopamine neuronal loss and/or survival [(Crocker *et al* 2003a,b) and reviewed by Jackson-Lewis and Przedborski (Przedborski *et al* 2000)]. In this chapter, we will discuss the role of calpains in an MPTP model of PD.

2.1.3 Pesticides

Since the application of MPTP as a model of PD, pesticides and herbicides which structurally resemble MPP+ (the active metabolite of MPTP), have been investigated for both their neurotoxic and synergistic effects on dopamine neuron loss. Paraquat is a commercially available herbicide which has been shown to impair mitochondrial function by inhibition of complex I (Shimada *et al* 1998). Systemic administration of paraquat has been reported to replicate the hallmarks of PD: loss of SNpc neurons, depleted striatal dopamine levels and impaired motor function (Brooks *et al* 1999; Thiruchelvam *et al* 2000). Rotenone, an insecticide also with structural similarities to MPP+ has also regained attention as a putative neurotoxin for modeling PD (Freidrich 1999). Specifically, the demonstration that chronic administration of low doses of rotenone (doses) to rats can produce motor deficits and selective degeneration of nigral dopamine neurons – that are accompanied by the formation of ubiquitinated intracellular inclusions (Betarbet *et al* 2000). *In vitro,* treatment of dopaminergic cells with rotenone also causes a reduction of mitochondrial function and increases expression of α-synculein (Sherer *et al* 2002). These

results have provided impetus for the use of rotenone as a model for the study of dopamine neuron dysfunction in PD. However, it is worth noting that the recent work on these agricultural chemicals contrasts previous work with these compounds that failed to demonstrate a specific or significant toxicity for dopamine neurons (Perry *et al* 1986). Nevertheless the recent characterization of chronic rotenone administration as a model of PD has reignited interest into the epidemiological correlation between PD incidence and pesticide use and lends support for a hypothesis that environmental chemicals may be involved in the etiology of PD (Olanow and Tatton, 1999).

2.1.4 Medial Forebrain Bundle Axotomy

Mechanical transection of the nigrostriatal pathway at the medial forebrain bundle (MFB) results in the degeneration of SNpc neurons and striatal dopaminergic fibres over the course of several weeks. In this model it is thought that the separation of the nigral neuronal soma from trophic retrograde support from their striatal afferents triggers a program of delayed neuronal death (Tomac *et al* 1995). Indeed, MFB axotomy elicits the activation of cell death programs observed in early post-natal periods that are not seen in adult dopamine neurons (Crocker *et al* 2001b). The idea of trophic deprivation is also supported by the demonstration that the death of nigral neurons can be prevented by the administration of glial cell line-derived neurotrophic factor (GDNF) (Beck *et al* 1995; Tseng *et al* 1997), a potent growth factor for dopamine neurons (Henderson *et al* 1994). Further, by cutting the nerve fibres rather than administering an exogenous substance it has been suggested that this form of injury evokes an intrinsic mode of cell death that may depict the natural processes involved in the loss of SNpc neurons during PD. Nevertheless, identifying cell death-related signaling molecules that are involved in more than one model of SNpc neuron injury increases our confidence that the role of any given protein is not merely context-specific but may represent an important mediator of dopamine neuron loss in PD. One example of a protein associated with neuronal loss in several models of PD is the stress-response transcription factor c-Jun (Crocker *et al* 2001a; Xia *et al* 2001).

2.1.5 Inflammation Models of PD

One of the more recent developments in the field of PD is the finding that direct injection of the bacterial endotoxin, lipopolysaccharide (LPS) into the substantia nigra induces inflammation and replicates many of the features associated with PD; microglial activation, SNpc neuron loss and reduced levels of dopamine in the striatum (Castano *et al* 1998).

Mesencephalic dopamine neurons seem particularly sensitive to inflammatory injury since cultured hippocampal or cortical neurons are resistant to neurodegeneration induced by LPS (Kim *et al* 2000). Interestingly, Jenner and colleagues have also demonstrated that direct injection of LPS induces significant oxidative stress in dopamine neurons and mimics the inflammatory changes observed in PD brain (Iravani *et al* 2002). Specifically, microglial activation is pronounced in the postmortem PD brain. Findings based upon the observation that LPS-induced inflammation in the ventral midbrain of rodents can provoke SNpc degeneration suggests that elevated expression of pro-inflammatory cytokines, such as interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNFα) or interleukin-6 (IL-6) is detrimental to the survival of nigral dopamine neurons (Gayle *et al* 2002). Central administration of TNFα to mice has also been reported to reduce expression of TH and modify behaviour (Aloe and Fiore, 1997), and TNFα-induced cell death is enhanced by expression of a mutant form of α-synuclein, a candidate gene for PD (Stefanova *et al* 2003). These results suggest that reported changes in cytokine expression in PD (modeled by LPS) may be causally related to neuropathology in PD. However, the microglial response to LPS *in vivo* and induction of pro-inflammatory cytokine mRNA is not necessarily correlated with production of secreted mature cytokines (Depino *et al* 2003). This finding, the authors' point out, is analogous to CNS infection by prions and may indicate that the microglia responses to dopamine neuron injuries are associated with a priming of the immune response to subsequent injuries or infections (Depino *et al* 2003). Consistent with this latter notion are very recent studies demonstrating that SNpc degeneration by dopaminergic toxins is accentuated by LPS: an effect that is mediated by microglia (Gao *et al* 2003a,b; Goralski and Renton, 2004). Taken together, inflammation mediated by reactive glia play a significant for in the neuropathology of PD. While we won't elaborate upon this point here, an eloquent and comprehensive review of the topic of glial involvement in PD was recently compiled by Hirsch and others (Hirsch *et al* 2003).

Another inflammation-related hypothesis pertaining to the etiology of PD is that of autoimmunity. Several studies have shown that degeneration of rat nigral dopamine neurons can be induced by stereotaxic injection of IgGs isolated from the serum of PD patients, but not from other neurodegenerative disorders (Chen *et al* 1998). Further, in much the same way that experimental autoimmune encephalomyelitis (an animal model for Multiple Sclerosis) can be induced by systemic administration of myelin peptides, Appel and colleagues have shown that immunization of guinea pigs with dopaminergic cells (MES 23.5) can produce an immune-mediated destruction of the SNpc (Le *et al* 1995). These intriguing studies addressing autoimmunity and PD indicate a possible role for adaptive immunity in

processes mediating cell loss in PD. The discovery that "auto"-antibodies can be generated in neurodegenerative diseases, such as antibodies against L-type calcium channels that can impair dopamine release in amyotrophic lateral sclerosis (ALS) (Offen *et al* 1998), and antibodies can be found in some cases of PD that recognize oxidatively modified proteins (Rowe *et al* 1998), lend further support to a potential function for the adaptive immune response in PD. How might this immune reaction be triggered in PD? An interesting angle to consider is the possibility that autoimmunity in PD may be an example of *molecular mimicry* (Levin *et al* 1998). As previously mentioned, pathogen infections have been linked to some cases of PD. Conceivably, antibodies developed in response to infections may erroneously recognize "self" antigens and therein trigger a deleterious inflammatory cascade directed at host tissues (Yamada *et al* 1996; Takahashi and Yamada, 2001).

2.1.6 Proteasome Inhibitors

The proteasome is a multimeric intracellular complex responsible for the breakdown of ubiquitinated peptides into amino acids. Accumulating evidence implicates the processes of ubiqutination and/or proteasomal function in the etiology of PD: (i) one of the hallmark pathologies of PD, the Lewy body, is a highly ubiquitinated cytoplasmic inclusion that is enriched with α-synuclein, a candidate gene for PD (Tofaris *et al* 2003), (ii) proteasomal function is reduced in sporadic PD (McNaught *et al* 2003), (iii) expression of the 20S component of the proteasome is reduced in PD (McNaught *et al* 2002a), (iv) administration of proteasomal inhibitors to cultured neurons causes cell death and formation of intracellular inclusions resembling Lewy Bodies (Rideout *et al* 2001; McNaught *et al* 2002c; Sawada *et al* 2004), (v) intranigral injection of proteasomal inhibitors can evoke SNpc degeneration (McNaught *et al* 2002b), (vi) dopaminergic neurotoxins induce ER stress that results in production of unfolded proteins (Elkon *et al* 2001; Holtz and O'Malley, 2003), (vii) deficits in mitochondrial function can lead to Lewy body formation (Trimmer *et al* 2004), and (vii) candidate genes for inheritable forms of PD (discussed below) are involved in the ubiquitin-proteasome pathway (Giasson and Lee, 2003). Collectively, these findings provide compelling evidence to suggest that when nigral dopamine neurons are stressed or injured their reduced ability to process proteins can precipitate neurodegeneration. In PD, therefore, the loss of SNpc neurons may be attributable to a fundamental defect in the ubiquitin-proteasome pathway. Though the presence of Lewy bodies is PD is definitive, the purpose of these inclusions or the reason they form in nigral dopamine neurons is unclear. Current hypotheses include: the purposeful sequestration of potentially harmful proteins as a neuroprotective response;

the accumulation of highly ubiquitinated proteins as a result of deficits in proteasome function; and the generation of particular pathologic proteins (either through de novo synthesis or modification in native conformations) initiate a systematic accumulation of proteins that results in perturbation in neuronal homeostasis. Whether the role of intracytoplasmic inclusion bodies is deleterious or a benign marker to the fate of dopaminergic neurons remains to be determined. Elucidating the requisite events that lead to their formation may reveal important insights into the etiology of PD.

2.1.7 Transgenic Models

Within the past decade, genetic linkage analyses for inheritable (familial) forms of PD have resulted in the identification of several candidate genes for PD [reviewed by (Dawson and Dawson, 2003)]. These include, but are not limited to: mutations in α-synuclein (autosomal dominant), parkin (early-onset autosomal recessive), DJ-1 (autosomal recessive), and UCH-L1 (autosomal recessive). Additional other genes have been associated with increased susceptibility to PD, such as tau, synphilin-1 and NR4A2 and the potential role of these genes are discussed below in section 2.2. Among the candidate genes causally linked to familial forms of PD, α-synuclein has garnered the most attention for the discovery that this protein is a main constituent of the highly ubiqutinated inclusions characteristic of PD: Lewy bodies (Spillantini *et al* 1997). While the precise function of α-synuclein is not known, the absence of a PD phenotype in the α-synuclein knockout mouse (Abeliovich *et al* 2000) suggests that the two point mutations associated with a familial form of autosomal dominant PD, A30P (Kruger *et al* 1998) and A53T (Polymeropoulos *et al* 1997), result in a pathological gain-of-function phenotype (Lim *et al* 2003). Several transgenic models of synuclein overexpression (WT or mutants) have been generated in mice. Most do not present with a specific PD-like nigral degeneration. However, some have shown degeneration of other neuronal populations as well as inclusion like phenotype. In the rat or monkey, virally-mediated overexpression of α-synuclein, either wildtype or containing either of the PD mutations, can reproduce nigral pathology with cytoplasmic inclusions accompanied with motor deficits (Kirik *et al* 2002; Lo Bianco *et al* 2002; Kirik *et al* 2003). The reason for the apparent and marked difference in physiological response to α–synuclein overexpression in different species is presently unclear.

The observation that other candidate genes for PD, parkin, a ubiquitin ligase, and UCH-L1, a ubiquitin hydrolase, suggest that there is a profound functional overlap of each candidate gene product with the ubiquitin-proteasome pathway. This obviously implicates some fundamental defect or failure in this intracellular system and points to the ubiquitination

pathway(s) as a unifying hypothesis for inherited forms of PD (Giasson and Lee, 2003). Recent reports on deletions of the parkin gene indicate that parkin deficiency is sufficient to perturb some dopaminergic functions, but insufficient to produce hallmark PD neuropathology (Goldberg *et al* 2003; Itier *et al* 2003).

2.2 A "Two-Hit" Hypothesis for PD

As in cancer, the recent awareness of the involvement and potential importance of the immune system in neurodegeneration has lead to the proposal that certain individuals may have a predisposition to develop PD, either through genetic inheritance or prior immune experiences. We refer to this notion as a "two-hit hypothesis" wherein certain individuals or populations (e.g. families) may have an increased propensity to develop PD due to a combination of either innate factors (e.g. genes) or previous experiences (e.g. viral infections) that when combined with subsequent exposure to another factor (e.g. environmental toxin) triggers the disease phenotype. In this model, the vast majority of any population would not develop PD when encountering the same "subsequent factor(s)". The characterization of those features that predispose individuals to develop PD (as there may be more than one susceptibility factor), as well as the identification of those factors considered triggers (the "second hit") will be central to our understanding of PD. Current hypotheses of PD etiology fit easily into the framework of our two-hit hypothesis for PD. For instance, genetic mutations could plausibly constitute a "first-hit" (vulnerability factor) and environmental exposure to agents, such as the pesticides (e.g. rotenone) could constitute the "second hit" (trigger factor) – the combination of these two events in the natural history of one's life could precipitate PD or represent risk factors for developing PD (Figure 3). This notion is garnering attention as increasing attention is being paid to the interactions of genes, inflammation and dopaminergic neurotoxins.

3. PROTEASES IN PARKINSON'S DISEASE

The identification of "apoptosis", or programmed cell death, as an active process requiring signaling through definable intracellular pathways has become an important development for the field of Parkinson's disease research over the past decade. Consequently, advances in the understanding of cell death have lead to novel hypotheses and enhanced understanding of how nigral dopamine neurons are lost in this neurodegenerative disease. However, the presence of apoptosis in PD is not unequivocal. Increasing

numbers of studies refute a role for classic apoptosis in PD or in animal models (Jackson-Lewis *et al* 1995; Nishi 1997; Banati *et al* 1998; Wullner *et al* 1999; Crocker *et al* 2001b; Kuhn *et al* 2003), and DNA chip experiments on animal models of PD have reported a surprising absence of changes in key apoptosis signaling molecules (Gu *et al* 2003), therein making the precise mode of cell death in PD presently unclear (e.g. "classic apoptosis" versus a similar biochemical process unique to dopamine neurons).

Clearly, accuracy in the clinical diagnosis of PD, aside from the variety of possible causes and PD-like conditions of this disease may dramatically influence histological outcome and determination of what cell death-related phenomena are involved. Similarly, variations in chemical lesion models of PD (e.g. dosing regime, neurotoxin used, animal age and strain) may also influence the identification of requisite cellular signals. Nevertheless, elucidating the relevant players in the signaling process(es) governing neuronal loss in PD may yield relevant therapeutic targets for intervening in pathology-associated pathways, and may too permit us to work "backwards" in establishing how these pathways are activated in disease: identifying possible causes of PD.

One aspect of cell death – in any context – is the involvement of proteolysis. That proteases are critical for the pathology of Parkinson's disease is now well established. As our knowledge of proteases has increased, so too has our appreciation of proteolysis in PD. As we will discuss in the following sections, proteases have been implicated in an increasing variety of pathophysiologies of PD including dopamine receptor-mediated signaling, plasticity and cell death. Our discussion will focus on the emerging role for the calcium-dependent proteases called calpains. We will begin by briefly summarizing the characteristic biochemistry, expression and regulation of these proteases, and then relate the expanding roles for the calpains in PD.

3.1 Calpains

Calpains are a highly conserved family of calcium-dependent cysteine proteases which have been implicated in a variety of physiological processes. In the CNS, calpains are widely expressed with distinct regional patterns of immunoreactivity (Li *et al* 1996). The most prominent neuronal populations expressing basal calpain-1-like immunoreactivity include the spinal cord, cerebellum, hippocampus and, most notably, the substantia nigra pars compacta (Siman *et al* 1985b; Perlmutter *et al* 1990). Calpains were first isolated from porcine skeletal muscle and characterized for their absolute requirement for calcium for activation (Dayton *et al* 1976a,b). This

family of non-lysosomal proteases is classified according to the structural homology and tissue-specific patterns of expression (for review see (Suzuki and Sorimachi, 1998)). There are two ubiquitously expressed calpains, termed μ-calpain (calpain-1) and m-calpain (calpain-2). μ-calpain and m-calpain were named for the μM and mM thresholds of calcium necessary for *in vitro* autolytic activation, respectively. However, it has been debated whether the concentration of calcium required for activation may differ *in vivo* since the relative concentrations of calcium very seldom occur under normal physiological conditions.

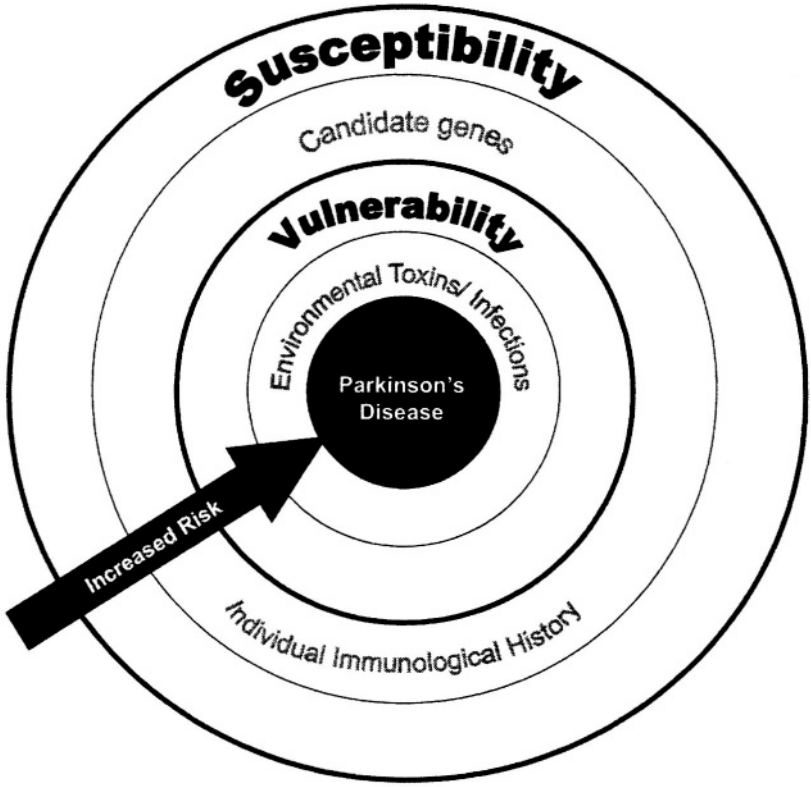

Figure 3: Diagram depicting the "two-hit" hypothesis for the etiology of Parkinson's disease. In this schematic, the circles represent varying degrees of risk associated with the development of Parkinson's disease. Each ring around the center ("Parkinson's disease") represents a feature or risk factor for PD. The outer rings depict the known "susceptibility" factors for PD that include (but are not limited to) inheritance of candidate gene or an individual's history of infection (see Section 2.1.5 above). The inner rings depict events or agents that can either induce PD or dramatically enhance one's risk of developing PD. For

instance, environmental exposure to factors such as pesticides or self-administration of MPTP-like compounds make one vulnerable to PD. In the case of individuals with susceptibility (the "first hit") subsequent exposure to "vulnerability" factors (the "second hit") is postulated to increase the likelihood of developing PD. While the identification of specific "susceptibility" or "vulnerability" factors listed in this figure (gray text) may indeed contribute to PD, this figure depicts an hypothesis for understanding the suspected interactions between risk factors associated with PD.

Structurally, the calpain zymogens are composed of a large "enzymatic" 80 kDa subunit, and a smaller 30 kDa "regulatory" subunit (reviewed in Suzuki and Sorimachi, 1998). The large subunit contains four highly conserved domains; a propeptide domain (I), a protease domain (II), a third domain (III) of unknown function, and a C-terminal calmodulin-like Ca^{2+} binding domain containing 4 helix-loop-helix (EF hand) motifs (IV). The EF-hand motifs are believed to be essential for calcium binding and calcium-dependent activation of the calpain heterodimer. While calpain-like enzymes are classified as "typical" or "atypical" based on the presence of large subunit homology domains, all calpain-like proteases contain an identical protease domain (II). In contrast, the small, regulatory subunit of calpains is identical among all species and tissue-specific isoforms. The regulatory subunit also contains a calmodulin binding domain (IV^1) and a glycine rich N-terminal domain believed to mediate the calpain heterodimer association with membranes. It is also postulated that association with membranes is a critical event in calpain activation.

In the presence of calcium, calpains translocate from the cytosol to intracellular membranes, where it is postulated that a combination of calcium and phosphatidylinositol (PIP_2) are required for autocatalysis of the N-termini of both the large 80 kDa and small 30 kDa subunits into 76 kDa and 18 kDa monomers that associate to create the active heterodimer. It has been suggested that the lipid interaction during this activation process may modify the calcium binding properties of calpain zymogen. Indeed, it appears that when procalpains are associated with membrane lipids, the amount of calcium required for zymogen activation is reduced to within a physiological range (Chakrabarti *et al* 1996). Consequently, it appears that membrane association is critical for the proper activity of calpains.

3.2 Mechanisms of Calpain Regulation

Calpains are regulated by the ubiquitously expressed endogenous inhibitor, calpastatin. This 110 kDa protein has four highly conserved calpain inhibitory domains (CIDs) which contain the consensus sequence TIPPEYR (Croall and McGrody, 1994). Transfection of this consensus inhibitory sequence of calpastatin is sufficient to inhibit calpain-mediated cellular degeneration (Huang and Forsberg, 1998). In terms of other known

cell death-related processes that may participate in PD, it is important to note that cross-talk between calpain and the caspase proteases – both associated with PD – have been reported (Neumar *et al* 2003). Specifically, the cysteine protease caspase-3 can cleave calpastatin *in vitro* (Wang *et al* 1998), thereby facilitating calpain activity. Conversely, procaspase-3 is also a reputed substrate of calpains (McGinnis *et al* 1999). Hence, activation of either of these proteolytic enzymes would presumably result in cross-activation of the other and positive feed-forward effect on cellular proteolysis. However, the role of caspase-3 in MPTP-induced cell death is not definite as activation of caspase-3 while reported by some (Turmel *et al* 2001) is not reported by others (Jellinger, 2002; Kuhn *et al* 2003). Hence, whether there exists an interplay between proteases in the SNpc neurons during PD is not known at this time. Nevertheless, we shall focus our discussion on the activation and actions of calpains as a unifying event linking many of the current hypotheses pertaining to neuronal loss and motor dysfunction in PD.

4. CALPAINS AND PARKINSON'S DISEASE

Mitochondria are a principal calcium store in neurons indicating that the perturbation of mitochondrial homeostasis in PD – and recapitulated in animal models – presupposes a role for calcium and calcium-related processes in the etiology of this disease. Several lines of evidence point to the inadequate management of calcium by mitochondria in PD. First, mitochondria from individual with PD poorly sequester or retain intracellular calcium and the PD-mimetic neurotoxin, MPP+, evokes the release of calcium from mitochondria (Frei and Richter, 1986; Chen *et al* 1995; Sheehan *et al* 1997). Moreover, the selective loss of SNpc neurons that do not express the calcium–buffering protein calbindin D supports the notion that during PD cells that can mitigate the impact of dysregulated calcium homeostasis are spared (Yamada *et al* 1990; McMahon *et al* 1998; Damier *et al* 1999). Furthermore, Hirsch and colleagues had noted an increase in calpain expression in the SNpc of PD cases, and additional studies by Banik and colleagues had also reported increased expression of calpains following MPTP intoxication in mice (Mouatt-Prigent *et al* 1996; Ray *et al* 2000; Chera *et al* 2002). Hence, we surmised that this collection of descriptive data pointed directly to an important functional role for activated calpains and calpain-mediated proteolysis in the pathology of PD and modeled in mice treated with MPTP (Crocker *et al* 2003b). Consistent with the previous work by others, we have shown that expression of calpains is not only increased by MPTP and in PD, but that these enzymes are also

active. Moreover, inhibition of calpains blocked MPTP-induced neuronal degeneration in mice and also prevented a decrement in locomotor performance (Crocker *et al* 2003b). From this work, we will now discuss our interpretation of the role(s) of calpain and the implication of their sustained activation in nigral dopamine neurons as a central feature of neuropathological signaling in PD.

The initial activation of calpains by MPTP exposure is most likely attributable to calcium increases from a variety of potential sources. Perturbation of cellular mitochondrial function can lead to numerous consequences, including an overall depletion in ATP level and a consequent increase in $[Ca^{2+}]_i$. Following an initial activation of calpains, we postulate that calpain activity is maintained in nigral dopamine neurons through a combination of calpain-mediated proteolysis and compromised mitochondrial function that facilitates sustained increases of intracellular calcium. Possible secondary, or "amplifying", sources of calcium include the endoplasmic reticulum (ER) (Ryu *et al* 2002) and/or voltage-gated calcium channels (Appel *et al* 1994). Together, these findings suggest that management of intracellular calcium, and thus regulation of calpain activation, is a prerequisite for dopamine neuron survival. Consistent with this idea are the additional observations that inhibition nimodipine-sensitive calcium channels can attenuate MPTP-induced nigral dopamine neuron loss (Kupsch *et al* 1996), and SNpc neurons that express the calcium buffer protein calbindin 28k are less vulnerable to degeneration in PD (Lavoie and Parent, 1991; Damier *et al* 1999). Hence, there is reason to suspect that an initial trigger of calpain activity can lead to a situation of positive feedback resulting in a sustained activation of these proteases in SNpc neurons. Indeed, we observed evidence of progressive and sustained activation of calpains in the nigral dopamine neurons of MPTP-lesioned mice as well as within surviving pigmented neurons of the SNpc of postmortem human PD cases (Crocker *et al* 2003b). This finding supports previous work by Hirsch and colleagues describing an increase in calpain enzyme expression in human PD SNpc (Mouatt-Prigent *et al* 1996). In addition, we have demonstrated that adenovirus-mediated expression of calpastatin could prevent neurodegeneration following MPTP in mice. Importantly, MPTP-induced PD in primates is not accompanied by any discernable changes in expression of calpastatin in the SNpc (Mouatt-Prigent *et al* 2000). This finding, along with the results demonstrating increased calpain enzyme expression in PD, suggest a possible model by which increased calpain levels is not compensated by increased calpastatin expression. This altered balance of calpain/calpastatin would potentially contribute to increased calpain activation in PD. Interestingly, as previously mentioned, calpastatin is a substrate of caspase-3. Since this enzyme, has been associated with MPTP-induced neurodegeneraton in PD (Eberhardt *et al* 2000; Hartmann *et*

al 2000), it is therefore unexpected that loss of calpastatin is not exacerbated in PD. However, this seemingly incongruous finding may indicate that either caspases were not abundantly activated following MPTP or that calpastatin is not a preferred *in vivo* substrate of caspase-3.

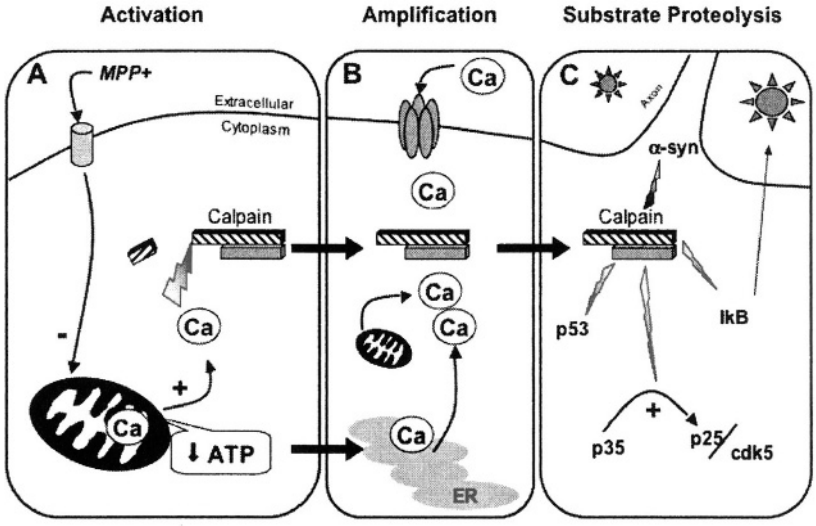

Figure 4: Proposed model for the role of calpains in dopamine neuron pathology in PD. Based upon our current understanding of the actions of MPTP as a dopaminergic neurotoxin and evidence from our *in vivo* studies involving this toxin and calpains, initial activation of calpains likely arises from the perturbation of mitochondrial function and loss of calcium homeostasis in this subcellular organelle (A). Evidence for activation of calpains in human PD has also been reported (Crocker *et al* 2003), though the source of calcium evoking the sustained activation of calpains in the MPTP-treated mouse or human PD condition is presently unclear. Here we propose that the initial activation of calpains is induced by a reduced capacity of the mitochondria to sequester calcium that is further amplified by persistent mitochondrial dysfunction and diminished levels of cellular ATP. (B) A reduction in cellular energy may precipitate loss of calcium from stores within the endoplasmic reticulum (ER), facilitate the influx of calcium from extracellular sources, and promote oxidative stress. This breakdown in cellular calcium homeostasis results in an increase and persistent level of calpain activity in dopaminergic neurons of the SNpc. (C) Calpain-mediated proteolysis results in dramatic alteration in substrate protein functions. For instance, proteolytic processing of the cyclin dependent kinase 5 co-activator protein p35 to a p25 isoform by calpains results in dysregulation of neuronal cdk5 activity that has been associated with neurodegeneration (Smith *et al* 2003). While the identification of calpain substrates within dopaminergic neurons in PD is incomplete, some putative substrate proteins listed in this figure have substantiated roles in PD-like pathology (see text for discussion).

How does the activation of calpains regulate other cell death processes reported to occur in PD? Currently, the downstream consequences of sustained calpain activation in dopamine neurons are not known. However, there is a growing list of possible candidate substrates for calpains that have been associated with calpains and neurodegeneration in a variety of neurodegenerative models (calapins reviewed in Carafoli and Molinari, 1998). Based upon previous work on calpains together with recent studies described for models of PD we can now develop schemata for the effects of calpain-mediated injury in nigral dopamine neurons (Figure 5c). In the following sections of this chapter we will describe proteolytic substrates of calpains that have been correlated with neurodegeneration in models of PD. As well, we will discuss how the actions of calpains on these substrates may influence what is known about other pathways involved in neurodegeneration associated with PD.

4.1 Calpain Substrates and the Pathology of PD

Autolytically active calpains show specific hydrolysis of predominantly short-lived proteins containing PEST sequences (Dice 1987). These substrates include, the structural proteins brain fodrin (Siman *et al* 1984) and α-spectrin (Roberts-Lewis *et al* 1994), the immediate early gene products c-Jun and c-Fos (Hirai *et al* 1991), and the signaling modulator protein kinase C (Cressman *et al* 1995) (Kikuchi and Imajoh-Ohmi, 1995). The proteolysis of substrates can dramatically influence the homeostasis of a cell. For example, fodrin can regulate the presentation of membrane receptors, so when cleaved by calpains the number of glutamate receptors on the extracellular surface is enhanced and therein sensitizing a cell to the effects of gluatamate (Siman *et al* 1985a). In general, the short half-life of calpain substrates along with the restricted nature of calpain cleavage of these target proteins has lead to the suggestion that calpains act to modify rather than completely catabolize substrate proteins. The transcriptional activity of c-Jun, for instance, is significantly reduced in the presence of activated calpain, suggesting that calpains may influence signal transduction modulating gene expression. For this reason, several physiological roles for calpains in neurons have been suggested, including the mediation of axonal regeneration (Shea *et al* 1995; Gitler and Spira, 1998) and effectuating neuronal death in certain neurodegenerative conditions (Carafoli and Molinari, 1998). While inhibition of calpains has been reported to be neuroprotective in a variety of neurodegenerative models, the identification of calpain substrate(s) important in neuronal death are poorly defined. In the following sections we shall outline a few proposed calpain substrates specifically related to neuropathology in PD.

4.1.1 p35

The cyclin dependent kinases (cdks) are a family of enzymes initially characterized for their regulatory role in cell cycle progression. That cell cycle related genes may be involved in the environment of the post-mitotic neuron has garnered much attention and spawned "the cell cycle hypothesis of neuronal death" (reviewed in Smith *et al* 2004). This hypothesis speculates that the activation of a cell cycle response in neurons at times of stress or injury is a fundamental cellular reaction that specialized, non-proliferative cell types, such as neurons, are incapable of managing. Consequently aberrant reactivation of cell cycle components in neurons may participate in neuronal death pathways associated with neurodegenerative diseases, including PD.

However, not all CDKs have predominate roles in cell cycle progression. In brain, one of the most abundantly expressed cdks is cdk5. Cdk5 is regulated by neuron-specific proteins p35 and p39. The active cdk5/p35 complex is crucial for normal brain development and deficiency of this activity leads to cortical lamination defects. However, recent evidence has also pointed to a pathogenic role of cdk5 in neurons. Interestingly, the mechanism by which a pathogenic activity of cdk5 is unmasked may be linked to calpain activation (Patrick *et al* 1999; Patzke and Tsai, 2002). When cleaved by calpain, p35 becomes a more stable p25 isoform that results in constitutive cdk5 activity and neurodegeneration (Patrick *et al* 1999; Cruz *et al* 2003). Alternatively, p25 has been shown to mislocalize to the nucleus where it inactivates a MEF2 mediated neuronal survival pathway (Gong *et al* 2003). Accordingly, cleavage of p35 by calpains may cause neuronal death by several potential means.

In PD, several lines of evidence support engagement of cdk 5 in nigral dopamine neurons. First, cdk5 is found to co-localize with hyperphosphorylated tau (τ), a cdk5 substrate, in the Lewy Bodies of nigral dopamine neurons (Arima *et al* 1999; Takahashi *et al* 2000; Ferrer *et al* 2001). We have recently found that MPTP administration induces an increase in cdk5 activity and production of the p25 isoform of p35 in the substantia nigra (Smith *et al* 2003). Inhibition of cdk5 activity either by intracerebroventricular administration of the cdk inhibitor, flavopiridol, or adenovirus-mediated expression of a dominant negative mutant of cdk5, attenuated the loss of SNpc neurons following MPTP (Smith *et al* 2003). Interestingly, blocking cdk5 activity using flavopiridol prevented MPTP-induced reductions in motor performance, prevented postsynaptic changes in gene expression in the denervated striatum all without restoring levels of striatal dopamine (Smith *et al* 2003). These findings are consistent with the selective nigral protection, attenuated striatal FosB induction and observed

functional neuroprotection achieved by calpain inhibition in the MPTP mouse model of PD (Crocker *et al* 2003b).

While studies have provided compelling evidence to support a role for cdk5 activity in neurodegeneration, the sequence of events following cdk5 activation that culminate in neurodegeneration are less clear. The colocalization of cdk in Lewy Bodies may suggest a role for cdk5 activity in their formation in disease. However, recent work on cortical neurons following administration of proteasomal inhibitors indicate that cdk function is related to neuronal death but their co-localization within intracellular inclusions (such as the Lewy bodies in PD) is not directly responsible for formation of these proteinaceous aggregations (Rideout *et al* 2003). Additional possible roles for cdk5 in PD include the modulation of striatal dopaminergic neurotransmission. Paul Greengard and colleagues have recently shown that inhibiting cdk5 activity in striatal neurons using roscovitine enhances dopamine release and increases NMDA receptor-mediated glutamate signaling (Chergui *et al* 2004). These effects of cdk5 inhibition were found to be both pre- and post-synaptically in striatal neurons that receive inputs from the cortex and SNpc (Chergui *et al* 2004). The coordinated effect of inhibiting cdk5 on neurotransmission is mediated by phosphorylation wherein the dopamine receptor signaling molecule, DARPP-32, when phosphorylated by cdk5 becomes an efficient inhibitor of protein kinase A (PKA) and increased dopamine release in the presence of cdk5 inhibition facilitates dopamine D1-receptor mediated NMDA activity (Bibb *et al* 1999; Chergui *et al* 2004).

From these studies on the role of cdk5, the possible contribution of calpains to the condition of hypodopamineregia in PD are indicated by the equivalent observations that inhibition of either calpains or cdk5 in the MPTP mouse model of PD mitigate SNpc neuron loss and prevent motor behaviour deficits without requiring replenishment of striatal dopamine levels. Taken together, our work would support a model of increased calpain in dopamine neurons leading to early disruptions in dopaminergic signaling that progress to neurodegeneration as a consequence of aberrant cdk5 activity.

4.1.2 α-synuclein

What effects PD-associated mutations in α-synuclein have on the natural function of this protein are not known, although it is postulated that by determining the pathological contribution(s) of PD candidate genes may provide clues to the etiology of sporadic PD. Since we have identified that calpain-mediated proteolysis plays an important role in the demise of SNpc neurons and motor decline in PD, it is therefore of particular relevance to the developing connection between genes linked to familial PD that

α–synuclein is a substrate of calpains (Mishizen-Eberz *et al* 2003). Recent studies have shown that overexpression of α–synuclein can evoke PD-like pathology (see above). These findings offer the intriguing possibility that active calpain-mediated proteolysis may underlie synucleinopathies. As there is presently no data regarding this question, but determining whether overexpression of α–synuclein evokes the activation of calpains, or whether wildtype α–synuclein is modified by calpains would provide a compelling link between calpains and α–synuclein-related pathologies in PD. Could calpain-mediated cleavage of α–synuclein contribute to accumulation of poly-ubiquitinated protein in PD? Could calpains mediate the degeneration of SNpc neurons produced by targeted α–synuclein overexpression? A possible caveat to this proposal is the recent demonstration that parkin activates calpains and reduces the neurotoxicity of wildtype α–synuclein expression in a hippocampal cell line (H19-7) (Kim *et al* 2003). That parkin, which physically associates with α-synuclein, was found to induce calpain (but not caspase)-mediated degradation of α–synuclein and the DNA repair enzyme, PARP, in neuronal cells *in vitro,* has lead to the suggestion that under certain circumstances increased parkin expression is cytoprotective and this protection ismediated by calpain proteolysis (Kim *et al* 2003). However, the preponderance of data implicates that activation of calpains in PD is part of neurodegeneration rather than part of a neuroprotective response to injury. The scheme put forth by Kim and others, however, is further complicated by additional data that indicate that mutations in α–synuclein associated with PD (A53T & A30P) create resistance to cleaveage by calpains (Mishizen-Eberz *et al* 2003). Thus our understanding of parkin function and its associated actions on α–synuclein and calpains are developing and future studies will provide additional data to define the biological functions of these protein interactions in dopamine neuronal loss in PD.

4.1.3 p53

The tumor suppressor protein p53 is a well-established fundamental regulator of cellular proliferation and is also recognized as a "pro-death" signal involved with neuronal death (reviewed by Morrison and Kinoshita, 2000). Evidence for involvement of p53 in the loss of dopamine neurons in PD comes from the demonstration that mice deficient of p53 are profoundly resistant to MPTP-induced neurotoxicity (Trimmer *et al* 1996). Moreover, Mattson and colleagues have demonstrated that synthetic inhibitors of p53 can preserve motor function and protect SNpc neurons from MPTP-induced injury (Duan *et al* 2002). Previous work has also shown that induction of p53 expression, which is usually transient, is sustained following MPTP (Mandir *et al* 2002). That p53 can be modified by phophorylation and

poly(ADP)-ribosylation has been proposed as a means for the stabilization – or protracted life of p53 following neuronal injury (Mandir *et al* 2002; Keramaris *et al* 2003). The precise mechanism by which p53 evokes cell death is not completely understood but its role as a transcription factor regulating expression of pro-apoptotic genes indicates that p53 may be an important proximal regulator of neuronal death. While initial reports have indicated that calpains cleave p53, (Gonen *et al* 1997; Pariat *et al* 1997) a recent report indicated that calpains can increase stability of p53, leading to neuronal death (Sedarous *et al* 2003). Blocking calpains either through expression of calpastatin or administration of MDL 28170 (a pharmacological inhibitor of calpains) resulted in increased neuronal survival and a profound reduction of p53 induction and expression (Sedarous *et al* 2003). Hence, there is evidence that one target of calpains related to neurodegeneration is p53. The mechanism by which this occurs is unknown but may involve modulating the numerous p53 stability factors known to regulate p53 activity, such as the ATM kinase (Mandir *et al* 2002; Keramaris *et al* 2003).

4.1.4 NFκB/IκB

The nuclear factor kappa B (NfkB) signalling pathway may also be regulated by calpains. The inhibitor of kappa B (IkB) is degraded by calpains leading to enhanced NFκB-dependent transcription (Shumway *et al* 1999). This has two potential biological outcomes important in PD. First, NFkB may regulate the survival/death of dopaminergic neurons themselves. Indeed, NFkB has been reported to mediate both survival and death of neurons, depending upon context (Aleyasin *et al* 2004). Second, NFkB can also mediate the inflammatory response in PD. For instance, many inflammatory genes, such as nitric oxide synthase (NOS) are regulated by (NFκB)-mediated transcription. Thus, activation of calpains can regulate expression of NFκB mediated inflammatory genes (Milligan *et al* 1996; Scholzke *et al* 2003). Certainly, recent studies by Przedborski and colleagues have elucidated a significant role for inflammatory mediators such as cyclooxygenase and nitric oxide in the demise of nigral dopamine neurons in the MPTP model of PD (Liberatore *et al* 1999; Wu *et al* 2002; Teismann *et al* 2003). Though a study by Teismann and others suggest that loss of NFκB subunit p50 is not be required for neuronal loss following MPTP (Teismann *et al* 2001), work by Schulz and colleagues have recently shown that neuroprotection of the SNpc by administration of peroxisome proliferator-activated receptor (PPAR) ligands is correlated with reduced expression of NFκB-mediated inflammatory genes (Dehmer *et al* 2004). Therefore, calpain inhibition may affect neuronal injury in PD at multiple

models, directly in the process of neuronal death, and indirectly through modulation of the inflammatory response.

5. COULD NEUROPROTECTION BE SUFFICIENT TO TREAT PD?

Within the CNS, the highly integrated network of nuclei that make up the basal ganglia are responsible for coordinating cortical outputs to syncronize and initiate movement. The nuclei of the basal ganglia include the caudate, the globus pallidus (GP), the subthalamic nucleus (STN), ventral tegmental area (VTA) and the substantia nigra (par compacta and pars reticulata, SNr). In PD, the primary afferent pathway from the SNpc to the caudate (in rodents the equivalent structure, called the striatum, encompasses both the caudate and GP) is lost. Degeneration of this nigrostriatal pathway results in reduced dopaminergic modulation of stimulatory (glutamatergic) cortical innervation of the striatum. The primary neuronal phenotype of the striatum is the medium spiny neuron which produces the inhibitory neurotransmitter, gamma aminobutryic acid (GABA). Thus, one consequence of SNpc neurodegeneration and loss of nigrostriatal afferents is an *increase* in the cortical innervation of the striatal GABA neurons and an overall *decrease* in the activity of nuclei receiving striatal afferents; (in humans) including the thalamus, globus pallidus, and subthalamic nucleus. It is also important to note that this organizational scheme is simplified and that levels of interconnectivity do exist. For instance, there are feedback loops of reciprocal nigro-striatal and striato-nigral innervation. Hence, the effects of SNpc degeneration likely involve signaling pathways that include, but are not limited to the loss of striatal dopaminergic innervation. Nevertheless, the ultimate aim of therapies for treating PD is the recovery of basal ganglia function control. In context of the neuroanatomy of the basal ganglia, the functional impact of inhibiting calpains in PD, as demonstrated in the MPTP mouse model may elucidate a functional reorganization of the basal ganglia in PD as a possible strategy to reacquire, or maintain, central motor control.

5.1 Inhibition of Calpains and Preservation of Motor Function in MPTP lesioned mice

One feature of our recent studies on the MPTP model of Parkinson's disease was the prevention of motor behaviour deficits in mice by inhibiting calpains (Crocker *et al* 2003b). The reason this observation was unexpected is that the levels of dopamine produced and/or released in the striata of

calpain inhibitor-treated mice were comparable to MPTP lesioned mice that displayed significantly reduced levels of motor activity. As well, we also found that changes in gene expression within the striatum that accompany dopaminergic denervation, increased expression of ΔFosB and neurotensin, were not significantly elevated in calpain inhibitor-treated MPTP-lesioned mice (Crocker *et al* 2003b). These findings suggest that inhibition of calpains and the protection of the dopaminergic neurons within the SNpc – without restoration of striatal dopamine levels – was sufficient to sustain normal motor function.

5.2 Nigral Management of Basal Ganglia Function

This model of functional preservation of basal ganglia function represents the possibility of inherent redundancy within the neuronal circuits that comprise the cortical reentrant loops that control motor function. Ostensibly, this also indicates that adaptive compensation in the PD brain could be achieved without requiring the replenishment of striatal dopamine levels but could be achieved via the modulation of other nuclei within the basal ganglia. Indeed, the notion of recovering motor functions is currently being addressed in some surgical strategies for remedying motor ticks in advanced PD. In these situations, surgical ablation of nuclei in the thalamus, for instance, can provide symptomatic recovery.

Other nuclei implicated in the control of basal ganglia output and locomotor activity that are also affected in PD include the locus cereleus (LC) and raphe nucleus (RN). Specifically, the RN sends serotonergic projections to both the striatum and substantia nigra (pars reticulata). Following a dopaminergic lesion, serotonergic innervation of the SNR is increased (Fox and Brotchie, 2000). The increased expression, and presumably, responsiveness of remaining SNpc neurons to serotonin receptor-mediated signaling - without counterbalanced dopaminergic innervation from the SNpc - has been proposed as a fundamental difference in the PD SNpc that accounts for locomotor dysfunction following dopaminergic neuron degeneration (Fox *et al* 1998; Bezard *et al* 2001). Thus, in this model, striatal function and motor recovery could be achieved through the preservation of the SNpc by providing positive feedback to the denervated striatum via counterbalancing localized serotoniergic influences in the SNR and consequently preventing the bias of cortical innervations on collateral striatal projections to other nuclei of the basal ganglia (Figure 2). While the primary site of calpain activation following MPTP administration in mice was the SNpc, we also observed indications that calpains were also activated in neurons of the denervated striatum. Systemic administration of calpain inhibitors (MDL 28170) may have therefore also modified the

activity of calpains in the striato-nigral or other striatal afferent pathways and attenuated dysregulation due to reductions in dopamine by preventing excessive calpain proteolysis.

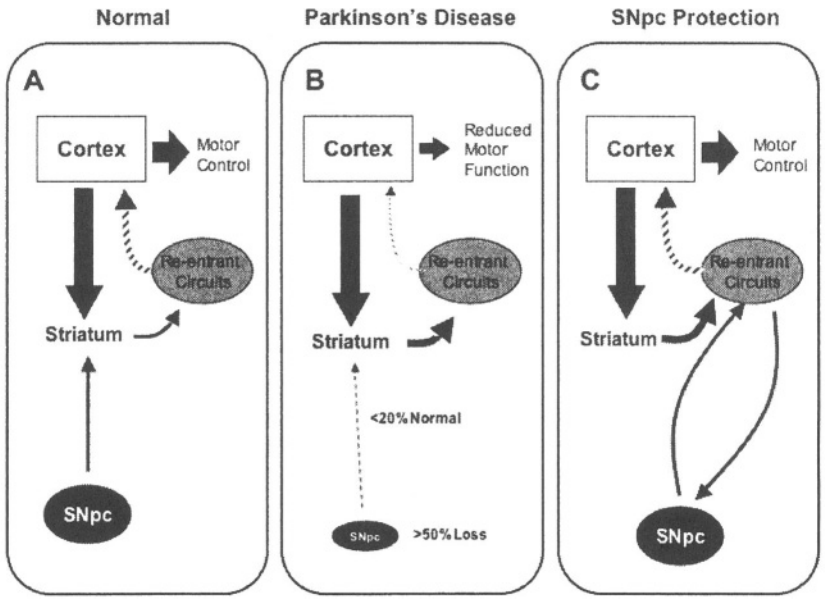

Figure 5: The development of motor dysfunction in Parkinson's disease is primarily attributed to the degeneration of nigral dopamine neurons and the commensurate depletion of striatal dopamine levels. (A) In the non-parkinsonian brain, dopaminergic modulation of striatal neurons that are innervated by cortical afferents regulate the voluntary control of movement through the series of anatomically connected nuclei that comprise the re-entrant circuits of the basal ganglia. (B) In PD, the loss of nigral dopamine neurons and diminished modulation of cortical innervation of the striatum results in a net reduction in motor function. (C) In the condition of SNpc neuroprotection yet without restoration of striatal dopamine, as observed with inhibition of calpains in MPTP-lesioned mice, the preservation of basal ganglia function *and* gross motor function is thought to be a consequence of dopaminergic modulation/feedback to nuclei of the basal ganglia, or ancillary loci, involved in the control of motor functions (for discussion see above, or Crocker *et al* 2003 for details). Hence, protection of the SNpc neurons may facilitate functional preservation in the condition of PD.

6. CONCLUSION

From our recent studies and the preceding discussion, it is becoming increasingly evident that calpains play a fundamental role in PD. From this several key issues germane to the study of PD and the understanding of

calpains in this neurodegenerative disorder arise. First; what can calpain activity tell us about the nature of PD pathology? Identification of the functional impact of calpain substrate proteolysis in SNpc neurons may yield important and fundamental insights into the etiology of PD. Second; is calpain activation a feature of all models of PD? Our findings on the MPTP mouse model of PD have shown that this rodent neurotoxin model recapitulates the biochemical changes in calpain expression and activities observed in human PD cases. Whether other models of PD, such as the 6-OHDA, rotenone or LPS models of PD reproduce similar changes in calpain expression and activity is not known at this time but would be of immeasurable value to our understanding of the ubiquitousness of calpain involvement in dopamine neuron loss. Third, the activities of calpains in PD likely involve more than cell death since it is also apparent that blocking calpains, even without restoring levels of striatal dopamine, that basal ganglia signaling is "normalized". Hence, do calpains contribute to both neurodegeneration (SNpc) and progressive neural dysfunction in other nuclei of the basal ganglia in PD? While we have studied and characterized the activation of calpains in the SNpc following MPTP administration, the outstanding issue of whether there is wider-spread induction and activation of calpains in the MPTP-lesioned brain has not addressed at this time. Given the significant behavioural differences we observed in MPTP-lesioned mice that were treated with calpain inhibitors, for instance, determining whether calpains are induced and/or activated in other nuclei of the basal ganglia, such as the thalamus, or perhaps even in other nuclei known to be affected in PD, such as the locus ceruleus would be of value. Moreover, establishing whether localized calpain inhibition into the SNpc alone, or perhaps other loci within the basal ganglia might reveal important roles for calpains and dysfunction in other nuclei as critical in PD.

In summary, the role of calpain-mediated proteolysis and its contribution to the demise of nigral dopamine neurons in PD is an emerging hypothesis for this neurodegenerative disease. Another feature of calpain involvement in the loss of nigral dopamine neurons in PD is the apparent gradual increase and sustained expression of this enzyme. We propose that calpain involvement in PD may enact the demise of dopamine neurons not through voracious proteolysis, but through a gradual decline in neuronal function attributed to selective calpain-mediated degradation or altered functionality of protein substrates. Future studies aimed at elucidating these proteolytic substrates of calpains, the functional impact of calpain activity in the basal ganglia and whether calpain activation is a ubiquitous feature of dopamine neuron degeneration in models of PD will provide additional support to the central role for calpains in PD. Furthermore, determining the precise mode of sustained calpain activation in nigral dopamine neurons in PD and the neuroanatomical re-organization implicit with SNpc neuroprotection in

models of PD will provide increased understanding into the etiology of PD and the potential therapeutic implications of calpains in this neurodegenerative condition.

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Chapter 3

Cysteine Proteases in Neuronal Death in Brain Ischemia

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1. INTRODUCTION

Prevalence of brain ischemia in the Western world is a major health problem leading to chronic disability and mortality. Current therapies, with a few exceptions, provide only symptomatic relief. An enormous increase in intraneuronal Ca^{2+} levels following ischemia activates Ca^{2+}-dependent deleterious enzymes for contribution to neuronal death and dysfunction. Besides, ischemic injury generates highly reactive free radicals and also triggers release of cytotoxic cytokines for activation of cysteine proteases. Accumulating evidence from different laboratories has already indicated a prominent role for the cysteine proteases of the calpain and caspase families in the pathogenesis of brain ischemia. Neuronal apoptosis occurs with activation of these cysteine proteases and administration of their inhibitors alone or in combination provide functional neuroprotection in various animal models of brain ischemia. It is anticipated that neuroprotection with cysteine protease inhibitors will be an integral part of the effective therapeutic strategy for the treatment of brain ischemia in the near future.

Brain ischemia is the major cause of chronic disability and the third leading cause of death in humans after cancer and coronary heart disease in the developed countries (Wolf *et al* 1986; Bonita 1992). About 85% of the strokes are caused by atherothrombotic occlusion of a blood vessel and 15% by intracranial hemorrhage in humans in the Western world (Bamford *et al* 1990). Each of these incidences results in acute neurological dysfunctions. Ischemic injury to the central nervous system (CNS) evokes a number of mechanisms for mediation of neuronal death (Ter Horst and Postigo, 1997).

Ischemic injury causes membrane depolarization and disrupts Ca^{2+} homeostasis in neuronal cells (Meyer 1989; Alps 1992). It is now known that Ca^{2+} enters neurons during cerebral ischemia via voltage-dependent ion channels as well as agonist-dependent and even non-specific ion channels following deterioration of cellular bioenergetics (Figure 1).

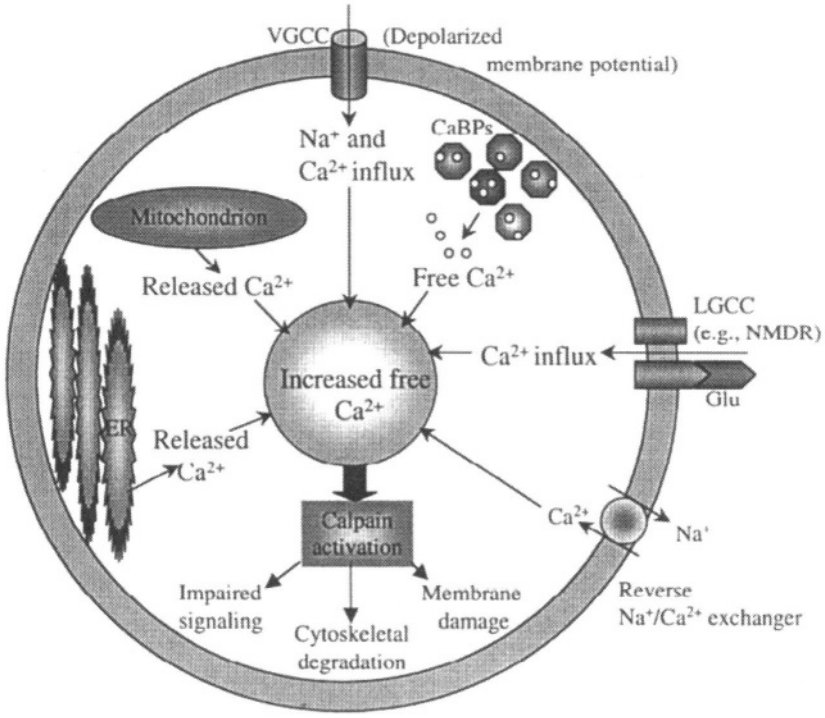

Figure 1: A central role of increased intraneuronal free Ca^{2+} in calpain activation. Various molecular mechanisms collectively contribute to a sustained increase in intraneuronal free Ca^{2+} for over activation of calpain leading to neuronal death. (a) Ligand-gated Ca^{2+} channels (LGCCs) are the primary sources of Ca^{2+} influx. (b) Voltage-gated Ca^{2+} channels (VGCCs) are the secondary sources of Ca^{2+} influx. (c) Ca^{2+} binding proteins (CaBPs), each capable of binding several Ca^{2+} ions, may be affected in brain ischemia altering Ca^{2+} affinity to contribute to a rise in intraneuronal free Ca^{2+} profile. (d) Sequestration of Ca^{2+} in endoplasmic reticulum (ER) and mitochondria are disrupted following ischemic injury. (e) Extrusion of Ca^{2+} from neurons fails due to an abrupt fall in ATP level following cerebral ischemia. Under ischemic condition, intracellular Na^{+} concentration rises with membrane depolarization and a ubiquitous Ca^{2+} transport mechanism works as a reverse Na^{+}/Ca^{2+} exchanger to export Na^{+} and import Ca^{2+} into the neurons. Finally, uncontrolled rise in intraneuronal free Ca^{2+} activates calpain, which degrades different classes of proteins and affects signal transduction mechanisms. Cleavage of calpain substrates and impairment of cellular signal transduction processes following ischemic injury triggers neuronal death.

The deleterious sequelae of cerebral ischemia may increase intraneuronal free Ca^{2+} levels leading to activation of Ca^{2+}-dependent proteases including calpain (Ray and Banik, 2003). Besides, reactive oxygen species and other cytotoxic factors are produced in course of cerebral ischemia prompting activation of Ca^{2+}-independent proteases such as caspases (Krupinski *et al* 2000). Cysteine proteases belonging to both calpain and caspase families are the major players in degradation of key cellular proteins for mediation of apoptotic death of neurons in the lesion and penumbra following ischemic stroke (Rami 2003). Therefore, calpain and caspase inhibitors are being examined extensively for neuroprotection in experimental ischemic stroke (Blomgren *et al* 2001; Zhang *et al* 2002; Pike *et al* 2004). Some limitations of these inhibitors must be conquered for their clinical evaluation. It should be mentioned here that the lysosomal cathepsins B and L are also cysteine proteases, which may contribute to some extent to the neuronal death after cerebral ischemia. However, description of this article will be limited to cysteine proteases belonging to the calpain and caspase families for their major role in the mechanisms of neuronal death and to experimental use of their current inhibitors for neuroprotection in brain ischemia.

2. CALPAIN ACTIVATION AND NEURONAL DEATH IN BRAIN ISCHEMIA

Excitotoxicity, elevation of intracellular Ca^{2+} levels, increases in free radicals, inflammatory responses via cytokines, and scarcity of survival factors following cerebral ischemia may lead to cell death, which can be broadly categorized into necrosis and apoptosis (Buja *et al* 1993). Necrosis is essentially accidental cell death and associated with cell swelling, loss of cellular energy, impairment of membrane integrity, and characterized by random fragmentation of genomic DNA; while apoptosis is energy-dependent and gene-directed cell death characterized by the presence of apoptotic bodies, shrunken cytoplasm, condensed chromatin, and internucleosomal fragmentation of genomic DNA (Server and Mobley, 1991). Light and electron microscopic studies following focal ischemic injury revealed that both apoptotic and necrotic cells co-exist in defined regions (Li *et al* 1995b). It is the severity of injury that determines whether cells die by apoptosis or necrosis (Beilharz *et al* 1995). Necrotic neuronal death predominantly occurs at the primary site of ischemic injury, while neuronal death that displays typical apoptotic features is predominantly found at the periphery of ischemic zone, also known as ischemic penumbra (Li *et al* 1995a,b).

Calpain, discovered by Guroff (Guroff 1964) in the CNS, is a ubiquitous intracellular Ca^{2+}-dependent cysteine protease (Ray and Banik, 2002).

Studies in various laboratories using cell culture models have confirmed that overactivation of calpain contributes to neuronal apoptosis (Jordan *et al* 1997; Ray *et al* 2000). Also, overactivation of calpain is known to be responsible for neurodegeneration in the animal models of CNS injuries such as spinal cord injury (Ray *et al* 2001; Carlson and Gorden, 2002; Ray *et al* 2003; Wingrave *et al* 2003) and traumatic brain injury (Saatman *et al* 1996; Kampfl *et al* 1997; Ray *et al* 2002). The elevation of intracellular Ca^{2+} levels following cerebral ischemic injury implied that Ca^{2+}-dependent enzymes, especially calpain, could be activated to contribute to neurodegeneration (Liebetrau *et al* 1999; Lipton 1999; Neumar *et al* 2001). The immunohistochemical demonstration of elevated intracellular levels of calpains in a rat model of reversible focal cerebral ischemia at various times of reperfusion strongly suggested a role for calpain in the pathogenesis of cerebral ischemia (Liebetrau *et al* 1999). Degradation of fodrin and microtubule-associated protein 2 (MAP2), the well known calpain substrates, preceded neuronal degeneration in cerebral ischemia indicating that calpain is indeed involved in the early events triggering the cascades leading to neuronal death (Blomgren *et al* 1995). Quantitative data also indicated that calpain-induced proteolysis occurred very early after the ischemic insult, correlated with earliest changes in cell hypotrophy, and preceded or occurred in tandem with evidence of significant cell loss (Bartus *et al* 1995). Recent studies suggest that calpains and caspases act synergistically for neuronal apoptosis following ischemic injury (Rami *et al* 2000; Yamashima 2000; Blomgren *et al* 2001; Zhang *et al* 2002).

Changes in the concentrations of calpain substrates such as MAP2, 200 kD neurofilament (NFP), tubulin, and myelin-associated glycoprotein (MAG) due to degradation at 4 h following occlusion of the left middle cerebral artery of rats indicated that calpain might be activated in cerebral stroke (Inuzuka *et al* 1990b). The mechanism of post-ischemic delayed death of nippocampal cornu Ammonis 1 (CA1) neurons involved Ca^{2+} mobilization, breakdown of phosphatidylinositol 4,5-bisphosphate (PIP2), and proteolytic activity of calpain in transient brain ischemia in monkeys (Yamashima *et al* 1996). Calpastatin is not only a potent calpain inhibitor but also a preferred calpain substrate. Hippocampal CA2 neurons are less vulnerable to ischemic stress than the adjacent CA1 neurons; and the mechanism of protection of CA2 neurons probably involves an elevated level of calpastatin in calpain regulation as a bait substrate to provide neuroprotection under ischemic conditions (Saido *et al* 1997). A recent study examined the temporal and spatial characteristics of calpain activity in brain after transient forebrain ischemia in Long Evans male rats, and found that between 24 and 48 h after reperfusion calpain activity in neurons progressed from the dorsal to ventral striatum, medial to lateral CA1 hippocampus, and centripetally expanded from watershed foci in the cerebral cortex (Neumar *et al* 2001).

3. NEUROPROTECTION WITH CALPAIN INHIBITORS IN BRAIN ISCHEMIA

Neuroprotection with calpain inhibitors is an attractive option for treatment of ischemic stroke. Multiple mechanisms that are currently known to contribute to the intraneuronal Ca^{2+} overload include Ca^{2+} entry through receptor-gated channels and voltage-sensitive channels, Ca^{2+} release from the intraneuronal stores, and Ca^{2+} extrusion and sequestration due to failure of energy-dependent mechanisms (Figure 1). The complexity of the intraneuronal Ca^{2+} overloading mechanisms immediately suggests that it may be efficacious to block pharmacologically a common consequence of intraneuronal Ca^{2+} overload rather than focusing on one of many Ca^{2+} overloading mechanisms. Because calpain activation occurs downstream of several initial events leading to intraneuronal Ca^{2+} overload (Figure 1), there exists an extended 'window of opportunity' for therapeutic use of calpain inhibitors for prevention of delayed neuronal death following ischemic injury. Indeed, calpain inhibitors administered at least 3 h after brain ischemia in rats could provide neuroprotection (Bartus *et al* 1994b; Siman *et al* 1996). Finally, the amount of calpain activation in normal brain is quite low, and robust calpain activation in brain is triggered by ischemic (Saido *et al* 1993) and traumatic (Kampfl *et al* 1996) injuries, indicating a possibility that administration of calpain inhibitors may preferentially block the pathological brain events without profoundly affecting the normal brain functions.

Post-ischemic administration of the calpain inhibitor MDL-28170 could ameliorate brain damage in animal models of ischemia (Li *et al* 1998; Markgraf *et al* 1998), confirming a prominent role for calpain in the pathophysiology of ischemia. Administration of E-64-c at a dose of 400 mg/kg twice a day for 3 days significantly inhibited depletion of MAP2, suggesting that calpain was involved in the degradation of MAP2 and that the use of calpain inhibitors could be a useful clinical approach for amelioration of cerebral ischemia (Inuzuka *et al* 1990a). Calpain inhibition with leupeptin prevented intense proteolysis of cytoskeletal proteins in rat brain ischemia with a marked reduction in the extent of death of hippocampal neurons, and protected neurons exhibited normal electrophysiological responses and retained their capacity for expressing long-term potentiation, a form of physiological plasticity thought to be involved in memory function (Lee *et al* 1991). The calpain inhibitors, calpain inhibitor I and leupeptin, prevented neuronal degeneration in the hippocampal CA1-subfield in transient global cerebral ischemia, which was induced in rats by clamping both carotid arteries and lowering the arterial blood pressure to 40 mmHg for 10 min (Rami and Krieglstein, 1993). Systemic administration (intravenous injections of cumulative doses of 30 mg/kg or 60 mg/kg) of the cell-penetrating calpain

inhibitor MDL-28170 exhibited significantly smaller volumes of cerebral infarction than saline-treated or vehicle-treated control animals, suggesting that targeting intracellular Ca^{2+}-activated mechanisms might represent a viable therapeutic strategy for limiting neurological damage after ischemia (Hong *et al* 1994a). The reversible calpain inhibitor AK275 over the concentration range of 10 to 200 μM (perfused supracortically at 4 μl/h for 21 h), could provide a reliable and robust neuroprotection to cortical neurons following focal brain ischemia in rats, demonstrating a potentially powerful process of treating stroke and other ischemic brain incidents (Bartus *et al* 1994a). Also, the calpain inhibitor AK295 in a dose-dependent manner protected neurons from focal brain ischemia in rats (Bartus *et al* 1994b). Activation of calpain and proteolysis of a neuronal cytoskelal protein, fodrin, precedes delayed neuronal death in hippocampal CA1 neurons. Selective death of hippocampal CA1 neurons in transient forebrain cerebral ischemia in gerbils was prevented by administration of a calpain inhibitor in a dose-dependent manner (Yokota *et al* 1999). It should be noted that despite promising efficacy of calpain inhibitors in experimental animal models of cerebral ischemia as mentioned above, these calpain inhibitors have not yet been evaluated in clinical trials.

4. CASPASE ACTIVATION AND NEURONAL DEATH IN BRAIN ISCHEMIA

Apoptosis with the activation of caspases may proceed simultaneously through two principal pathways following ischemic injury (Figure 2). The receptor mediated 'extrinsic', and mitochondria mediated 'intrinsic' pathways re-unite at the final phase of apoptosis for activation of caspase-3 (Cohen 1997). Increases in production of various stress factors such as reactive oxygen species (ROS), reactive nitrogen species (RNS), and Ca^{2+} influx into the CNS cells following injury induce the mitochondria-mediated intrinsic pathway of apoptosis (Dawson and Dawson, 1996; Lewen *et al* 2000). Accumulation of Ca^{2+} via ligand-gated Ca^{2+} channels (LGCCs) like α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor (Anzai *et al* 2003) as well as voltage-gated Ca^{2+} channels (VGCCs) (Park *et al* 2003) causes activation of calpain (Hong *et al* 1994b; Yamashima *et al* 1996; Neumar *et al* 2001), which is connected to the caspase cascade (Rami *et al* 2000; Schild *et al* 2001; Rami 2003) leading to delayed neuronal death in cerebral ischemia (Figure 2). The Bcl-2 family of proteins makes the critical decision of life-or-death in response to stress and injury (Adams and Cory, 2001). Apoptosis in the CNS is tightly regulated by the alterations in the levels of pro-apoptotic (e.g., Bax, Bad, Bcl-xS) and anti-apoptotic (e.g., Bcl-

2, Bcl-xL) members of the Bcl-2 family (Merry and Korsmeyer, 1997). Expression of Bcl-2 family members varies and can be regulated at the transcriptional (mRNA) level (Gillardon *et al* 1996; Mu *et al* 1996). Alteration in transcriptional expression of Bax and Bcl-2 leading to a high Bax:Bcl-2 ratio has been implicated in apoptosis in cerebral ischemia in rats (Gillardon *et al* 1996). Global ischemia also altered the transcriptional expression of Bcl-xS and Bcl-xL resulting in an increased Bcl-xS:Bcl-xL ratio that might be responsible for induction of apoptosis in hippocampal CA1 and CA3 neurons as well as in cortical neurons (Honkaniemi *et al* 1996).

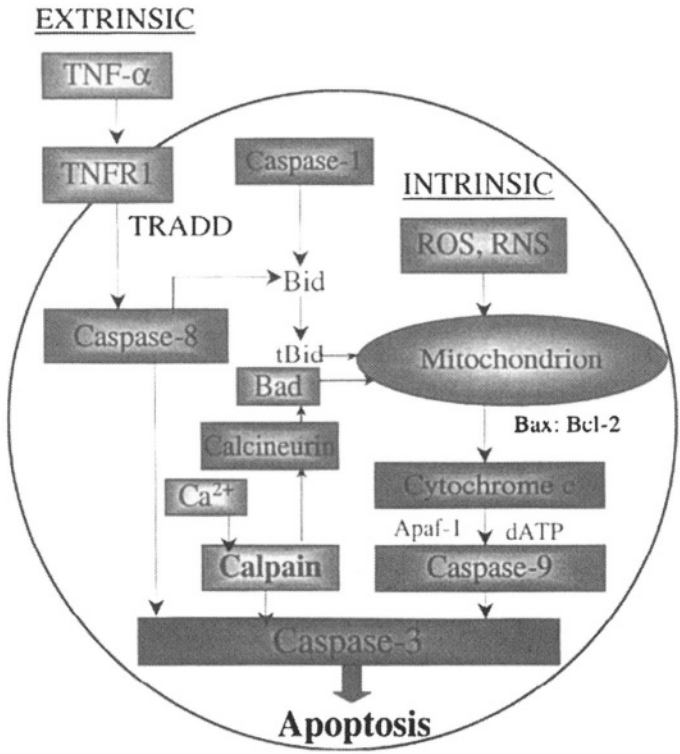

Figure 2: Molecular mechanisms for activation of calpain and caspase cascades leading to neuronal apoptosis in cerebral ischemia. Upregulation of cytotoxic cytokines such as **TNF-α** can stimulate cell surface receptors for induction of the 'extrinsic' caspase cascade leading to activation of caspase-8 and neuronal apoptosis. Caspase-8, directly and indirectly via mitochondria, activates caspase-3, the final executioner of apoptosis. Also, brain ischemia increases production of intracellular apoptosis signaling agents such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), which induce 'intrinsic' caspase pathways. Increased intraneuronal free **Ca^{2+}** activates calpain that in turn, directly and indirectly via mitochondria, activates caspase-3. Thus, all these proteolytic pathways converge to caspase-3 and culminate in neuronal apoptosis. Currently, calpain seems to be working upstream of capase-3 in the apoptotic process in ischemic stroke.

The extrinsic caspase cascade can be initiated by ligation of tumor necrosis factor-α (TNF-α) to TNF receptor 1 (TNFR1) that signals through the TNFR1-associated death domain (TRADD), an adapter molecule (Ashkenazi and Dixit, 1998). Once the trimerized death-inducing signaling complex (DISC) is formed, the adapter protein binds to an initiator such as procaspase-8 or procaspase-10 containing death effector domains (DEDs) for auto-activation of the initiator caspase (Muzio *et al* 1998). Activation of both caspase-8 and caspase-3 has been observed in both cerebral ischemia (Velier *et al* 1999) and spinal cord ischemia (Matsushita *et al* 2000). Investigation of neuronal apoptosis in a murine model of focal cerebral ischemia suggested that Bid was activated early in ischemia in a caspase-8-dependent fashion and was most potent activator of the mitochondrial pathway of apoptosis (Yin *et al* 2002). The intrinsic caspase cascade is triggered by mitochondria that receive apoptotic stimuli and release cytochrome c into the cytosol (Kluck *et al* 1997). When mitochondria release cytochrome c, they also release another protein, called either Smac (Du *et al* 2000) or Diablo (Verhagen *et al* 2000). This protein neutralizes a set of endogenous inhibitor-of-apoptosis proteins (IAPs) that otherwise suppress the activities of caspase-9 (Srinivasula *et al* 2001) and caspase-3 (Riedl *et al* 2001). Following the release from mitochondria, cytochrome c binds to the apoptotic protease activating factor-1 (Apaf-1), which has caspase recruitment domains (CARDs) (Zhou *et al* 1999). Apaf-1 with a conformational change in the presence of dATP can recruit procaspase-9 through the CARDs of both proteins leading to auto-activation of caspase-9, which then converts procaspase-3 to its active form (Hu *et al* 1999; Zou *et al* 1997).

Both caspase-dependent and caspase-independent apoptotic pathways in ischemic stroke have been well documented (Zhan *et al* 2001; Ferrer *et al* 2003; Ferrer and Planas, 2003). Various studies showed increased mRNA expression and activation of caspase-8 and caspase-9 (initiator caspases) and caspase-3 (effector caspase) leading to apoptosis in cerebral ischemia (Harrison *et al* 2001; Benchoua *et al* 2001; Cao *et al* 2002). Accumulating evidences from different laboratories strongly suggest synergistic activation of calpain and caspase-3 for mediation of apoptosis following ischemic stroke (Blomgren *et al* 2001; Zhang *et al* 2002; Pike *et al* 2004). Because calpain pathway as well as extrinsic and intrinsic caspase cascades ultimately converge to caspase-3 for mediation of apoptosis (Figure 2), inhibition of only one target upstream of caspase-3 may not provide enough neuroprotection. Additionally, inhibition of caspase-3 itself can provide sufficient neuroprotection in ischemic lesion and penumbra. Current investigations in this direction seem to be highly promising.

5. NEUROPROTECTION WITH CASPASE INHIBITORS IN BRAIN ISCHEMIA

Attenuation of apoptotic neuronal death directly with caspase inhibitors is an attractive therapeutic strategy in ischemic stroke (Onteniente *et al* 2003). Inhibition of the caspase family proteases provided neuroprotection following cerebral ischemia in gerbils (Himi *et al* 1998), mice (Hara *et al* 1997; Endres *et al* 1998), and rats (Loddick *et al* 1996). Interestingly, a prolonged therapeutic window existed for inhibition of caspase activity as pre-ischemic treatment or post-ischemic treatment with the caspase-3 inhibitor (z-DEVD-fmk), up to 9 h after induction of ischemic injury provided neuroprotection that was long lasting (21 days) in a murine model of brain ischemia (Fink *et al* 1998). Studies in rodent models of focal and global cerebral ischemia showed that pre-ischemic and post-ischemic treatments with the pan-caspase inhibitor (z-VAD-fmk) or caspase-3 inhibitor (z-DEVD-fmk) could block the activation of caspases to reduce neuronal injury after focal but not global cerebral ischemia (Li *et al* 2000). A similar but independent investigation reported previously that infusion of z-DEVD-fmk significantly attenuated caspase-3-like enzymatic activity and blocked delayed death of hippocampal CA1 neurons after global cerebral ischemia, however, did not prevent impairment of induction of long-term potentiation in post-ischemic hippocampal CA1 neurons, suggesting inability of caspase inhibition alone in preserving functional plasticity in neurons (Gillardon *et al* 1999).

Moreover, the synergistic effect of calpain and caspase inhibitors in protecting hippocampal neurons from ischemic damage suggests that there is a cross-talk between calpain and caspase for apoptosis during cerebral ischemia (Rami *et al* 2000). This concept has later been confirmed by a different group of investigators demonstrating that M826 (a small and reversible caspase-3 inhibitor) could selectively and potently inhibit caspase-3 activation and apoptosis related DNA fragmentation but it did not prevent calpain activation in the cortex after hypoxia-ischemia in neonatal rats (Han *et al* 2002). Inhibition of the caspase family proteases prevents cell death in a number of models of neurodegeneration including ischemic stroke but the sparing of neurons does not always correlate with long-term functional recovery, possibly due to the limitations of the currently available caspase inhibitors (Bilsland and Harper, 2002). So, recently there is an effort in reducing unacceptable molecular features of caspase inhibitors without loss of their anti-apoptotic potency (Becker *et al* 2004).

6. CONCLUSIONS

Multiple cellular and molecular mechanisms may contribute to neurodegeneration following brain ischemia. Investigations from different laboratories indicate that proteases, particularly cysteine proteases, play a major role in mediation of delayed neuronal death in the site of ischemic injury and ischemic penumbra. Overload of intraneuronal Ca^{2+} due to disruption of Ca^{2+} homeostasis in neurons following an ischemic attack invokes pathophysiological processes with activation of Ca^{2+}-dependent deleterious enzymes including calpain for acute and delayed neuronal death. Besides, oxidative stress and inflammatory responses may cause activation of various caspases, especially caspase-3, for mediation of neuronal apoptosis. Inhibitors of calpain and caspase-3 alone or in combination are showing promising neuroprotective effects in cell culture and animal models of cerebral ischemia. Investigations are underway for improvement in the structure and therapeutic efficacy of cysteine protease inhibitors, which may therefore be useful in the future for prevention of neurodegeneration in ischemic stroke in humans. Inclusion of neurotrophic factors in the therapeutic strategy for treating ischemic stroke may also help regeneration mechanisms. Finally, it is becoming clear from various investigations that an effective treatment for ischemic stroke will require a cocktail of several pharmacological and biological agents. In the future, inclusion of inhibitors of cysteine proteases seems to play an important role in effective prevention of neurodegeneration in brain ischemia.

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Chapter 4

Proteases in Traumatic Brain Injury

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1. INTRODUCTION

Traumatic brain injury (TBI) may be caused by a penetrating insult, or by rapid acceleration/deceleration of the brain resulting in cerebral contusion and/or axonal shearing, often accompanied by hemorrhage and hypoxia. This primary injury not only causes direct physical damage, but also initiates a complex series of physiological and biochemical reactions that lead to secondary neurodegeneration and associated functional deficits (McIntosh 1994; Yakovlev and Faden, 1995). Because these secondary injury changes evolve over time, they are potentially preventable. Understanding the mechanisms involved may lead to treatments that preserve tissue and limit functional impairment (Laurer and McIntosh, 2001; Faden 2002).

2. MECHANISMS OF TRAUMATIC BRAIN INJURY

Secondary injury includes a variety of physiological and biochemical changes that result in neuronal cell death and tissue destruction (for review see Panter and Faden, 1992; McIntosh 1994; Yakovlev and Faden, 1995; Lewen *et al* 2000; Raghupathi *et al* 2000; Morganti-Kossmann *et al* 2001). Physiological changes include reductions in blood flow and cerebral metabolism, development of edema and increased intracranial pressure, breakdown of the blood-brain barrier, and immune/inflammatory activation. The latter results both from direct infiltration of blood cells into the brain parenchyma from which they are normally isolated, as well as from acute

reactions of resident brain cells (astrocytes, microglia) to injury. Biochemical disturbances include ionic imbalances (e.g. excess intracellular sodium and calcium), neurotransmitter release (e.g. glutamate/excitotoxicity), changes in gene expression, generation of eicosanoids and lipid degradation by-products, and oxidative stress due to production of a variety of free radicals. Importantly, many of these biochemical reactions can be linked to the activation of proteases, providing support for the concept that proteases play a critical role in cell death, tissue loss and behavioral deficits after TBI.

TBI-induced neuronal cell death may morphologically resemble either necrosis or apoptosis. Necrosis is typified by failure to maintain energy stores and ionic gradients, organelle swelling, and eventually, cell lysis, which releases enzymes and debris that are potentially toxic to surrounding cells, and provokes an inflammatory response. Generally, necrosis occurs relatively early after injury (minutes to hours) in the cortical region that receives the impact. Signal transduction pathways causing necrosis have not been unequivocally defined, as this cell death phenotype has been largely perceived as a passive response to energy failure and associated loss of ionic homeostasis. However, recent evidence suggests that necrosis is not necessarily a passive process, and may involve activation of specific biochemical pathways (Tavernarakis and Driscoll, 2001; Syntichaki *et al* 2002). In contrast to necrosis, apoptotic cell death evolves over a longer time frame (hours to weeks), and may involve many individual cells scattered widely within a brain region. Apoptosis is a coordinated cascade that is often both energy and protein synthesis dependent and characterized morphologically by chromatin/nuclear condensation or fragmentation, plasma membrane blebbing, and cellular shrinkage, without inflammatory involvement (Clarke 1990).

A growing number of studies indicate that neuronal death may result in phenotypes that are neither classically apoptotic nor necrotic (Kitanaka and Kuchino, 1999; Formigli *et al* 2000; Sperandio *et al* 2000). Such unconventional cell death phenotypes are also present after TBI, where cells with mixed features of apoptosis and necrosis have been observed (Singleton and Povlishock, 2004). Studies also suggest that the type and temporal course of cell death after TBI depends on a variety of factors, including sex, age and injury severity (Rink *et al* 1995; Conti *et al* 1998; Newcomb *et al* 1999; Pohl *et al* 1999; Kupina *et al* 2003; Prins and Hovda, 2003). Characterization of these phenotypic differences in cell death and their related mechanisms is important for the development of optimal clinical treatment strategies for head trauma patients. Important in this regard is that blockage of pathways involved in one type of cell death may enhance pathways involved in other forms of cell death.

3. EXPERIMENTAL MODELS OF TBI

Several different models have been developed for experimental studies of TBI mechanisms. The most widely-used and clinically relevant models include injury induced by lateral fluid-percussion (LFP), controlled cortical impact (CCI), or impact-acceleration (IA). LFP injury causes transient reductions in blood flow, and a focal contusion with mixed apoptotic and necrotic cell death in the ipsilateral cortex, followed by delayed damage to surrounding cortical and subcortical structures (McIntosh *et al* 1987; Cortez *et al* 1989; Sato *et al* 2001). In contrast, the CCI model primarily induces a focal contusion at the site of impact, which is characterized by early necrosis (within minutes), persistent reductions in blood flow, and delayed, regional apoptosis (Dixon *et al* 1991; Kochanek *et al* 1995; Fox *et al* 1998; Newcomb *et al* 1999). Impact-acceleration models cause widely disseminated, diffuse cell death and axonal injury, rather than a contusion (Foda and Marmarou, 1994).

4. CYSTEINE PROTEASES

Calpains, caspases and most cathepsins are papain superfamily cysteine proteinases that are derived from inactive precursors (zymogens). Though they differ in terms of signals that activate their parent zymogens, they share many substrates. In addition, they have important roles in the maintainence of cell function under normal conditions, but contribute to cell death pathways when overactivated. Thus, the activity of these proteases is tightly regulated by highly selective endogenous inhibitors (e.g. calpastatin, inhibitor of apoptosis proteins, cystatins/stefans). In recent years the roles of calpains and caspases in TBI have been extensively investigated. In contrast, there is less information on the involvement of cathepsins in TBI, but they have been implicated in secondary injury mechanisms.

4.1 Calpain

Calcium-activated neutral proteinases (calpains) are cytosolic endopeptidases with calcium binding properties (Guroff 1964; Sorimachi *et al* 1997). More than a dozen different calpains have been identified; most are ubiquitously distributed (for general review see Vanderklish and Bahr, 2000; Wang 2000; Huang and Wang, 2001; Ray *et al* 2003). Only calpains I (CAPN1, μ-calpain, micro-calpain) and II (CAPN2, m-calpain, milli-calpain) have been studied with regard to TBI; they are activated by micro- or milli-molar concentrations of calcium, respectively, due to different affinities of their calcium-binding domains (Mellgren 1987). Nevertheless, calcium levels

required for activation in live cells may be reduced by association with lipid components of cell membranes, and/or by a specific calpain activator protein found in brain, blood and skeletal muscle (Coolican and Hathaway, 1984; Melloni *et al* 1998). In addition, calpains I and II (hereafter referred to as calpains) may be activated autolytically (Suzuki *et al* 1981; Pontremoli *et al* 1985). Calpain activity is regulated endogenously by calpastatin, which is a reversible, highly selective calpain inhibitor that is itself degraded by active calpains (Melloni *et al* 1996). Thus the calpain-calpastatin ratio critically determines calpain activity (Salamino *et al* 1992).

Calpains preferentially modify cytoskeletal proteins such as actin, spectrin, neurofilaments (NF) and microtubule associated protein 2 (MAP2) (Nelson and Traub, 1982; Stracher 1999). Other substrates include plasma membrane proteins (adhesion molecules, phospholipases), myelin proteins, kinases, proteins involved in intracellular calcium signaling (Ca^{+2} ATPase, calcineurin, L-type Ca^{+2} channels, NMDA receptors), and proteins with regulatory roles in apoptosis (p53, Bax, Bcl-2, poly(ADP)-ribose polymerase) (Nelson and Traub, 1982; Shields and Banik, 1999; Stracher 1999; Wang 2000).

Calpains and caspase 3 cleave α-spectrin into 145 kd and 120 kd signature fragments, respectively (Nath *et al* 1996b; Wang *et al* 1998). The presence of these distinctive cleavage products can be detected with immunoblotting methods or specific antibodies as a measure of enzyme activation (Saatman *et al* 1996a; Springer *et al* 1997; Pike *et al* 1998). Alternatively, calpain activity has been expressed as the ratio of active (76-kDa) to precursor (80 kD) isoforms (Fujitani *et al* 1997; Grynspan *et al* 1997).

Calpain activation after TBI is likely a result of increased intracellular calcium subsequent to excitotoxic glutamate stimulation (for review see Vanderklish and Bahr, 2000). Temporal studies of calpain activation have been carried out in several different experimental TBI models (Saatman *et al* 1996a; Pike *et al* 1998). Rapid and sustained calpain activation (15 minutes - 24 hours) was observed in cortical tissue after CCI injury (Kampfl *et al* 1996; Newcomb *et al* 1997). The largest increase (15 minutes - 6 hours) in such activity preceded most neuronal death (Kampfl *et al* 1997). Active calpains were localized to neuronal dendrites, soma and axons (Newcomb *et al* 1997). Similar results were observed after LFP injury (Saatman *et al* 1996a).

Calpain activity varies regionally after experimental TBI, with the largest increase observed in the injured cortex, though delayed and prolonged activation also occurred in regions that are not directly injured, such as the hippocampus, thalamus and striatum (Pike *et al* 1998; Saatman *et al* 1996a). These data support the potential involvement of calpains in necrotic death and axonal injury after TBI, and they also suggest that calpains may be involved in delayed, secondary apoptotic cell death as well. Calpain-mediated cleavage

fragments of α-spectrin were also present in cerebrospinal fluid from 24-72 hours after experimental injury (Pike *et al* 2001). Thus, it has been suggested that calpain activation in CSF may serve as useful biomarker of neurodegeneration in humans (Pike *et al* 2001). Consistent with this view, calpain-mediated α-spectrin breakdown products have been found in human cerebrospinal fluid after cerebral hemorrhage (Lee *et al* 1993).

Calpain inhibition improves functional outcome and/or reduces lesion volume after TBI. Continuous intravenous infusion of the calpain inhibitor AK295 for 48 hours after injury improved both motor and cognitive deficits after LFP (Saatman *et al* 1996b). However, in a subsequent study identical administration of AK295 failed to reduce calpain-mediated α-spectrin breakdown, lesion volume or apoptotic cell death (Saatman *et al* 2000). In studies to evaluate the therapeutic window for calpain modulation, the calpain inhibitor SJA6017 improved behavioral outcome when given as late as 4 hours after diffuse injury in the mouse (Kupina *et al* 2001), though this treatment also failed to reduce calpain-mediated α-spectrin breakdown (Kupina *et al* 2001). Such studies are promising, but additional work is needed to verify that the benefit provided by these inhibitors results from calpain inhibition rather than other actions of these compounds. In this regard, it should be emphasized that drugs such as calpain inhibitor II, MDL-28170 and SJA6017, like many other calpain inhibitors, lack selectivity and have limited cellular permeability. Indeed, as summarized in Table 1, most of the pharmacological inhibitors of cysteine proteases are only relatively selective for individual proteases.

Calpains may contribute to the pathophysiology of injury through a variety of mechanisms, but most studies to date have focused on their potential contribution to the cytoskeletal degradation that follows traumatic insults. MAP2 and NF are not only key components of the neuronal cytoarchitecture but also preferred calpain substrates. Indeed, degraded forms of low molecular weight NF observed after TBI specifically resemble those produced by calpain cleavage, rather than caspase 3 or cathepsins B or D (Posmantur *et al* 1998).

Shortly after TBI, calpains are activated and MAP2 is reduced in the dendritic compartment (Taft *et al* 1992; Posmantur *et al* 1996). In addition, localized ionic imbalances within the axon may lead to increases in calcium, calpain activation, and subsequent compaction and degradation of NF (Buki *et al* 1999; Povlishock *et al* 1999; Maxwell *et al* 1997; Posmantur *et al* 2000). In head injured patients, calpain activation was concurrent with reductions in NF in the corpus callosum (McCracken *et al* 1999). Administration of calpain inhibitor II also markedly attenuated NF degradation in neural dendrites and soma, and reduced the lesion in ipsilateral cortex after CCI (Posmantur *et al* 1997). This treatment also decreased diffuse axonal damage in the corpus callosum and subcortical white matter.

Table 1. Specificity of cysteine protease inhibitors*

Inhibitor	Primary Inhibition	Secondary Inhibition	References
z-VAD-fmk	caspases 1,3,6,7	calpain	Blomgren *et al* 2001 Schotte *et al* 1999 Waterhouse *et al* 1998 Wolf *et al* 1998
		cathepsins B/H/X	Rozman-Pungerčar *et al* 2003 Vancompernolle *et al* 1998
		AIF	Susin *et al* 1996
z-DEVD-fmk	caspase 3,6,7	calpain cathepsin B/X	Knoblach *et al,* in press Rozman-Pungerčar *et al* 2003 Schotte *et al* 1999 Vancompernolle *et al* 1998
z-YVAD-fmk	caspase 1	cathepsins B/X	Rozman-Pungerčar *et al* 2003
Calpain inhibitor II (ALLM)	calpain	cathepsins B/L	Wang *et al* 2001 Squier *et al* 1994
		proteasome chymotrypsin	Wang *et al* 1994 Sasaki *et al* 1990
MDL28170	calpain	cathepsins B/L	Wang *et al* 2001
SJA6017	calpain	cathepsins B/L	Wang *et al* 2001
AK 275	calpain	cathepsin B	Carafoli *et al* 1998
z-FA-fmk	cathepsin B/L	caspases 2, 3, 6, 7	Lopez-Hernandez *et al* 2003 Eldadah *et al* 1997

* Widely used parmacological inhibitors as discussed in the text are listed. The list is not all-inclusive.

Similarly, infusion of the calpain inhibitor MDL-28170 reduced axonal damage induced by an impact-acceleration model of TBI (Buki *et al* 2003). Alternatively, unrelated but beneficial treatments that improve function in experimental injury models also reduce calpain activity and NF degradation. Examples include alterations in free Mg^{+2} with diet or drugs, or treatment with cyclosporine A (Okonkwo *et al* 1999; Saatman *et al* 2001).

Calpains are activated in *in vitro* models of cell death that mimic a variety of TBI-induced mechanisms. These include cell death induced by axonal stretch, oxygen-glucose deprivation, calcium overload, and metabolic inhibition (Wang *et al* 1996; Brana *et al* 1999; Pike *et al* 2000; Pang *et al* 2003). In these models as well as *in vivo,* calpains are strongly associated with necrotic cell death and related increases in intracellular calcium (Wang *et al* 1996; McGinnis *et al* 1999b). Moreover, calpains cleave and inactivate Apaf-1, as well as caspases 7, 8, 9, thereby inhibiting both the intrinsic and extrinsic pathways of apoptosis (Chua *et al* 2000; Wang 2000; Bizat *et al* 2003). However, recent studies also indicate that calpains may be involved in certain types of neuronal apoptosis. Calpain activation contributes to apoptosis induced by staurosporine, trophic factor deprivation, or potassium

withdrawal (in cerebellar granule cells) (Nath *et al* 1996a; Nath *et al* 1996b). Calpains may also activate the pro-apoptotic inducer Bax, and generate activating or inactivating cleavages of caspase 3, as well as activation/inactivation of p53 (McGinnis *et al* 1999a; Sedarous *et al* 2003). The reasons for these opposite effects are presently unclear, but they are hypothesized to reflect steps in complicated feed-back pathways that are not yet defined (Wang 2000; Sedarous *et al* 2003).

Indeed, calpains likely serve as important modulators of intracellular signaling, rather than only as degradative proteases. This view is supported by the observation that calpain-mediated cleavage tends to result in limited proteolysis of its substrates, rather than digestion (Vanderklish and Bahr, 2000; Wang 2000). In this regard, calpains may assist with the regulation of short-lived signal transduction proteins, such as Jun, Fos, c-myc and cyclin D1, which are involved in transcription and cell-cycle regulation (Carafoli and Molinari, 1998; Small *et al* 2002).

Most studies on the role of calpains in cell death have focused on neurons, but calpains are also expressed in many other cell types, including glia and muscle, where they may be a factor in additional secondary injury mechanisms and pathophysiology (Lee *et al* 2000; Nakanishi 2003a). For example, subarachnoid hemorrhage activates calpains in vascular smooth muscle cells, and this activation leads to smooth muscle contractions and vasospasms in cerebral vessels that can be reversed by calpain inhibitors (Minami *et al* 1992; Sato *et al* 1997). Intravenous injection of calpain inhibitors also reduces blood-brain barrier breakdown associated with subarachnoid hemorrhage, but the mechanism underlying this process remains unknown, and likely multifactorial (Germano *et al* 2002). Nevertheless, taken together such findings suggest that calpain activation may also contribute to the pathophysiology of TBI through secondary reductions in cerebral blood flow and breakdown of the blood-brain barrier.

4.2 Caspases

Cysteine-dependent aspartate-specific proteases (caspases) are cysteine proteases that require an aspartate residue in the substrate P1 site. Caspases subserve important roles in both apoptotic cell death and inflammation (for review see Eldadah and Faden, 2000; Nicholson 2000; Denault and Salvesen, 2002). More than a dozen caspases have been identified, and as a group, they are remarkably conserved across species (Denault and Salvesen, 2002). They exist in the cytosol as inactive zymogens that are cleaved and reorganized into active heterotetramers in response to the appropriate death signals. Caspases have been divided into 3 main functional groups (Eldadah and Faden, 2000). Caspases 2, 8, 9 and 10 are categorized as “initiator” caspases because of

their apical position in apoptotic protease cascades, and caspases 3, 6 and 7 are designated as "executioner" caspases because they function as relatively downstream effectors of apoptosis, and cleave proteins that maintain the integrity of the nucleus and the plasma membrane, resulting in morphological manifestations of apoptosis. Caspases 1, 11 and 12 are involved in cytokine synthesis as well as cell death, and have thus been called "inflammatory"caspases.

There are multiple caspase activation pathways. An "intrinsic" cascade is initiated at the level of the mitochondrion with release of cytochrome c and apaf-1; the resulting multimolecular complex (apoptosome) activates caspase 9, which subsequently activates caspase 3 (Chang and Yang, 2000). An extrinsic cascade is initiated by ligand binding of cell surface "death" receptors of the TNF/nerve growth factor superfamily (Fas/TNFR1/APO-1), and trimerization/association of these receptors with death adaptor proteins that activate caspase 8. Caspase 8 then activates caspase 3 (Chang and Yang, 2000).

Caspases have many substrates, including cytoskeletal (actin, spectrin, tubulin) and structural proteins (lamin) that are also substrates for calpain (for review see Wang 2000; Fischer *et al* 2003). Other important proteins cleaved by caspases include DNA repair enzymes (PARP, DNA-protein kinase), and regulators of apoptosis (Apaf-1, Bax, Bid), cell adhesion (cadherins and catenins), and cell cycle (cyclins A and E, Rb, p21), as well as DNA binding proteins (NF-κB, SP1, CREB), many kinases, and proteins linked to specific neurodegenerative disorders (amyloid precursor protein, presenilins, Parkin, Huntingtin). Like calpain, caspases do not completely degrade their substrates but rather, generate peptide fragments with altered activity.

Caspase activity is tightly controlled by a number of regulatory proteins that operate at different levels of the activation cascade. Thus, the amount of caspase activation reflects interactions between these regulatory factors and the strength of the apoptotic stimulus (Cain *et al* 2002). Endogenous caspase inhibitors include decoy receptors, competitive adaptor molecules, and specific inactivator proteins such as CrmA, p35, and the inhibitor of apoptosis (IAP) family of proteins (IAPs-1 and -2, NIAP, XIAP, survivin and others) (for review see Stennicke *et al* 2002). Several heat-shock proteins (Hsp-70, Hsp-90, Hsp-27), also prevent activation of caspase 9 through interactions with the apoptosome (Cain *et al* 2002). Additional regulation is achieved by proteins that promote caspase activation by inactivating endogenous caspase inhibitors. Examples include the second mitochondrial activator of caspases (Smac) and OMI/Htra2, which inactivate IAPs (Srinivasula *et al* 2001; Suzuki *et al* 2001).

Caspases have been extensively investigated with regard to TBI, and both extrinsic and intrinsic caspase activation pathways are involved in secondary

injury and cell death. A schematic diagram of caspase pathways that have been demonstrated in models of TBI and human head injury is shown in figure 1.

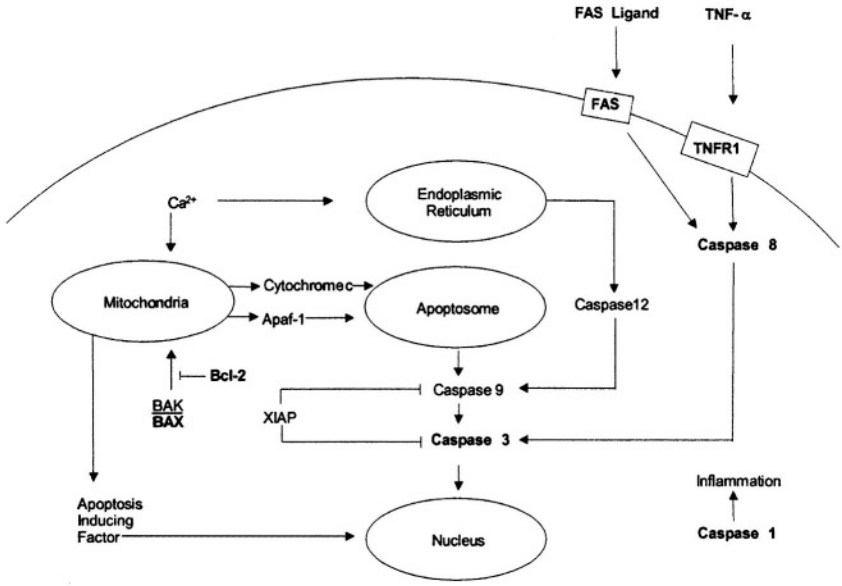

Figure 1. Components of intrinsic and extrinsic caspase activation pathways that are associated with traumatic brain injury are shown in the diagram. Proteins listed in bold typeface have been documented in cases of human head injury, as well as in experimental models of traumatic brain injury. See text for additional details.

Support for the intrinsic pathway comes from studies showing release of Apaf-1 and cytochrome c after TBI, as well as inactivation of XIAP and increases in the bax:bcl-2 ratio in injured cortical tissue (Clark *et al* 1997; Buki *et al* 2000; Keane *et al* 2001; Yakovlev *et al* 2001; Sullivan *et al* 2002; Raghupathi *et al* 2003). In addition, caspases 9 and 3 were activated in injured cortex from ~1- 48 hours after LFP or CCI (Yakovlev *et al* 1997; Beer *et al* 2000b; Buki *et al* 2000; Knoblach *et al* 2002). Such activation correlated regionally (e.g. hippocampus, thalamus) with increases in TUNEL staining and DNA fragmentation (~24 - 168 hours), and was preferentially activated in neuronal dendrites and axons, as well as the soma (Beer *et al* 2000b; Buki *et al* 2000; Knoblach *et al* 2002). This suggests that activation of caspase 9 and 3 may contribute to synaptic loss and axonal injury, as well as apoptotic cell death (Buki *et al* 2000).

Increases in Bcl-2, DNA fragmentation, caspase 3 and leakage of caspase 3 into CSF have been observed after TBI in humans (Clark *et al* 1999; Clark

et al 2000; Harter *et al* 2001). The presence of extracellular caspase 3 in primary cell cultures and in CSF from TBI patients may suggest additional novel roles for this protease in reorganization/modification of the extracellular matrix that not have not yet been considered (Hentze *et al* 2001).

Components of the extrinsic activation cascade also appear to be involved in TBI. TNF-α, Fas receptors, Fas Ligand, and caspase 8 are increased and/or activated over a time course similar to that observed for caspases 9 and 3 (Knoblach *et al* 1999; Beer *et al* 2000a; Beer *et al* 2001; Knoblach *et al* 2002; Qiu *et al* 2002). The distribution of active caspase 8 was somewhat broader than caspase 9, as it was found in neurons, astrocytes and oligodendrocytes (Beer *et al* 2000a; Beer *et al* 2001), though this has not been entirely consistent (Knoblach *et al* 2002). Nevertheless, clinical studies indicate that Fas Ligand expression and caspase 8 activation increased after TBI in humans (Ertel *et al* 1997; Zhang *et al* 2003).

Inflammatory and immune reactions contribute substantially to secondary injury processes. Caspase 1, a so-designated inflammatory caspase, is associated with TBI in humans (Clark *et al* 1999), yet experimental evidence to support an important role for this caspase in TBI remains mixed. Caspase 1 was reported to be activated after CCI in the mouse (Fink *et al* 1999). In addition, lesion volumes and free radical generation were reduced in dominant negative caspase 1 inhibitor mice (Fink *et al* 1999). In contrast, however, caspase 1 activation decreased after LFP in the rat, whereas mRNA for caspase 1 increased (Yakovlev *et al* 1997). These differences may be species-related, as inflammatory mechanisms in mouse may differ from rats (Colton *et al* 1996; Kuhn and Wrathall, 1998).

An alternative intrinsic caspase activation scheme has been described that involves activation of caspase 12 by the endoplasmic reticulum (ER) in response to stress induced by glucose deprivation, disrupted calcium homeostasis (and calpain activation) or free radical damage (Morishima *et al* 2002). Activated caspase 12 may subsequently activate caspase 9 without assistance from the apoptosome or release of cytochrome c (Morishima *et al* 2002). Caspase 12 activation in the cortex and hippocampus correlates with the severity of CCI injury in the rat (Larner *et al* 2004). However, caspase 12 may not be significantly involved in cell death in humans, although it may contribute to inflammation (Saleh *et al* 2004).

In general, caspase inhibition strategies improve function and reduce lesion size in experimental TBI. However, results from such studies are mixed, probably due to differences in dosage, administration, and possibly species. Intracerebroventricular (icv) injection of the pan-caspase inhibitor z-VAD-fmk, or the caspase 3 selective inhibitor z-DEVD-fmk improved functional recovery after LFP in rats (Yakovlev *et al* 1997; Knoblach *et al* 2002). In addition, icv pretreatment with z-VAD-fmk, or the caspase 1 selective inhibitor Ac-YVAD-cmk, reduced lesion volume after CCI in mice

(Fink *et al* 1999). However, intraparenchymal infusion of z-DEVD-fmk over several days after combined CCI/hypoxia reduced lesion size, but did not improve functional outcome (Clark *et al* 2000), and icv pretreatment with z-Boc-fmk, another pan-caspase inhibitor, had no effect on lesion volume after CCI (Sullivan *et al* 2002).

Recent studies indicate that z-VAD-fmk and z-DEVD-fmk are only relatively selective for caspases, as they inhibit both calpains and cathepsins at micromolar concentrations (see Table 1). Moreover, such non-specific activity may contribute to the efficacy of some of these compounds in live cell preparations and *in vivo* (Vancompernolle *et al* 1998; Waterhouse *et al* 1998; Wolf *et al* 1999; Blomgren *et al* 2001; Rozman-Pungercar *et al* 2003; Knoblach, *in press).* Therefore, mechanistic conclusions derived from the use of these compounds should be interpreted with caution.

Caspase activation (caspase 3, 8 or 9) in injured cortical neurons does not completely co-localize with TUNEL staining (Clark *et al* 2000; Knoblach *et al* 2002). In addition, caspase 3 activation is not as prevalent as is TUNEL labeling. These findings may, in part, reflect differences between the time course of caspase activation and nuclear degradation and/or non-specific attributes of TUNEL labeling (e.g. necrosis) (Charriaut-Marlangue and Ben-Ari, 1995; Grasl-Kraupp *et al* 1995). They may also indicate that non-caspase mediated apoptosis contributes to TBI-induced cell death. Consistent with this view, apoptosis-inducing factor (AIF) translocates to the nucleus from 2 - 72 hours after TBI and this event is associated with DNA fragmentation (Zhang *et al* 2002).

Extrinsic and intrinsic caspase pathways are interactive. Thus, caspase 3 may activate caspases 2, 6, 10, 8 and 9 through feedback amplification (Slee *et al* 1999). The pro-apoptotic bcl-2 family member bid may also function as a point of intersection between intrinsic and extrinsic caspase cascades. Cleavage of Bid by caspase 8 produces a truncated form of this protein (tBid) that promotes cytochrome c release and activation of caspase 9 (Luo *et al* 1998). In addition, tBid may also activate caspase-independent apoptosis, via release of AIF (van Loo *et al* 2002). Caspase 8, caspase 9 and tBid increased in the injured cortex over a similar time frame (6 hours - 3 days), which suggests that extrinsic/intrinsic cross-talk may indeed occur after injury (Franz *et al* 2002).

There is also evidence for cross-talk between cysteine proteases in general. Calpain may release active cathepsins into the cytosol via targeted degradation of lysosomal membranes (Yamashima 2000). In addition, calpain may prevent activation of the intrinsic pathway by cleaving caspases 3 and 9 and Apaf-1 (Chua *et al* 2000; Bizat *et al* 2003). However, caspase 3 can cleave calpastatin, thereby enhancing the activity of calpain (Porn-Ares *et al* 1998). Cysteine proteases may also interact indirectly. For example, caspase 3 may cleave and inactivate plasma membrane pumps, which leads to a rise in

intracellular calcium, activation of calpain and eventual necrosis (Schwab *et al* 2002). In this regard, it is notable that caspase activation has been observed as early as 1 hour after TBI in cortical regions that eventually comprise the necrotic core and cavity of the lesion (Knoblach *et al* 2002). Thus, such "secondary necrosis" may contribute to TBI. Indeed, it is not entirely clear which cysteine protease dominates secondary injury processes. A direct comparison of α-spectrin cleavage fragments indicated that calpain-mediated proteolysis was both greater in magnitude and more prolonged than that due to caspase 3, in a CCI model that preferentially shows a necrotic profile (Pike *et al* 1998; Newcomb *et al* 1999). Possibly, caspases may predominate in milder or more diffuse injuries, but additional studies are needed to correlate protease activation with the type of cell death and injury severity.

Lastly, there are marked age-dependent differences in caspase activation and susceptibility to apoptotic cell death in the CNS that may impact treatment strategies for pediatric and adult TBI. Several proteins of the intrinsic pathway (Apaf-1, caspase 3) are highly expressed in neonatal rats, and comparatively down-regulated in adults (Yakovlev *et al* 2001). This may, in part, explain why neuronal death after TBI in very young animals is primarily apoptotic, whereas in adults it is primarily necrotic (Bittigau *et al* 1999).

4.3 CATHEPSINS

Proteases of the endosomal-lysosomal system degrade many proteins and cellular organelles as part of normal turnover. Endosomal-lysosomal dysfunction is associated with aging and chronic neuronal degenerative conditions such as Alzheimer's disease and lysosomal storage disorders (for review see Nixon *et al* 2001; Bahr and Bendiske, 2002). Recent evidence also links alterations in endosomal-lysosomal function to mechanisms involved in acute CNS injuries such as cerebral ischemia and TBI.

In acute injury processes, lysosomes were historically described as "suicide bags" that would rupture and spill their lethal contents internally and/or extracellularly as part of end stage necrosis (De Duve and Wattiaux, 1966). This type of acute release is likely to be involved in the primary mechanical insult of TBI. However, recent work points to lysosomes and the endoplasmic reticulum as potentially important modulators of mitochondrial death/survival signaling in apoptotic pathways, as well. Such data are intriguing, as they challenge the conventional view that lysosomes are passively involved in necrotic cell death. Moreover, they may suggest new avenues for treatment of TBI.

Cathepsins, so-named from a Greek root word meaning "to digest", are among the most abundant lysosomal proteases (Nakanishi 2003b). Cathepsin substrates in the CNS include myelin basic protein, neurofilaments, beta-amyloid, pro-forms of several hormones and neuropeptides, and the extracellular matrix, where cathepsins play important roles in tissue reorganization and invasion, as well as angiogenesis (for review see Turk *et al* 2000; Brömme and Kaleta, 2002; Turk and Guncar, 2003). In contrast to calpains and caspases, cathepsin proteolysis is aimed at terminal protein degradation, rather than cleavage to modulate substrate activity, although there are exceptions to this generalization (Brömme and Kaleta, 2002). In addition, cathepsins are optimally active in the relatively acid environment found in lysosomes (particularly cathepsin D), even though some retain activity in neutral conditions, or are stabilized in neutral biological environments (cathepsin B) (Mort and Buttle, 1997; Dash *et al* 2003). In certain diseases, active cathepsins also preside in the extracellular compartment, where they contribute to degradation and rearrangement of the extracellular matrix (Turk *et al* 2000). Endogenous inhibitors of cathepsins include stefins (stefin A/cystatin A, stefin B/cystatin B), cystatins (cystatin C, S and others) and kininogens, and activity *in vivo* likely reflects a balance between enzyme activation and inhibition (for review see Kos and Lah, 1998).

Only a few studies directly indicate an involvement of cathepsins in TBI or related *in vitro* models of acute injury. However, considerable evidence suggests that these proteases may be important mediators of secondary injury. Recent studies utilizing microarray techniques revealed that mRNAs for a number of cathepsins increase after TBI (Tang *et al* 2002; Natale *et al* 2003). Cathepsin D, B and L mRNAs were elevated from several hours to 3 days after CCI or LFP in rodents. Moreover, active forms of these proteases were detected by immunoblotting experiments with a similar time course (Knoblach, unpublished observation). Cathepsins B, L, C, and D are also selectively upregulated after spinal cord trauma or root avulsion injury (Ellis *et al* 2004; Hu *et al* 2002).

In addition, data from other models of acute neuronal injury indicate that increases in cathepsin expression/activation may contribute to secondary cell death. For example, expression of cathepsins B and L was upregulated in selectively vulnerable neurons after cerebral ischemia, and infusion of relatively selective synthetic cathepsin B/L inhibitors (CA-074, CP-1) improved functional outcome and reduced neuronal death (Yamashima *et al* 1998; Tsuchiya *et al* 1999; Seyfried *et al* 2001). Intravenous infusion of the endogenous cathepsin B/L inhibitor stefin A also reduced infarct volume after ischemia (Seyfried *et al* 1997, 2001). In models of acute excitotoxicity induced by kainic acid, the activity of cathepsins D and E was upregulated in degenerating neurons (Hetman *et al* 1995, 1997; Tominaga *et al* 1998).

Together these findings suggest a pathophysiological role for cathepsin activation in acute injuries such as TBI.

Further support comes from the observation that many currently available inhibitors of calpain or caspase also inhibit certain cathepsins (Table 1), which suggests that some of the beneficial effects attributed to these compounds may, in part, reflect cathepsin inhibition. In addition, factors with established pathophysiological roles in TBI also evoke release of active cathepsins from the lysosomal compartment. For example, calpain has been co-localized with compromised lysosomes after cerebral ischemia in the monkey, where it may degrade lysosomal membranes and release cathepsins into the cytosol (Yamashima *et al* 1998; Yamashima 2000). Also, lysosomes contain high levels of iron, and therefore are highly sensitive to damage initiated by free radicals (Ollinger and Brunk, 1995). Oxidative stress induced by TBI may therefore induce cathepsin release, in part, through this mechanism. Lysosomal release may also be initiated by TNF-α, a secondary injury factor that contributes to neuronal death and functional impairment after TBI (Knoblach *et al* 1999; Guicciardi *et al* 2000; Werneburg *et al* 2002).

Cathepsins have been linked to early stages of caspase-dependent and caspase-independent cell death pathways in non-neuronal cells. Many of the molecular components of these pathways are also operative in TBI. For example, in hepatocytes, apoptosis induced by TNF-α involves sequential activation of caspase 8, release of cathepsin B, truncation of Bid, release of cytochrome c and activation of caspases 9 and 3 (Guicciardi *et al* 2000; Reiners *et al* 2002; Werneburg *et al* 2002). In lymphocytes, released cathepsin D activates Bax, and Bax then induces the release of apoptosis-inducing factor (AIF) (Bidere *et al* 2003). In liver cells, cathepsin B activates caspase 11, and in HeLa cells cathepsin B directly activated nuclear degradation (Schotte *et al* 1998; Vancompernolle *et al* 1998). Since a variety of cell types utilize the cathepsins in apoptotic pathways, it is likely that they may contribute to apoptosis in neurons and/or after TBI. Potential roles for cathepsins in neuronal cell death pathways are summarized in figure 2.

Several studies implicate cathepsins in neuronal cell death, *in vitro*. For example, cathepsin D is upregulated after neuronal apoptosis induced by camptothecin or staurosporine, and decreases after necrosis induced by glutamate (Adamec *et al* 2000). However, treatment with a cathepsin D inhibitor had no effect on either type of cell death. Cathepsin D was also increased in PC 12 cells after serum deprivation, and was associated with decreased survival (Shibata *et al* 1998). In contrast, cathepsin B decreased after serum deprivation, and survival was improved by cathepsin B overexpression, suggesting a pro-survival role for cathepsin B (Shibata *et al* 1998).

Invading blood cells (monocytes and macrophages), astrocytes and microglia may also represent sources of cathepsin-related damage after TBI. Neutrophils, macrophages and microglia release a variety of cathepsins as part of inflammatory activation, which may degrade the extracellular matrix and contribute to vasogenic edema through endothelial damage and breakdown of the blood brain barrier (Buck *et al* 1992; Nakanishi and Yamamoto, 1998; Kingham *et al* 1999; Nakanishi 2003a; Takuma *et al* 2003). Activated microglia have also been shown to induce caspase-dependent neuronal apoptosis directly via release of cathepsin B (Kingham *et al* 1999; Gan *et al* 2004).

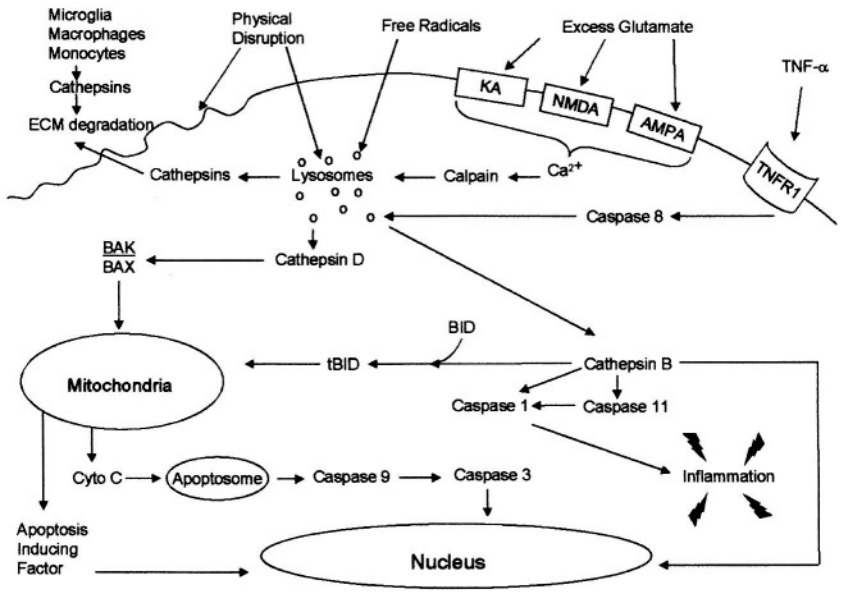

Figure 2. Potential roles for cathepsins in cell death induced by traumatic brain injury are represented graphically. The pathways that are depicted have not directly been associated with traumatic brain injury, but they are supported by observations from non-neuronal cells, or studies of cerebral ischemia. Degradation of the extracellular matrix (ECM) by cathepsins may lead to secondary cell death. See text for additional details.

5. MATRIX METALLOPROTEINASES

Matrix metalloproteinases (MMPs) are soluble endopeptidases that are secreted into the extracellular environment where they are involved in migration, development, plasticity and normal turnover of the extracellular matrix (ECM). More than 20 MMPs have been identified, but in the CNS the most important are MMP-2 (gelatinase A), MMP-3 (stromelysin) and MMP-9 (gelatinase B) (for review see Lukes *et al* 1999; Lo *et al* 2002; Rosenberg 2002). These proteases are expressed and activated in neurons, glia and endothelial cells in response to inflammation and/or cytokine stimulation (e. g. IL-1, TNF-α), oxidative stress, growth factors, and changes in the ECM. The activity of MMPs is regulated by their level of expression, activation of the proenzyme, and inhibition by endogenous tissue inhibitors of metalloproteases (TIMPs). There are 4 different TIMPs (Gardner and Ghorpade, 2003). Of these, TIMP-1 is inducible and its expression is controlled by cytokines and growth factors, similar to MMPs. TIMP-1 regulates the activity of MMP-9, but also is independently involved in a variety of other events, including angiogenesis and protection from some forms of neuronal cell death (Gomez *et al* 1997; Tan *et al* 2003).

The role of the MMP/TIMP system in acute brain injuries has recently been reviewed (Lo *et al* 2002), and extensively studied with regard to cerebral ischemia. MMPs are upregulated after ischemia, and administration of MMP inhibitors reduces edema and subsequent tissue damage (Wang *et al* 2002; Lee *et al* 2004). In addition, both breakdown of the blood brain barrier and lesion volume were reduced after cerebral ischemia in an MMP-9 knock-out mouse, in comparison to wild-type controls. Only a few studies of MMPs have been performed in clinically relevant models of TBI, though they indicate that TBI primarily results in upregulation of MMP-9, and also MMP-2. Synthesis of MMP-9 was increased from 1-4 days after cortical contusion injury; TIMP-1 was also increased over a slightly more delayed time course (von Gertten *et al* 2003). Studies in MMP-9 knock-out mice suggest that this increase contributes to pathophysiology, as MMP-9 knock-outs had smaller lesions after TBI than did wild-type controls (Wang *et al* 2000). In general, metalloproteinases are induced by cellular stresses (i.e. oxidative) that lead to activation of mitogen-activated kinase cascades (MAPK), including those mediated through p38, Jun N-terminal (JNKs), and extracellular-regulated kinases (ERKs) (Rosenberg 2002). Thus, treatment with a MAPK/ERK inhibitor reduced blood-brain barrier breakdown, edema and lesion volume after CCI in the mouse (Mori *et al* 2002). In addition, upregulation of MMP after TBI was shown to be mediated through an ERK/p38 MAP kinase signaling pathway (Wang *et al* 2002).

MMPs may contribute to secondary injury cascades in several different ways. Injury-induced release of cytokines (e.g. IL-1) may induce the

expression/activation of MMPs which degrade the blood brain barrier, and thus contribute to vasogenic edema and extravasation of blood cells into the brain parenchyma (Vecil *et al* 2000). Injury-induced activation of MMPs may also lead to secondary neuronal apoptosis due to disruption of the ECM, as well as to damage of white matter, since certain myelin proteins (e.g. myelin basic protein) are MMP substrates (Rosenberg 2002). MMPs are also involved in neuronal plasticity and reorganization of the ECM, and therefore they may potentially contribute to chronic adaptive changes that promote recovery after TBI as well, though this concept is, at present, largely unexplored.

Age-dependent differences in injury-induced expression of MMPs have been noted; injury-induced MMP activation in rodents is greater and more prolonged with increased age (Kyrkanides *et al* 2001).

6. SERINE PROTEASES

This diverse group of proteases is involved in a wide variety of normal or pathophysiological processes. Components of the plasminogen activator axis (tissue-type and urokinase-type plasminogen activators, their serpin inhibitors, and thrombin, among others) are by far the best studied serine proteases with regard to TBI. These proteases have recently been the subject of several comprehensive reviews (Gingrich and Traynelis, 2000; Lo *et al* 2002; Xi *et al* 2003), and their interaction with protease-activated-receptors (PARs) is reviewed in chapter 13 of this volume. Therefore, we confine our discussion to a brief mention of several other serine proteases that have recently been specifically associated with TBI.

Migration of activated neutrophils from the cerebrovasculature into the parenchyma after TBI correlates with blood-barrier damage and increased cerebral edema (Carlos *et al* 1997; Whalen *et al* 2000). The neutrophil-derived serine proteases elastase and cathepsin G have recently been shown to contribute to these events through degradation of the extracellular matrix (Armao *et al* 1997; Bonnefoy and Legrand, 2000). In addition, there is an emerging role for these proteases as regulators of cytokine activity (i.e. TNF-α, IL-1β, TGFβ) and growth factor availability (Bank and Ansorge, 2001). For example, in TBI patients, influx of neutrophils and release of elastase and cathepsin G in injury foci was correlated with shedding of IL-2 and IL-6 receptors; follow-up studies indicated that elastase and cathepsin G specifically solubilized these receptors (Bank *et al* 1999). Additional studies are needed to assess whether modulation of cytokine activity by this mechanism has a significant impact on secondary inflammation and tissue loss.

Chymotrypsin-like serine proteases (CTSP) and their endogenous serpin inhibitor alpha1-antichymotrypsin (alpha1-ACT) may also be involved in secondary injury after TBI. Alpha1-ACT is increased in glia within 3 hours of TBI in humans (Hausmann and Betz, 2001). In addition, it is expressed by reactive astrocytes from hours to days after experimental penetrating brain injury (Abraham *et al* 1993). Chymotrypsin-like serine protease (CTSP) activity increased progressively from 4 -72 hours after TBI (Movsesyan *et al* 2001). Intracerebroventricular injections of the CTSP inhibitor N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK) over the first 24 hours after injury blocked this increase in CTSP activity. However, TPCK had no effect on cognitive function, and exacerbated motor recovery (Movsesyan *et al* 2001). Moreover, TPCK was toxic to neurons in culture, suggesting that CTSP may be necessary for neuronal survival (Movsesyan *et al* 2001). In contrast, intraperitoneal administration of TPCK improved behavioral function and provided neuroprotection after cerebral ischemia (Hara *et al* 1998). Additional studies are needed to explain these discrepancies.

7. CONCLUSIONS

TBI initiates a complex cascade of biochemical reactions that lead to tissue loss and neurological impairment. Proteases are critically involved in these processes. Future studies are needed that utilize complementary methods (activity assays, immunoblot, immunohistochemistry) to evaluate the role of these enzymes within the context of potential specific cell death pathways after TBI. In addition, concurrent analysis of protease expression and activation, together with that of endogenous inhibitors will provide a more complete view of the dynamic changes that regulate protease-mediated effects, as activity *in vivo* likely reflects a balance between enzyme activation and inhibition. It may also be important to consider interactions between cysteine proteases as a group, since they share multiple points of intersection. Furthermore, shifts in the balance between proteases has been observed in chronic neurodegeneration, and may occur after TBI as well (Nixon 2000).

Studies to address the specificity issues that confound the interpretation of data obtained with widely available protease inhibitors are also needed. Development of knock-out animals that may complement pharmacological approaches has met with partial success. Cathepsin and MMP knock-outs are viable; however, knock-outs of the most important caspases (e.g. caspase 3, 9) are generally lethal (Kuida *et al* 1996, 1998; Wang *et al* 2000; Reinheckel *et al* 2001). Other approaches that have been underutilized to date include overexpression of endogenous inhibitors and use of siRNA constructs,

perhaps in conditionally dependent vectors. Ultimately, more selective reversible inhibitors are needed.

Given the fundamental and interactive role of multiple proteases in cell death processes after TBI it seems unlikely that selective inhibition of a single protease, or even protease class, will provide optimal benefit. Rather, combination strategies or less selective agents that inhibit multiple protease classes are likely to be most effective. It will also be important to continue to compare and contrast potential differences in the activation of proteases and cell death pathways that are linked to age, sex or type of injury, since these variables appear to considerably alter response patterns.

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Chapter 5

Proteases in CNS Infection and Inflammation

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1. INTRODUCTION

In the past the nervous system has long been regarded as a site of immunologic privilege. This conception was based on the assumptions that (a) an anatomically tight interface between blood vessel wall and the neural parenchyma, the so called blood-brain barrier (BBB), forms a strict separation between the systemic immune compartment and the nervous system, and that (b) other immunologic mechanisms such as the expression of major histocompatibility complex (MHC) molecules, T-cell surveillance, and lymphatic drainage are missing. Accumulated experimental knowledge indicates that these notions are no longer tenable. We know that immune mediators occurring in the nervous system are recruited from the systemic lymphoid organs, and that endogenous cells of the nervous system, such as astrocytes and microglia, play key roles in triggering and regulating the immunologic machinery in the nervous system. Much of our present understanding of the pathogenesis in multiple sclerosis (MS) and other disorders of the central nervous system (CNS) is based on information gained through studying experimental animal models. Whereas the investigation of human tissue samples – obtained by biopsies or post mortem – can provide only a focused view at one given time point in the disease process of which the precise pathobiological history cannot be ascertained, such animal models offer an excellent tool to study the pathogenesis and evolution of the entire course of the disease.

Experimental autoimmune (allergic) encephalomyelitis (EAE) is one of the most intensively studied models in immunology and has contributed

profoundly to our understanding of autoimmunity in general and helped to clarify basic aspects of neuroimmunology, such as immune surveillance in the nervous system, physiological regulation of inflammation, and effector mechanisms of neural damage (Gold *et al* 2000). It can be induced in susceptible species by active immunization with myelin proteins or by the adoptive transfer of encephalitogenic T-cells. Histomorphologically EAE is characterized by inflammation, demyelination, and axonal damage, and therefore widely is used as an animal model for MS.

MS is the prototypic inflammatory demyelinating disorder of the CNS (Lassmann 2000). The increasing application of new powerful technologies during recent years revealed new players in the pathogenesis of this disabling disease (Hemmer *et al* 2002). Large-scale sequencing of mRNA from MS brain plaques (Chabas *et al* 2001), gene microarray analysis of transcripts from MS brain, and proteomic analysis of the immune response unravelled a far more complex picture of the underlying immune cascade (Robinson *et al* 2002; Steinman *et al* 2002).

Also for infectious disease of the CNS, especially bacterial meningitis (BM), a substantial body of evidence indicates that immunological reactions are critically involved in the process of tissue destruction.

During recent years our knowledge about the putative involvement of various proteases in the infected as well as the inflamed CNS has increased. In this chapter the role of certain proteases in the pathogenesis of inflammatory and infectious CNS diseases will be reviewed. Most of the presented are based on findings in MS and BM, which does not reflect a preferential expression of these enzymes in these two categories of CNS disorders. The reason is more historical, since most of the fundamental research on inflammatory responses within the CNS were conducted in both fields of neurological disorders, especially in MS.

2. CASPASES

Caspases represent a recently identified family of cysteine-dependent proteases which are mainly implicated as initiators and effector molecules of programmed cell death or apoptosis. Caspases are expressed within the cytoplasma as proenzymes, with the catalytic domain impaired by a prodomain that is usually cleaved by another member of the family, by a pro-apoptotic activator with an associated death domain (FADD) such as Fas- and tumor necrosis factor (TNF)-receptors, or by the intracytoplasmatic apoptotic proteases activating factor (Apaf), among other factors. Once the prodomain is removed an active tetramer is formed.

Among the caspase family, the most prominent members investigated are caspase-1, caspase-8, caspase-9, and caspase-3. The first three are considered as initiator or upstream caspases, while the last one is the main downstream or effector enzyme towards most of the apoptotic pathways converge. Caspase activation is strictly regulated by the control of multiple inhibitory proteins, such as FADD-like ICE inhibitory protein (FLIP), Bcl-2 and inhibitor of apoptosis proteins (IAP) (Thornberry and Lazebnik, 1998). Besides their strong impact on apoptosis caspases exhibit various other functions. For example, caspase-1, also known as interleukin-converting enzyme (ICE), has been shown to play a major role in cytokine processing, e.g. of the proinflammatory interleukin (IL)-1β and IL-18 (Fantuzzi and Dinarello, 1999). Such an activity might be of critical relevance in perpetuating the immune response within an inflammatory environment.

2.1 Caspases in CNS Inflammation

Caspases in MS appear to exhibit pathogenetic relevant functions on at least three different levels: cell death, cytokine production, and impairment of apoptosis. Besides inflammation, demyelination and axonal loss represent two histopathological hallmarks of MS. During recent years various groups intensively investigated the potential relevance of apoptosis in oligodendroglia and neurons during the course of MS. Most of these studies revealed increased expression patterns of caspase-1, caspase-3, and caspase-11, suggesting that these proteases may play a role in apoptotic processes associated with MS pathogenesis. However, since caspases, e.g. caspase-1, are also involved in processing of inflammatory cytokines their putative contribution to the inflammatory reaction within the CNS should be considered. Finally, clearance of encephalitogenic T-cells by apoptosis is a characteristic feature of terminating the immune response in the CNS. Impaired functioning of caspases during this pathway and consequently a reduced frequency of apoptosis of self-reactive lymphocytes has been proposed as an explanation for the chronicity of inflammation in MS.

Studying the cerebrospinal fluid (CSF) as a compartment close to the inflammatory response within the CNS, caspase-1 has been found to be significantly increased in MS patients on the protein level (Franciotta *et al* 2002). Such an increase was also seen on the mRNA level in peripheral blood mononuclear cells from MS patients, interestingly with peak expression levels one week before an acute clinical attack, suggesting that caspase-1 may be critically involved in cytokine processing (Furlan *et al* 1999a). Further studies were conducted in caspase-1 deficient mice. While in the wildtype control animals caspase-1 mRNA expression was upregulated and paralleled by the expression of IL-1β, interferon (IFN)-γ and TNF-α, knock-out mice were less

susceptible for EAE paralleled and mediated, at least in part, by a reduced IFN-γ production and probably a defective Thl development (Furlan *et al* 1999b). In a rat model of EAE increased expression of caspase-1 could be localized to infiltrating cells as well as to neurons, however, the expression of this caspase did not correlate with any increase of apoptotic bodies (Ahmed *et al* 2002), suggesting a non-apoptotic function or an increased downstream inhibition of apoptosis in this model.

Caspase-1 was found to be expressed in MS lesions, however, these observations make a functional role in cytokine-processing more likely than an involvement in apoptotic pathways (Ming *et al* 2002): Caspase-1 mRNA was detected to be increased in MS lesions as well as in the so called normal appearing white matter (NAWM), and active protein was localized to oligodendrocytes and macrophages. When the nuclear fragmented DNA-sensitive staining TUNEL (characteristic of apoptosis and some forms of cell death) was performed in these samples, no co-localization with caspase-1 was observable, making a link to an effective induction of apoptosis less likely. A major substrate for caspase-1 is IL-18, formerly known as IFN-γ inducing factor (IGIF) (Fantuzzi and Dinarello, 1999). Recently it was described that oligodendrocytes are able to express IL-18 (Cannella and Raine, 2004), thus the presence of active caspase-1 in this cell population may be related to an increased processing of this cytokine and subsequently inducing the release of IFN-γ in surrounding lymphocytes, contributing to a proinflammatory environment within the CNS.

Less data are available for the other upstream caspases, which appear more related to the induction of apoptosis. Caspase-8 is upregulated during the clinical course of EAE but is not expressed by oligodendroglia (Hisahara *et al* 2001). Caspase-9 has been implicated in oligodendrocyte death via glutamate receptor overactivation, which has been suggested to be relevant in the pathogenesis of MS (Sanchez-Gomez *et al* 2003). However, no studies have been investigating the expression of caspase-9 in MS lesions so far. During recent years strong interest has been formed on murine caspase-11 as an upstream enzyme in demyelination of EAE and a common activating point for both caspase-1 mediated cytokine processing and induction of apoptosis (Hisahara *et al* 2003). This enzyme, which human closest homolog (60%) is caspase-4, is able to activate both caspase-1 and caspase-3 in inflammatory settings through two different pathways (Kang *et al* 2002). It is strongly upregulated in mouse EAE, and caspase-11 deficient mice are less susceptible to the induction of the disease (Hisahara *et al* 2001).

Caspase-3 expression has been described in both oligodendrocytes and neurons in clinically active EAE as well as *in vitro* (Hisahara *et al* 2001; Meyer *et al* 2001; Ahmed *et al* 2002). In one EAE model with IL-12 induced relapses there was an increase on caspase-3-positive neurons including large axonal portions only in the third relapse coincident with the presence of DNA

fragmentation at that time point (Ahmed *et al* 2002). Such a late onset of neurodegeneration appears similar to the one described in relapsing-remitting MS, where successive inflammatory episodes are thought to result in neuronal death. However, the expression of caspase-3 in MS patients is less clear. CSF from MS patients has shown pro-apoptotic properties when applied to a primary neuronal culture, inducing a caspase-3 dependent cell death, which can be blocked when caspase inhibitors were added (Cid *et al* 2003). The extent of the induced apoptosis related directly to the degree of increased disability of each individual patient and is probably based on the presence of proinflammatory cytokines. On the other hand neuronal apoptosis in MS lesions could not be correlated to caspase activity (Lucchinetti *et al* 2000). In a recent study of an acute newly formed MS lesion in which oligodendroglial nuclear fragmentation morphologically suggestive of apoptosis was described, only 2% of these cells stained positive for caspase-3 (Barnett and Prineas, 2004). The expression of caspase-3 was also absent in another type of autoimmune driven pathology with neuronal death, the paraneoplastic encephalomyelitis (Bernal *et al* 2002), suggesting that caspase-3 independent cell death may be a common feature in immune mediated inflammation in the CNS.

Studies with caspase inhibitors in animal models have been able to show a clear reduction and even a blockade of most apoptotic processes related to inflammation. Oligodendrocyte death after different insults is inhibited *in vitro* by both wide spectrum and more restricted caspase inhibitors targeting caspase-8 and caspase-9, respectively (Ming *et al* 2002; Benjamins *et al* 2003). *In vivo* similar effects were achieved with a putative caspase-1 inhibitor (Furlan *et al* 1999b). However, specificity of these inhibitors is not absolute. Moreover, it has been showed that most of these small peptides are also able to significantly inhibit not only caspases but also cathepsins even at low concentrations (Rozman-Pungercar *et al* 2003). Therefore such inhibitors can be used to ensure the role of caspases in the investigated aspect, but not to define the specific identity of an enzyme.

Caspases appear not only to be involved in cell death of CNS resident cells but also in apoptosis of autoreactive T-lymphocytes. During fetal development most immature autoreactive B- and T-cells are induced to die by apoptosis to avoid autoimmune self-reactions. In adults this system is maintained and autoreactive cell clones are suppressed when no co-stimulatory factors act along with the presented antigen in the CNS through activation of Fas receptor and caspase-8-dependent apoptosis by resident glia (Holtzman *et al* 2000; Pender and Rist, 2001). The failure of this system may be at the origin of the relapsing aspect of MS, since self-reactive lymphocytes from MS patients are less susceptible to become apoptotic (Pender 1998; Macchi *et al* 1999; Zang *et al* 1999). This view is supported by the observation that peripheral blood mononuclear cells from MS patients exhibit

increased mRNA levels not only of Fas receptor, Fas ligand and caspase-8, but also of FLIP, an inhibitory protein that interferes with caspase-8 cleavage by Fas receptor, disturbing the signal transduction and the triggering of apoptosis (Gomes *et al* 2003). *In vitro* studies demonstrated a correlation between the overexpression of FLIP in both T- and B-lymphocytes and the clinical stage of the disease (Semra *et al* 2001; Seidi and Sharief, 2002).

2.2 Caspases in CNS Infection

The role of caspases in infectious diseases of the CNS is better understood than in MS. Caspase-1 protein expression is observable in the animal model of BM as well as in patients (von Mering *et al* 2001; Koedel *et al* 2002). In experimental meningitis caspase-1 expression was directly related to the IL-1β concentration in the CSF and in caspase-1 deficient mice clinical disease severity was diminished and paralleled by a decrease in CSF pleocytosis and cytokine concentrations (Koedel *et al* 2002), pointing the critical role of caspase-1 in proinflammatory cytokine processing. These results were mimicked when the broad spectrum caspase inhibitor z-VAD-fmk was administered after the infection of the animals (Koedel *et al* 2002), underlining the crucial role of caspases in the pathogenesis of BM. In the CSF of BM patients protein levels for caspase-1 were found to be significantly higher compared to those levels measured in samples obtained from patients diagnosed as viral meningitis, MS, or non-inflammatory neurological disorders (Franciotta *et al* 2002). Moreover, caspase-1 protein levels in the CSF correlated negatively with the clinical outcome of affected patients.

In post mortem studies in patients with BM around 60% of the neurons stained positively for activated caspase-3, and most of these cells also revealed apoptotic features (Gerber *et al* 2001). Interestingly, apoptosis and caspase-3 expression were only detectable in the dentate gyrus, while other brain areas suffered from necrosis and did not express this protease. This regional caspase-3 expression was also found in animal models (Braun *et al* 1999; Gianinazzi *et al* 2003). Moreover, the infecting agent might drive the apoptotic program, as infant rats of the same litter infected with *S. pneumoniae* or group B streptococcus showed divergent patterns of cell death, with a more necrosis-biased process induced by the second pathogen group (Bifrare *et al* 2003). This remains a puzzling observation, since the meningitis pro-apoptotic activator, although still unidentified, is thought to be related to the detrimental effects of the host immunological system rather than the infectious agent, based on the observation that the blockade of leukocyte extravasation in animal models reduced substantially neuronal death (Braun *et al* 1999). In the same study, Braun and co-workers showed the efficacy of

caspase inhibitors in the reduction of neuronal death, not only delaying neurodegeneration, but even preventing it.

In BM, as well as in MS, the pro-apoptotic signal is believed to be originated in the host immunological system, either through heterocellular contact and cell-mediated cytotoxicity via Fas-ligand or via soluble factors such as TNF-α (Dinkel *et al* 2004). In contrast, a virus can activate such a pro-apoptotic cascade involving members of the caspase family through the viral coat and capside proteins. This mechanism has been detected e.g. for the West Nile virus (Yang *et al* 2002), rabies virus (Faber *et al* 2002) and the human immunodeficiency virus (HIV) (Patel *et al* 2000; Garden *et al* 2002). All viral proteins were able to induce caspase-mediated apoptosis both *in vivo* and *in vitro,* but the molecular basis of this activation is poorly understood so far. *In vitro* studies confirmed the efficacy of caspase inhibition in blocking neuronal virus-induced apoptosis (Richardson-Burns *et al* 2002).

3. CALPAINS

Calpains form a small group of heterodimeric cysteine-dependent proteases active only in the presence of ionic calcium (Ca^{2+}). For more than 25 years there were only two described proteases: μ- and m-calpain, according to their requirements of intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$), and a specific inhibitor termed calpastatin. However, during the last years more than ten DNA sequences have been detected with homology to the known calpains. At present, only calpain-3b among all new calpains has been isolated as a functional enzyme and most studies remain focussed on μ- and m-calpains. These two enzymes represent cytoplasmic proteases. Transient micromolar increases of $[Ca^{2+}]_i$ activate μ-calpain, while m-calpain needs millimolar increments as well as unidentified activating factors to reduce the real $[Ca^{2+}]_i$ need, far beyond physiological levels. The Ca^{2+}-induced conformational change enables an activation by autoproteolysis that allows calpains to cleave substrates at leucine, valine or isoleucine residues, and their inhibition is mediated by highly selective calpastatins. As calpains cleave several proteins producing very characteristic fingerprints, their degradation products have been used as indirect markers of enzyme activity. Calpains have been related to cytoskeletal remodelling, as well as the modification of diverse kinases and transcription factors. Nevertheless, despite a large number of putative substrates, no clear physiological role has been assigned by now. At present, calpains have been implicated to be involved in necrosis and disturbed Ca^{2+} homeostasis (Goll *et al* 2003).

3.1 Calpains in CNS Inflammation

The pathogenetic relevance of calpains in inflammatory demyelination is subject of an ongoing discussion. Two functional roles have been proposed: On the one hand many myelin proteins, including myelin basic protein (MBP) and myelin associated glycoprotein (MAG), are potential substrates for calpain proteolysis, consequently a putative involvement of calpains in CNS demyelination has been suggested (Deshpande *et al* 1995). On the other hand calpains have been implicated in mediating cell death as well (Yamashima 2000). An increased expression of μ-calpain has been detected in MS plaques at both mRNA and protein levels, as well as an indirect activity measure, defined as characteristic calpain-cleaved fodrin (a cytoskeletal protein) fragment production both in plaques and in NAWM (Shields *et al* 1999). Furthermore, increased calpain immunoreactivity was localized to infiltrating inflammatory cells, activated microglia, and resident astroglia. Similar expression patterns were observed in rat EAE, where increased expression of calpain and calpastatin were detected in all analyzed cell types, including immunocompetent cells as well as oligodendrocytes (Shields and Banik, 1998; Shields *et al* 1998). The same group demonstrated that calpain expression and activity, as well as the presence of MBP degradation products paralleled clinical disease severity in EAE (Schaecher *et al* 2002). However, no characteristic fingerprint has been detected so far in MS samples (Schaecher *et al* 2001). Moreover, killigen, an extracellular protein that inhibit calpains efficiently, is found to be expressed in CNS and it is even upregulated during CNS inflammation, thus, theoretically free calpains may be inactive in MS (Goll *et al* 2003; Takano *et al* 2003). However, the functional link between the increase of calpains and the observed demyelination is still missing.

A role of calpains in cell death appears more likely, since calpain inhibitors block oligodendrocyte death *in vitro* (Benjamins *et al* 2003). In recent years a role for glutamate overactivation and related exitotoxicity has been proposed in oligodendroglial and neuronal death in MS (Pitt *et al* 2000; Matute *et al* 2001; Kanwar *et al* 2004). Activation of glutamate receptors leads to an increase of $[Ca^{2+}]_i$ that in pathological conditions induces cell death characterized by a mixed apoptosis-necrosis profile. As a result, activation of caspase-3 and calpain in oligodendrocytes could be demonstrated *in vitro* based on characteristic spectrin fragments (Liu *et al* 2002). This mixed apoptosis-necrosis profile may be the result of a cross-talk between the caspase and calpain systems. Calpains are able to digest procaspase-3 *in vitro,* impairing its later efficient activation and obstructing controlled apoptosis, leading to a necrotic-related death profile (Neumar *et al* 2003). On the other hand, active caspase-1 and caspase-3 can degrade inhibitory calpastatins and cleave pro-calpains (Wang 2000). Apparently, $[Ca^{2+}]_I$ drives the pathway: via the activation of glutamate receptors in oligodendrocytes a mild

increase of $[Ca^{2+}]_i$ may result in activation of the caspase route, with an indirect activation of calpains in later steps, whereas a more severe increase of $[Ca^{2+}]_i$ may trigger early calpains activation and the start of the necrotic process (Figure 1).

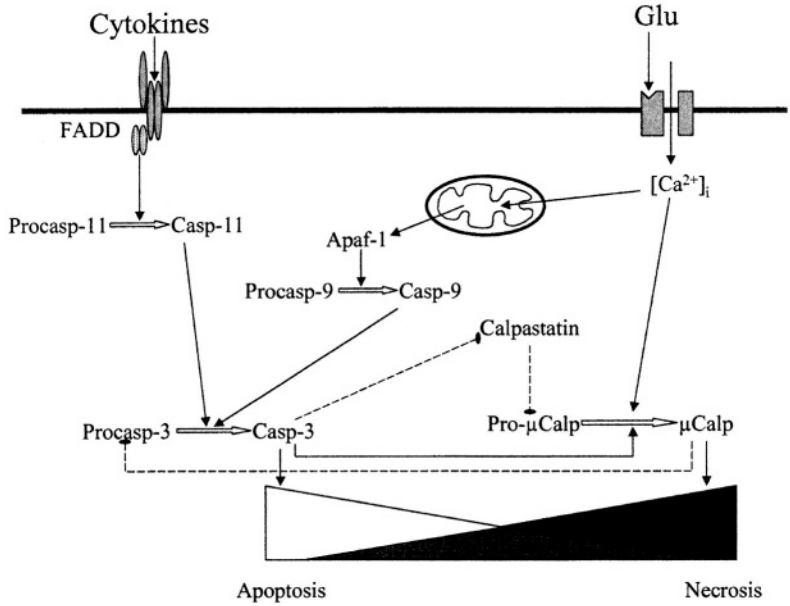

Figure 1: Proposed imbalance between apoptotic and necrotic cell death of oligodendroglia in multiple sclerosis. Solid arrows indicate pathways of activation, broken arrows represent inhibitory ones. Induction of caspase dependent (Casp) pathways is triggered in oligodendroglia mainly by FADD-associated cytokine receptor activation, while glutamate (Glu) receptors may induce μ-calpain (μCalp) activation through an increase in intracellular calcium concentration ($[Ca^{2+}]_i$), although this increase can also activate a caspase cascade through mitochondria-mediated release of apoptotic protease activating factor (Apaf-1). Caspase-3 can degrade calpastatin, thus enhancing μ-calpain activation, as well as activating μ-calpain itself. On the other hand μ-calpain can impede effective caspase-3 activation. In a pure active caspase-3 profile cell death would present apoptotic features, while in a pure active μ-calpain one it would be necrotic; the imbalance between both pathways would lead to a mixed scenario with varying degrees of each death mechanism involved.

3.2 Calpains in CNS Infection

Only little knowledge is available on the role of calpains in CNS infection. A single experiment using a broad spectrum calpain inhibitor improved clinical disease severity in experimental meningitis, apparently by blocking the activation of NF-κB (Koedel *et al* 2000). However, due to the unspecificity of ALLN, the inhibitor employed, little can be concluded, as it can inhibit also among others the large proteasome system, which has been

proposed also to participate in cell death and thus might be partially responsible of its effect (Drexler 1998). With respect to virus infection, HIV coat glycoprotein gp120 has been proposed to act as an inducer of necrotic cell death *in vitro,* with increased $[Ca^{2+}]_i$ and concomitant calpain activation in neuroblastoma cell-lines (Corasaniti *et al* 1996), but no follow-up studies have been performed *in vivo* in animal models or in humans.

4. CATHEPSINS

In contrast to cytosolic caspases and calpains, cathepsins are monomeric proteases located in lysosomes. Most members of the cathepsin family are cysteine-proteases, although some members are serine- and aspartate-proteases. More than 15 cathepsins have been described in human so far, some of which are ubiquitously expressed, such as cathepsin B, cathepsin D and cathepsin L. Cathepsins are, as other proteases, initially expressed as inactive precursors, and become active after proteolytic removal of the N-terminal prodomain (Johnson 2000; Turk *et al* 2001). This proteolytic step can be mediated by other proteases or by an autocatalyctic activation at acidic pH, as it can be found in the lysosomal environment. As the total cathepsin concentration inside lysosomes can exceed 1 mM, there are robust endogenous controls to avoid accidental intracellular digestion. Stefins, a group of protease inhibitors related to cystatins and kininogens, which are able to inhibit released cathepsins, represent part of such control mechanisms. Besides the physiological protease action on endocytosed structures, including degradation of membrane proteines and prohormone cleavage, a major role has been assigned to cathepsins in the antigen processing to produce natural epitopes being presented via MHC class II molecules by antigen presenting cells (APCs) (Turk *et al* 2001). While an antigen can be degraded by many proteases, only some of them will produce "natural" immunogenic peptides that trigger an effective immune response of B- and T-cells.

4.1 Cathepsins in CNS Inflammation

Various hypotheses suggest cathepsins to be implicated at least at three different levels in the immunopathogenesis of MS: (1) Cathepsins digest antigens and as such may cleave putative autoantigens, such as MBP and MAG; (2) as they may be released by cytotoxic T-cells, cathepsins may contribute to demyelination of the CNS; (3) because of a disturbance of lysosomal membrane integrity in oligodendrocytes and neurons, cathepsins may be released in the intracellular space, overcoming the inhibitory system and participating in oligodendroglial and neuronal cell death.

Autoreactive T-lymphocytes against several myelin epitopes can be isolated from the peripheral blood from MS patients. One putative autoantigen is MBP, with the fragments 85-99 and 116-123 as the main immunogenic epitopes (Lutton *et al* 2004). The cysteine-proteases cathepsin S and legumain, an asparagine-specific endoprotease, have been implicated in the production of antigenic peptides, as demonstrated in lymphoblastoid cell lines (Beck *et al* 2001). Recent studies, using human primary B-cells as a model for antigen presentation, revealed a central role for the serine-protease cathepsin G for the effective cleavage of MBP, eliminating the immunodominant determinant of MBP (Burster *et al* 2004). Interestingly, the degradation of intact MBP by cathepsin G was not restricted to a lysosomal environment, but was also performed by the soluble protease. As such, this protease may have a critical function in controlling the immune response.

Cathepsins are able to digest not only MBP, but also other myelin proteins such αB-crystallin (Bajramovic *et al* 2000), while their competence to degrade MAG is still a matter of discussion (Stebbins *et al* 1998; Paivalainen *et al* 2003), and no studies have been conducted with respect to MOG. Activated macrophages and microglia are able to release cathepsin B, cathepsin S, and cathepsin L (Liuzzo *et al* 1999) and in MS white matter lesions increased expression of cathepsin B can be found (Bever and Garver, 1995). Similarly, increased expression levels have been reported in the CSF from MS patients, paralleled by clinical disease severity (Nagai *et al* 2000). Cathepsin S and cathepsin L may not be involved in antigen presentation (Paivalainen *et al* 2003; Burster *et al* 2004), but they are able to degrade MBP and MAG if secreted within the CNS. However, further studies are warranted to define the role of these proteases in the inflamed CNS in greater detail.

The third hypothetical role of cathepsins in MS relates to cell death, in association with caspases and calpains. Although no direct studies have been conducted in oligodendroglia, it is known that *in vitro* stimulated microglia secrete cathepsin B and may induce neuronal apoptosis in a caspase-3-dependent fashion (Kingham and Pocock, 2001). This induction was blocked, although not fully, by specific antibodies and cathepsin B inhibitors. It has been shown that cytosolic cathepsin B is able to cleave the caspase-11 prodomain, in turn activating caspase-3 (Yamashima 2000). The inflammatory environment in a MS lesion could activate microglia and increased expression of cathepsin B has been demonstrated in MS lesions (Bever and Garver, 1995). Thus, it appears plausible that the extracellular presence of this protease may trigger apoptosis in oligodendroglia and neurons in a similar way. Furthermore, the CSF from MS patients contained increased levels of free cathepsin B along with a reduction of its endogenous extracellular inhibitor cystatin C (Nagai *et al* 2000), suggesting the presence of active enzyme in the CNS of MS patients. Finally, not only microglial-released

extracellular but also the intracellular cathepsins may be involved in cell death. Activation of calpains reduces the stability of lysosomal membranes, resulting in the release of its content into the compromised cell (Yamashima 2000). Whether this effect may be relevant in the cell death of oligodendrocytes and neurons in MS needs to be determined in further studies.

4.2 Cathepsins in CNS Infection

Virtually no studies have been conducted addressing the specific role of cathepsins in CNS infection, beyond the general physiological antigen presenting process already discussed for MS. Apparently, the cathepsin system is not specially disturbed during meningitis, as the levels of both cystatin C and cathepsin B remained inside the normal ranges when CSF from aseptic meningitis, a virus-related non-fatal infection, were analyzed (Nagai *et al* 2000).

Table 1. Putative roles of intracellular proteases

Protease	Pathology studied	Suggested function	Reference
Caspase-1	Multiple sclerosis	Cytokine production	Furlan *et al* 1999a
	EAE	Cytokine production	Furlan *et al* 1999b
	Bacterial meningitis	Cytokine production	von Mering *et al* 2001
Caspase-8	Multiple sclerosis	Induces T-cell apoptosis	Gomes *et al* 2003
Caspase-11	EAE	Induces apoptosis of oligodendrocytes	Hisahara *et al* 2001
	EAE	Activates cytokine production	Hisahara *et al* 2001
Caspase-3	EAE	Effector in apoptosis of neurons	Ahmed *et al* 2002
	EAE	Effector in apoptosis of oligodendrocytes	Hisahara *et al* 2001
	Bacterial meningitis	Effector in apoptosis of neurons	Gerber *et al* 2001
μ-Calpain	Multiple sclerosis	Proteolysis of myelin components	Shields *et al* 1999
	In vitro study	Oligodendrocyte death	Benjamins *et al* 2003

EAE = Experimental autoimmune encephalomyelitis

5. MATRIX METALLOPROTEINASES

The matrix metalloproteinases (MMPs) represent a large subfamily of endoproteinases that share structural domains. All enzymes have a catalytic zinc-binding domain in common that includes a sequence motif HEXXH in which Glu acts as a catalytic base (Woessner Jr 1994). At least 23 members have been identified so far that are grouped together because of their sequence homology, resulting in structural and enzymatic similarities (Yong *et al* 2001); these subfamilies comprise the collagenases, gelatinases, stromelysins, matrilysins, metalloelastase, and membrane-type metalloproteinases. The membrane type MMPs are bound to the cellular surface (Takino *et al* 1995), whereas all other MMPs are secreted into the extracellular space by a wide range of cell types as latent pro-enzymes requiring activation to expose the active catalytic site (Birkedal-Hansen 1995). The regulation of MMPs expression and activity is a highly complex process strictly controlled at various levels (Ries and Petrides, 1995): gene transcription, pro-enzyme activation, and activity of endogenous tissue inhibitors of metalloproteinases (TIMPs). Once synthesized MMPs are secreted as inactive zymogens. The activation of most of these propeptides involves sequential exogenous or endogenous cleavage steps, destabilizing the cysteine-zinc interaction, modifying the enzyme conformation, and allowing further exogenous or autocatalytic processing to the final active form (Murphy *et al* 1994). MMPs and other proteinases, such as plasmin, are known to modulate this process. The activated forms are subject to inhibition by TIMPs (Overall 1994), which are ubiquitously expressed in the extracellular milieu and form a complex, of 1:1 stoichiometry, with the endoproteinases (Stetler-Stevenson *et al* 1989).

MMPs have broad, but not necessarily overlapping substrate specificities. They can degrade all protein components of the extracellular matrix (ECM), such as collagen, elastin, fibronectin, and laminin (Chandler *et al* 1997). Therefore, a finely tuned regulation is essential: any imbalance in favor of inhibitors can lead to fibrotic processes whereas any increase in enzymatic activity will result in tissue destruction or cell invasion (Birkedal-Hansen 1995).

5.1 Matrix Metalloproteinases in CNS inflammation

An emerging body of evidence suggests an involvement of MMPs in the pathogenesis of inflammatory demyelination of the CNS (Yong *et al* 1998; Kieseier *et al* 1999b). In various animal models strong evidence has been provided that MMPs at multiple checkpoints are key to the pathogenesis of inflammatory demyelination (Pagenstecher *et al* 1998). The intracerebral injection or induction of MMP-2, MMP-7, MMP-8, and MMP-9 in the CNS

results in breakdown of the ECM, leukocyte recruitment, and opening of the BBB in rats (Rosenberg *et al* 1992, 1994; Anthony *et al* 1998). In EAE, increased MMP-9 levels are detectable in the CSF of diseased animals (Gijbels *et al* 1993) and immunohistochemistry localized this MMP to infiltrating mononuclear cells and to the perivascular space (Kieseier *et al* 1998) (Figure 2). Thus, MMP-9 appears to be critically involved in the process of BBB damage as well as transmigration of immunocompetent cells into the CNS. Young mice that are genetically deficient for MMP-9 are relatively resistant to EAE induction compared with wild-type mice (Dubois *et al* 1999).

Studies investigating the clinical course of EAE revealed in addition increased mRNA expression patterns of MMP-7, MMP-9, MMP-12, and MMP-14 coincident with peak disease severity, emphasizing a key role of these proteases not only during disease induction but also in perpetuating the inflammatory response in the CNS (Clements *et al* 1997; Kieseier *et al* 1998; Brundula *et al* 2002). Inhibition of MMP activity suppressed the development and abrogated clinical EAE in a dose-dependent way (Gijbels *et al* 1994; Hewson *et al* 1995; Matyszak and Perry, 1996; Liedtke *et al* 1998; Brundula *et al* 2002; Popovic *et al* 2002). Thus, MMP inhibitors might act by preventing the influx of inflammatory cells across the BBB, and by blocking the activation of other proinflammatory mediators, such as cytokines.

In vitro studies confirmed that MMPs are crucial in facilitating the transmigration of circulating leukocytes into the nervous system and transferred the concept to human cells (Leppert *et al* 1995; Stüve *et al* 1996). A systematic analysis of 23 members of the MMP family revealed a distinctive pattern of MMP expression in various leukocyte populations (Bar-Or *et al* 2003). Interestingly, MS patients revealed higher levels of MMP-2 and MMP-14, expressed in monocytes, compared to healthy controls, suggesting that monocytes might be prominent contributors of the inflammatory response in the nervous system.

Within the CNS all resident cell types are potential sources of MMPs: *in vitro,* astrocytes, microglia, neurons (reviewed in Gottschall and Deb, 1996), and oligodendrocytes (Uhm *et al* 1998) are capable of expressing various members of the MMP family. In acute demyelinating MS lesions MMP-9 can be detected by immunohistochemistry and localized to macrophages and astrocytes. The latter continue to express this enzyme in chronic lesions. Moreover, white matter perivascular mononuclear cells are found to be positive for this protease (Cuzner *et al* 1996; Maeda and Sobel, 1996). Other MMPs identifiable in MS plaques include MMP-1, MMP-2, and MMP-3, all of which are expressed by macrophages, whereas the latter two are also expressed by astrocytes (Maeda and Sobel, 1996). MMP-12 protein was localized to foamy macrophages with parenchymal and perivascular distribution patterns in actively demyelinating MS lesions. Other CNS cells,

microglia and astrocytes, stained only occasionally positive with the antibody used (Vos *et al* 2003). However, detection and localization of MMPs in the MS lesion does not necessarily provide information about their proteolytic activity. As inhibition of the activated forms of MMPs by TIMPs and other proteinase inhibitors plays a crucial role in MMP regulation, further studies are needed to elucidate the functionality of these enzymes.

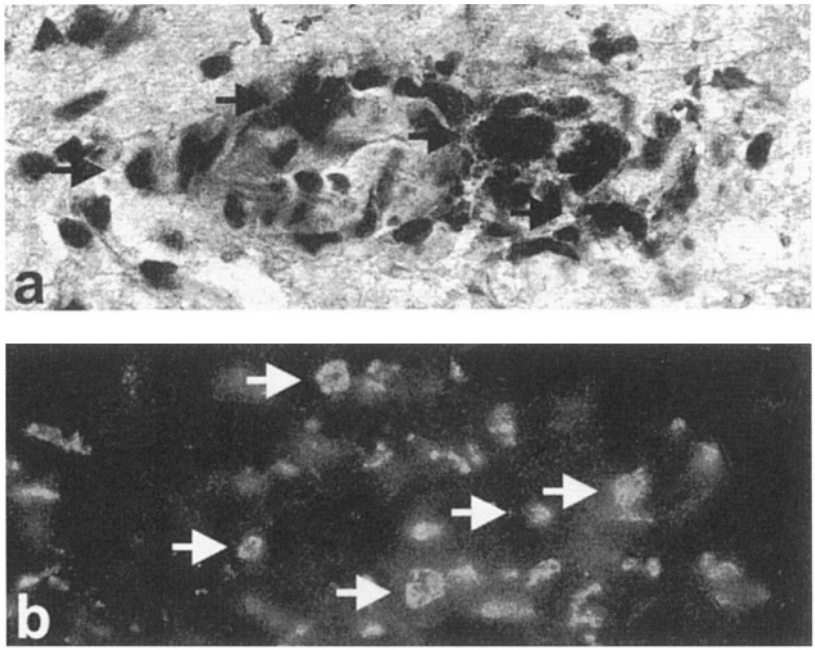

Figure 2: (a) In experimental autoimmune encephalomyelitis, MMP-9 can be localized to perivascular mononuclear cells (arrows); (b) by in situ zymography gelatinolytic activity, e.g. MMP-9, can be visualized, exhibited by mononuclear cells invading nervous tissue.

During clinical exacerbations of MS increased activity of MMPs in the CSF has been demonstrated: MMP-9 levels are significantly elevated in the CSF of MS patients compared to control subjects (Gijbels *et al* 1992; Mandler *et al* 2001) and are primarily related to the CSF cell count (Yushchenko *et al* 2000). CSF levels of α1-antitrypsin, a proteinase inhibitor, are either decreased or not elevated in MS patients (Pearl and Mullins, 1985). Since MMP-9 levels are raised in these patients, an increase of proteolytic activity within the CNS would be expected. Furthermore, tissue-type plasminogen activator, which generates plasmin that in turn converts the inactive MMP zymogen by sequential cleavage into an active protease, is expressed by mononuclear cells in the blood vessel walls in

early MS lesions (Cuzner *et al* 1996). It is therefore possible that both a reduction of an inhibitor and an increase of an activator could potentiate the proteolytic action of MMPs within the CNS of MS patients. Evidence suggests that MMPs are involved in BBB breakdown in MS. Raised CSF levels of MMP-9 are associated with a disturbed BBB as demonstrated by gadolinium-enhanced magnetic resonance imaging (MRI). In addition, treatment with high-dose methylprednisolone, a drug known to downregulate the transcription of MMPs, reduced both gadolinium-enhancement and CSF levels for MMP-9. The selective diminution of MMP-9 activity in the CSF of patients with contrast enhancement on brain MRI, is interpreted to result from either a reduction of MMP or an increase of TIMPs production (Rosenberg *et al* 1996). Consequently, in both settings proteolytic activity would be enhanced giving rise to disruption of the basal lamina around capillaries, thereby paving the way for inflammatory cells and other soluble substances into the CNS. In MS patients MMP-9 levels were found to be elevated during clinical relapses in the CSF (Leppert *et al* 1998) and in the serum (Lee *et al* 1999). High mean serum MMP-9 levels were associated with significantly more T1-weighted gadolinium enhancing MRI-lesions, underlining the potential impact of this MMP on BBB disruption.

Thus, although the contribution of each individual MMP to the pathogenesis of immune mediated demyelination of the CNS remains elusive in most parts, collective evidence supports the view that MMPs are critically involved in BBB damage, leukocyte trafficking, cytokine activation, and myelin damage within the inflamed CNS (Figure 3).

5.2 Matrix Metalloproteinases in CNS Infection

MMPs contribute to the pathogenesis of BM. Various studies in different animal models revealed an upregulated expression of the mRNAs of MMP-3, MMP-8, MMP-9, MMP-12, MMP-13, and MMP-14 in the brain parenchyma of diseased animals, whereas MMP-7 and MMP-2 are not induced (Kieseier *et al* 1999a; Leib *et al* 2001). On the protein level, MMP-8 and MMP-9 are strongly increased within the CNS tissue and can be detected in the CSF of diseased animals (Paul *et al* 1998; Leppert *et al* 2000). MMP-8 appears to degrade predominantly parenchymal ECM components. In analogy to the observations in immune-mediated demyelination, MMP-9 is thought to play a key-role in BBB disruption in BM, as it is capable to degrade components of the major basal membrane, such as laminin and collagen IV. Upregulation of MMP-9 starts as early as 15 min after initiation of experimental BM (Leib *et al* 2001). As cellular sources resident brain cells as well as immunocompetent cells derived from the systemic immune compartment, mainly neutrophils and monocytes, could be

defined in pneumococcal meningitis (Meli *et al* 2003). Proteolytic activity of neutrophils invading the CNS could be visualized in the subarachnoid space and in the brain parenchyma (Meli *et al* 2004).

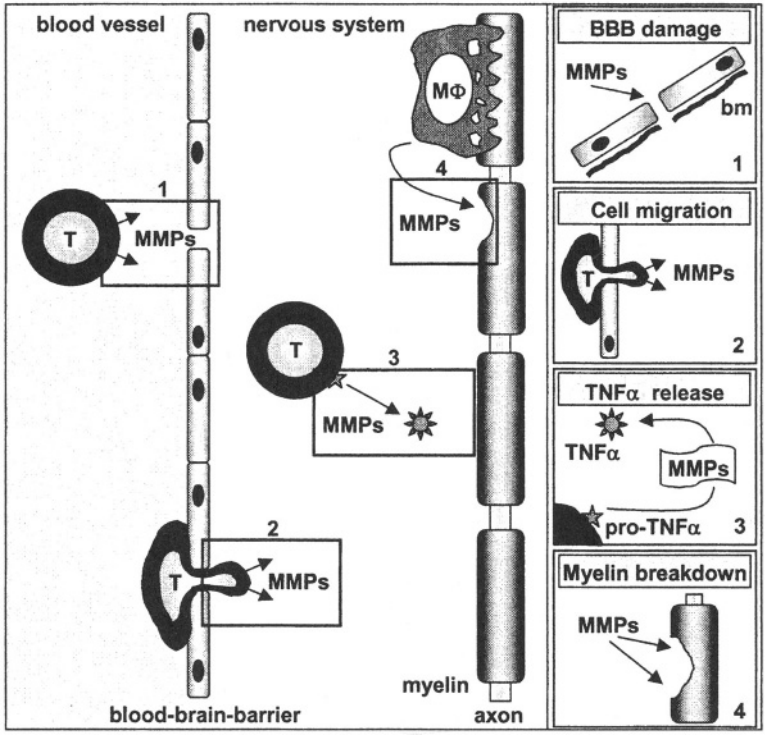

Figure 3: Matrix metalloproteinases (MMPs) exhibit at least four different deleterious roles in the pathogenesis of inflammatory demyelination of the CNS: (1) opening of the blood-brain (BBB) by disruption of extracellular matrix components of the basement membrane (bm), (2) migration of inflammatory cells across the BBB into the neuropil, (3) enhancement of the release of the pro-inflammatory cytokine, such as tumour necrosis factor-α (TNFα), and (4) direct degradation of the myelin sheath.

In a rat model of meningococcal meningitis induced by the intracisternal injection of heat-killed meningococci the occurrence of MMP-9 activity in the CSF paralleled the disruption of the BBB, an increase in intracranial pressure, and CSF pleocytosis. The MMP inhibitor batimastat (BB-94) significantly reduced the BBB disruption and the increase in intracranial pressure suggesting that MMPs are involved in the alterations of BBB permeability during experimental meningococcal meningitis (Paul *et al* 1998). In pneumococcal meningitis the synthetic MMP inhibitor GM-6001 reduced cortical injury in an infant rat model (Leib *et al* 2001). Another

inhibitor attenuated the incidence of seizures in treated animals and was neuroprotective in the cortex (Meli *et al* 2004). The mechanisms how MMPs are involved in neuronal death are not well understood at present. However, in MMP-9 deficient mice the induction of pneumococcal meningitis did not reveal any differences in the clinical course of the disease, the amount of leukocyte recruitment into the subarachnoid space, and bacterial titers in the brain (Bottcher *et al* 2003). Whether technical issues or species differences might account for these discrepancies remains unclear at present.

Expression patterns of MMPs in patients with meningitis have been analyzed by various groups (Paul *et al* 1998; Leppert *et al* 2000; Shapiro *et al* 2003; Lee *et al* 2004). In a systematic investigation MMP-8 and MMP-9 were found to be upregulated in the CSF obtained from patients with BM, in analogy to the observations from the animal studies (Leppert *et al* 2000) (Figure 4). CSF levels of MMP-9 in cases of BM were 10- to 1000-fold higher than in cases of viral meningitis (Kolb *et al* 1998) and MS (Leppert *et al* 1998). That MMP-9 plays a critical role in BBB disruption in human BM can be postulated by correlating the CSF protein content. However, because of the rapid kinetic changes of MMPs and the sustained BBB disruption in fully established BM, this correlation is restricted to early phases of the disease (Leppert *et al* 2000).

Current data available point to a critical role of MMP-9 and MMP-8 as effector molecules in the pathogenesis of meningitis, especially in BBB disruption and neuronal injury. Thus, MMPs may be interesting targets for an adjunctive therapy in BM.

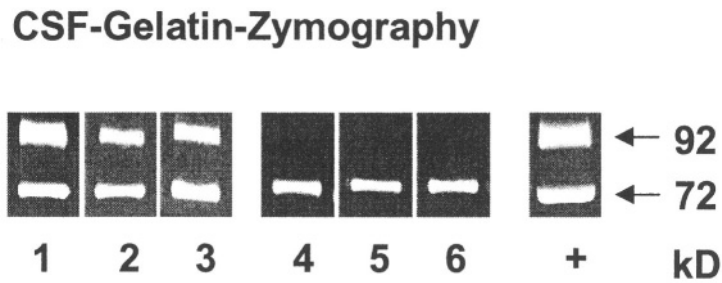

Figure 4: In the CSF of patients with bacterial meningitis (lane 1) and multiple sclerosis (acute relapse, lanes 2 & 3) increased expression of MMP-9 at the size of 92 kD can be detected, whereas the expression level of MMP-2 at the size of 72 kD remains unchanged compared to non-inflammatory controls (lanes 4-6).

6. METALLOPROTEASE-DISINTEGRINS (ADAMS)

Metalloprotease-disintegrins (ADAMs) represent a family of membrane-anchored glycoproteins that have been implicated in fertilization, myogenesis, neurogenesis, and protein ectodomain shedding (Schlondorff and Blobel 1999). At present a total of 34 ADAMs have been identified in a variety of organisms (see the following URL for details: http://www.people.virginia.edu/%7Ejw7g/Table_of_the_ADAMs.html). Nearly half of these enzymes contain a catalytic-site consensus sequence (HEXXH) in their metalloprotease domain, thus they are predicted to be catalytically active (Weskamp *et al* 2002). These proteases appear to play a role in protein ectodomain shedding. The first identified sheddase, the TNF-α converting enzyme (TACE) is an ADAM (ADAM-17) (Black *et al* 1997; Black 2002). Moreover, ADAMs are implicated to act in cell-cell adhesion through interactions with integrins (Eto *et al* 2000; Nath *et al* 2000) or syndecans (Iba *et al* 2000). Given the spectrum of proteolytic and adhesive properties ADAMs may represent proteases critically involved in immune responses in the CNS.

6.1 ADAMs in CNS Inflammation

Only few data have been published on ADAMs in the inflamed nervous system so far. ADAM-10, initially cloned from bovine brain, was found to be expressed in a wide variety of tissues (Howard *et al* 1996). In the human CNS astrocytes were identified as a cellular source of ADAM-10 under inflammatory and non-inflammatory conditions. Moreover, in chronic active MS lesions ADAM-10 could also be localized to perivascular macrophages (Kieseier *et al* 2003) (Figure 5). These observations suggest that a yet to be specified inflammatory reaction within the CNS might regulate the expression of ADAM-10 in MS. Furthermore, the functional implications of an upregulation of ADAM-10 as well as the constitutive expression of this protein in the CNS remain to be determined. Although purified ADAM-10 is able to degrade protein components of the myelin sheath, such as MBP, *in vitro* (Amour *et al* 2000), the relevance of such an activity *in vivo* remains difficult to estimate at present. It is tempting to speculate that ADAM-10 might be involved in the critical process of demyelination in MS pathogenesis, especially since macrophages appear as one of its cellular sources, however, presently available data do not strongly suggest an involvement of ADAM-10 in the process of demyelination, since this protease was found to be expressed primarily by cells located within the perivascular space.

Astrocytes and endothelial cells have been identified as cellular sources of ADAM-17 in human brain tissue without obvious pathology (Goddard *et*

al 2001). In MS lesions ADAM-17 was found to be expressed primarily by invading T-lymphocytes suggesting that infiltrating cells perpetuate the ongoing inflammatory reaction through the release of ADAM-17 by shedding pro-TNF-α into its active form (Kieseier *et al* 2003). A pivotal role of TNF-α in the pathogenesis of inflammatory demyelinating diseases of the CNS has been proposed, although its impact may vary in different stages of MS evolution and activity (Liu *et al* 1998). In MS lesions TNF-α was found to be expressed by astrocytes and macrophages (Hofman *et al* 1989; Selmaj *et al* 1991). The view that this cytokine exhibits a deleterious effect in the pathogenesis of MS has been challenged more recently. Although the requirement for the proinflammatory activities of this cytokine for disease initiation has been documented in TNF-α deficient mice (Körner *et al* 1997), other groups could demonstrate that TNF-α is not essential for the development of inflammatory demyelination in the CNS (Frei *et al* 1997). Moreover, an anti-inflammatory role has been proposed as well (Liu *et al* 1998). Currently, it is suggested that the appropriate context of mediators at any given time point during the course of the disease may well determine whether the effect of TNF-α is protective or harmful. Hence, TNF-α is critically involved in the pathogenesis of autoimmune demyelination and as such ADAM-17, as the primary protease activating pro-TNF-α, consequently appears to play a substantial role in the pathogenesis of MS as well.

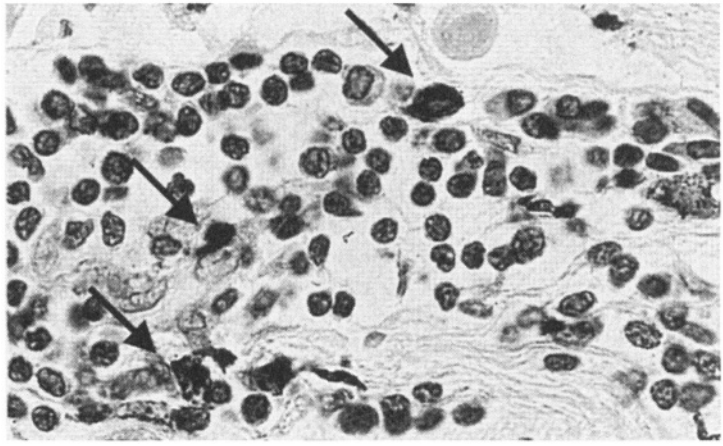

Figure 5: ADAM-10 is expressed by perivascular mononuclear cells (arrows), identified as CD68 positive macrophages, in acute multiple sclerosis lesions.

6.2 ADAMs in CNS Infection

Only little knowledge is available on the expression of ADAMs in CNS infection. One hallmark of human BM and corresponding animal models is apoptotic neuronal death in the hippocampus, which is clinically associated with learning difficulties of the survivors (Nau *et al* 1999). The exact mechanisms underlying this apoptosis in the hippocampal dentate gyrus are not well understood so far, however, collective evidence suggests that ADAM-17 plays a critical role in this process. Effective inhibition of the cleavage of membrane bound TNF-α, using the protease inhibitor BB1101, attenuated apoptosis in the hippocampus and, functionally, preserved learning performance of rats (Leib *et al* 2001). Thus, the combined inhibition of MMPs and TACE represents a putative therapeutic strategy to prevent brain injury and neurological sequelae in BM, at least in the animal model.

In patients with BM increased protein expression of ADAM-17 are detectable in the CSF (Kieseier *et al* 2003), however, the immunological implication of this observation remains elusive at present.

7. CONCLUSION

There is an emerging body of evidence implicating a central role of proteases in the inflammatory immune response of the nervous system. Collective data suggests that these enzymes might contribute to the process of inflammation as well as demyelination. Functionally, they seem to be involved in the recruitment of inflammatory cells, in damaging the BBB, in axonal and neuronal toxicity as well as myelin damage, and in modulating the inflammatory mechanism. These multifarious actions are part of the complexity of the immune response within the infected and inflamed CNS.

It is hoped that the exponential progress in research of cellular and humoral immunity during the last years will increase our knowledge about the enzymatic cascades involved in inflammatory reactions. Based on this grown understanding new synthetic modulators of proteolytic activity should be designed which hopefully will enlarge our therapeutic armentarium for the treatment of these diseases of the CNS in the future.

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Chapter 6

Dipeptidyl Peptidase IV Regulates T Cell Function in CNS Inflammation

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1. INTRODUCTION

The ectoenzyme dipeptidyl peptidase IV (DP IV, CD26, EC 3.4.14.5) was first described by Hopsu-Havu and Glenner (1966) 38 years ago. DP IV is an exopeptidase catalyzing the release of N-terminal dipeptides from oligo- and polypeptides preferentially with proline, hydroxyproline and, with less efficiency, alanine in the penultimate position. The post proline-cleaving substrate specificity makes DP IV relatively unique among other proteases. Among the rare group of proline-specific proteases, DP IV was originally believed to be the only membrane-bound enzyme specific for proline as the penultimate residue at the amino-terminus of the polypeptide chain. However, recent studies have identified a number of additional molecules bearing DP IV-like enzymatic activity. Among them are the DP IV-like exopeptidases fibroblast activation protein (FAP), expressed at fibroblasts, and attractin, highly expressed on activated T cells (for review see: Sedo and Malik, 2001).

The cDNA encoding human DP IV predicts a protein of 766 amino acids with 9 potential glycosylation sites. In the plasma membrane, DP IV occurs as a homodimer with a total molecular mass of 220–240 kDa (Fleischer 1994). The human DP IV gene is localized on chromosome 2q24.3 and is composed of 26 small exons.

Distribution and function of DP IV are only partially known and complex and became even more complex by the existence of DP IV-like enzymes. DP IV is ubiquitously distributed with exceptional high expression in intestine, kidney and liver (Fleischer 1994). In 1977, Lojda demonstrated for the first time DP IV activity on human peripheral blood lymphocytes. Later, it was discovered that DP IV is identical with the leukocyte surface antigen CD26. Subsequently at the 4th Workshop on Leukocytes Differentiation Antigens a number of monoclonal antibodies recognizing DP IV were subsumed under the term CD26 (Fleischer 1994).

The physiological functions of DP IV greatly differ depending on the type of tissue where it is expressed. For example, within the gastrointestinal system the main function of DP IV is probably the delivery of proline-containing dipeptides for reutilization by final digestion of nutrients. Pauly (1996) expanded this function by reporting DP IV-mediated processing of gastrointestinal hormones such as glucagon-like peptide-1(7-36) and glucose-dependent insulinotropic polypeptide.

Within the hematopoietic system, DP IV is expressed on the surface of resting and activated T, B, and NK cells. In general, CD26 expression is upregulated following stimulation of T cells with mitogen, antigen, anti-CD3 antibodies or IL-2, B cell stimulation with staphylococcal proteins and IL-2 stimulation of NK cells. Thus, activated antigen-specific CD4+ T cell clones express high levels of DP IV/CD26 (for review see Kähne *et al* 1999).

Although extensively studied, the role of CD26 in T cell biology has not yet been clarified. The wide range of DP IV/CD26 activity within the hematopoietic system is related to soluble mediators and cellular functions. DP IV exopeptidase activity has been shown to play a role in the activation or inactivation of biological peptides (De Meester *et al* 1999). DP IV/CD26 has attracted great interest due to its ability to process and either inactivate or alter the specificity of many chemokines, including CXCL10 (IFN-inducible protein 10 kDa; IP-10) and CXCL12 (stromal cell-derived factor-1; SDF-1) (Lambeir *et al* 2001), and has been described to be preferentially expressed by T helper-1 (Th-1) (Rogge *et al* 2000) and T regulatory-1 (Tr-1) cells (Cavani *et al* 2000). Thus, processing of CXCL10 and CXCL11 (IFN-inducible T cell α-chemoattractant; I-TAC) by DP IV/CD26 results in reduced CXCR3-binding and more than 10-fold reduced chemotactic potency (Proost *et al* 2001). On the other hand, DP IV/CD26 differentially regulates the chemotaxis of T cells and monocytes toward the chemokine CCL5 (regulated on activation, normal T expressed and secreted; RANTES), enhancing directed T cell migration by a factor of two (Iwata *et al* 1999). Of note, anergic T cells have been reported to exert antigen-independent inhibition of cell-cell interactions via chemokine metabolism. Instrumental to this effect may be the increased cell

surface expression and enzymatic activity of CD26, which may act by metabolizing chemoattractants bound to the endothelial/epithelial cell surface (James *et al* 2003). Apart from processing polypeptides, DP IV/CD26 can interact either as a receptor or ligand with various proteins playing a role in immune responses. The enzyme has been described as a collagen receptor (Hanski *et al* 1985) and adenosine deaminase (ADA) binding protein (Kameoka *et al* 1993). Furthermore, it appears to be associated with CD45 (Torimoto *et al* 1991). Previous studies have shown that CD26 directly binds to the cytoplasmic domain of CD45 (Ishii *et al* 2001).

Finally, several groups have shown a key role of DP IV in the regulation of differentiation and growth of T lymphocytes (Torimoto *et al* 1991; Ansorge *et al* 1991; Dang *et al* 1990; Reinhold *et al* 1997).

2. THE ROLE OF DP IV/CD26 IN LYMPHOCYTE ACTIVATION

The development of synthetic inhibitors of DP IV enzymatic activity facilitated the study of the cellular functions of this enzyme on the surface of immune cells. In 1985, Schön and coworkers showed for the first time that N-Ala-Pro-O-(nitrobenzoyl-)hydroxylamine, which irreversibly inhibits DP IV, is capable of suppressing the proliferation of human peripheral blood mononuclear cells (PBMC) stimulated with mitogens. This was the first evidence that DP IV plays a critical role in the regulation of DNA synthesis of immune cells and that the enzymatic activity of DP IV is involved in this process.

In the meantime, a multitude of biochemically distinct synthetic compounds inhibiting DP IV and/or DP IV-like enzymes have been studied under different stimulation conditions using a variety of target cells. The most potent reversible inhibitors produced and tested in our laboratories were Lys[Z(NO_2)]-thiazolidide, -piperidide, and -pyrrolidide which cause a 50 % inhibition of DP IV in the range of 2 - 3 μM.

The inhibitory effects of these inhibitors on leukocyte proliferation and cytokine production were confirmed in different T cell models. Previously, we reported the dose-dependent suppression of DNA synthesis of pokeweed mitogen (PWM)- and phytohemagglutinin (PHA)-stimulated human PBMC and purified T cells (Reinhold *et al* 1997a). The same holds true for myelin basic protein (MBP)-stimulated T cell clones derived from patients with multiple sclerosis (MS) (Reinhold *et al* 1998). These cells also express high levels of DP IV/CD26 on their surface.

Likewise, mouse splenocytes and thymocytes express high levels of DP IV/CD26 (Vivier *et al* 1991). We could show that Lys[Z(NO_2)]-thiazolidide and Lys[Z(NO_2)]-pyrrolidide inhibit the enzymatic activity of freshly isolated murine splenocytes and thymocytes as well as the DNA synthesis of activated mouse splenocytes and thymocytes (Reinhold *et al* 1997b). In addition, the antigen-specific proliferation of murine lymph node cells, T cell lines and clones can be suppressed by these inhibitors (Steinbrecher *et al* 2000; Steinbrecher *et al* 2001).

Besides their influence on DNA synthesis, inhibitors of DP IV enzymatic activity also exhibit strong suppressive effects on the production of various cytokines. It was shown that Lys[Z(NO_2)]-thiazolidide, -piperidide, and -pyrrolidide inhibit the production of IL-2, IL-10, IL-12, and IFN-γ of PWM-stimulated PBMC and purified T cells (Reinhold *et al* 1997a) as well as the production of IL-2, IL-6 and IL-10 of PHA-stimulated mouse splenocytes and Con A-stimulated mouse thymocytes (Reinhold *et al* 1997b). These inhibitors also reduce in a dose-dependent manner IFN-γ, IL-4, and TNF-α production of myelin basic protein (MBP)-stimulated T cell clones from MS patients (Reinhold *et al* 1998). As shown by competitive RT-PCR, also the levels of IL-2 and IFN-γ mRNA in mitogen-stimulated T cells were found to be decreased after exposure of cells to Lys[Z(NO_2)]-thiazolidide (Arndt *et al* 2000).

Interestingly, these inhibitors of DP IV enzymatic activity elicit the enhanced production and secretion of latent transforming growth factor-ß1 (TGF-ß1). To determine whether this "immunoinhibitory" cytokine is involved in the suppression of DNA synthesis and cytokine production induced by DP IV inhibitors, the concentrations of latent TGF-ß1 in supernatants of PWM-stimulated PBMC and T cells were assayed in the presence or absence of DP IV inhibitors. Interestingly, these inhibitors induce a three to fourfold increased secretion of latent TGF-ß1, as measured with a specific TGF-ß1 enzyme-linked immunosorbent assay (ELISA) and in the MvlLu bioassay (Reinhold *et al* 1997a). Levels of TGF-ß1 mRNA were found 2- to 3-fold increased in PHA-stimulated T cells in presence of Lys[Z(NO_2)]-thiazolidide (Kähne *et al* 1999). Previously, it had been demonstrated that TGF-ß1 exhibits the same inhibitory effects as DP IV inhibitors on DNA synthesis and cytokine production (Reinhold *et al* 1994; Reinhold *et al* 1995). A neutralising chicken anti-TGF-ß1 antibody was capable of abolishing the DP IV inhibitor-induced suppression of DNA synthesis of PWM-stimulated PBMC and T cells, suggesting that TGF-ß1 might have key functions in the molecular action of DP IV/CD26 concerning the regulation of DNA synthesis and cytokine production. Moreover, TGF-ß1 as well as inhibitors of DP IV enzymatic activity have been demonstrated to down regulate the mRNA expression of DP IV/CD26 itself (Arndt *et al* 2000).

3. NATURALLY OCCURRING MOLECULES INFLUENCING DP IV/CD26

If modulation of T cell activity by compounds inhibiting DP IV is a pathway with physiological importance, endogenous as well as exogenous molecules with an inhibitory function on DP IV may play a role in regulating immune responses *in vivo.* Interestingly, inhibition of the enzymatic activity of purified porcine kidney DP IV was observed with a number of non-substrate oligopeptides containing a N-terminal Xaa-Xaa-Pro- sequence, e. g. HIV-1 Tat(1-86) and Met-IL-2(1-6) (Hoffmann *et al* 1995; Wrenger *et al* 1997). The short N-terminal nonapeptide of HIV-1 Tat, Tat(l-9), also inhibiting human DP IV, interferes with the proliferation of tetanus-toxoid-stimulated peripheral blood mononuclear cells (Wrenger *et al* 1997). The exchange of aspartic acid at position two in Tat(1-9) by tryptophan results in a peptide which strongly enhanced DP IV inhibition and suppression of DNA synthesis. The inhibitory effect of W^2-Tat(1-9) is in the range of those of the inhibitors TMC-2A and TSL-225, (K_i values of 5.3 μM and 3.6 μM, respectively, compared to 2.12 μM for W^2-Tat(1-9)) (Tanaka *et al* 1998). Thus, the N-terminal motif XWP turned out to be important for DP IV inhibition.

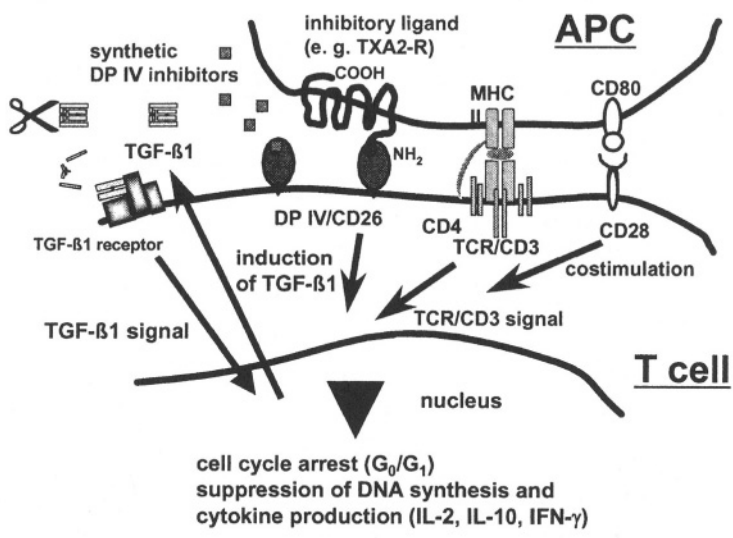

Figure 1: Model of the TGF-ß1-mediated modulation of T cell activation following inhibition of DP IV/CD26 by synthetic inhibitors or inhibitory ligands such as the thromboxane A2 receptor.

By database searches for the N-terminal XWP motif, the thromboxane A2 receptor (TXA2-R) sequence was identified. TXA2-R is a broadly distributed G protein-coupled receptor with seven putative transmembrane helices locating the MWP-bearing N-terminus (29 amino acids) at the outer site of the plasma membrane. The N-terminal nonapeptide of TXA2-R, TXA2-R(l-9), exerts comparable inhibitory effects as W^2-Tat(1-9) on DP IV activity and on DNA synthesis of activated PBMC (Wrenger *et al* 2000). Interestingly, TXA2 receptors have been identified on peripheral blood monocytes. Monocytes participate in the activation of T lymphocytes by presenting antigens via the major histocompatibility complex II (MHC II) to the T cell receptor (TCR). At the same time multiple molecular interactions between ligand-receptor pairs of antigen presenting cells and T cells occur (e. g. CD2-CD58, CD28-CD80), requiring a close contact between both cells. Therefore it is highly probable that the N-terminal part of TXA2-R expressed on monocytes is able to interact with T cell-expressed DP IV during antigenic T cell activation.

We speculate that TXA2-R might be an endogenous ligand of DP IV, contributing to the aforementioned complex molecular interplay modulating T cell activation. A hypothetical mechanism of the modulation of T cell activation by inhibition of DP IV via synthetic inhibitors or inhibitory ligands such as the TXA2-R is shown in Figure 1.

4. TARGETING DP IV IN CNS INFLAMMATION

Due to their blocking effects on T cell activation, it is conceivable to assume that compounds inhibiting DP IV could be used for immunosuppressive therapy *in vivo.* The role of DP IV/CD26 and the effect of synthetic DP IV inhibitors were thus examined in several animal models of human diseases *in vivo* (Table 1). In these studies, various mechanisms have been suggested to account for the therapeutic effects of DP IV/CD26 inhibitors *in vivo.*

Korom and coworkers (1997a; 1997b; 1999) analyzed the influence of the pro-pro-diphenyl-phosphonate (prodipine) on the immune cascade triggered by organ transplantation. The treatment abrogated acute rejection, resulting in prolonged allograft survival in a rat cardiac transplantation model. The inhibition of DP IV was associated with severely impaired host cytotoxic T cell responses.

Table 1: *In vivo* studies examining inhibitors of DP IV enzymatic activity

Model	References
Rat cardiac transplantation	Korom *et al* 1997a; 1997b;1999
Collagen- and alkyldiamine-induced arthritis	Tanaka *et al* 1997; 1998
Experimental autoimmune encephalomyelitis (EAE)	Steinbrecher *et al* 2000; 2001

Other studies demonstrated that different synthetic inhibitors of DP IV enzymatic activity like Ala-boro-Pro, Lys[Z(NO_2)]-thiazolidide, Ala-Pro-nitrobenzoyl-hydroxyl-amine, TMC-2A and TSL-225 suppressed collagen- or alkyldiamine-induced arthritis in a dose-dependent fashion (Tanaka *et al* 1997; Tanaka *et al* 1998). Based on these results, the authors conclude that these inhibitors have antiarthritic effects, although the precise mechanisms of their therapeutic effects remain to be elucidated.

DP IV/CD26 has attracted interest as a potential target in developing anti-inflammatory therapy for multiple sclerosis (MS). As shown on other T cells, CD26 is expressed on myelin-reactive T cell lines from patients with MS (Reinhold *et al* 1998). In addition, patients with MS have an increased median percentage of CD26+ cells among CD4+ T cells in the peripheral blood. CD26 expression decreases somewhat in peripheral blood and cerebrospinal fluid after oral high-dose methylprednisolone treatment Constantinescu *et al* 1995; Hafler *et al* 1985; Sellebjerg *et al* 2000a; Sellebjerg *et al* 2000b). Others found correlations between changes in the CD26-expressing T cells and lesion activity on magnetic resonance imaging in MS patients with relapsing-remitting and chronic progressive disease, consistent with the presumed function of CD26 in T cell activation (Khoury *et al* 2000). Jensen *et al* (2004) recently studied CD4+ T cell activation in patients with clinically isolated syndromes suggesting an initial attack of MS. They demonstrated that the percentage of blood CD26+ CD4+ T cells was increased in these patients, and correlated with magnetic resonance imaging disease activity and clinical disease severity. In contrast, the percentage of CD25+ CD4+ T cells in cerebrospinal fluid correlated negatively with the cerebrospinal fluid concentration of myelin basic protein and the presence of IgG oligoclonal bands. Moreover, gene expression profiling in MS patients and healthy controls identified higher average expression of DP IV/CD26 in peripheral blood mononuclear cells of MS patients (Bomprezzi *et al* 2003). Finally, the DP IV-like ectopeptidase attractin, expressed on T lymphocytes and glia cells, is discussed to be involved in processes of myelination (Kuramoto *et al* 2001).

For these reasons, we addressed the role of CD26/DP IV in murine experimental autoimmune encephalomyelitis (EAE), a well characterized CD4+ T-cell mediated autoimmune disease leading to CNS inflammation and demyelination in susceptible strains of rodents (Steinbrecher *et al* 2000; Steinbrecher *et al* 2001; see also chapter 5 by B.C. Kieseier and F. Bernal).

We demonstrated that the clinical signs of EAE can be suppressed by Lys[Z(NO$_2$)]-pyrrolidide *in vivo* both in a preventive and therapeutic fashion. CNS inflammation associated with acute EAE was reduced. DP IV inhibition *in vivo* did not eliminate autoreactive T cells as encephalitogenic T cells were isolated from both healthy mice treated with Lys[Z(NO$_2$)]-thiazolidide and from diseased control mice. In addition, DP IV inhibition *in vivo* did not suppress antigenic priming. We could show, however, that the protective effect of DP IV inhibition is caused by a modulation of T cell effector function. DP IV activity was detected on the cell surface of all autoreactive T cell clones examined. The inhibitors Lys[Z(NO$_2$)]-thiazolidide and -pyrrolidide had strong antiproliferative effects *in vitro* on the T cell clones examined, on both an encephalitogenic Th1 clone and a nonencephalitogenic Th2 clone. Lys[Z(NO$_2$)]-thiazolidide and -pyrrolidide also suppressed the proliferation of lymph node cells and, importantly, their secretion of TNF-α and, to a lesser extent, IFN-γ. It is widely accepted that EAE can be mediated by Th1 CD4+ T cells typically secreting IFN-γ, TNF-α, and lymphotoxin. We therefore suggest that the inhibition of T cell proliferation and effector functions including proinflammatory cytokine secretion may in part be responsible for the *in vivo* effect. More importantly, we found an up-regulation of latent TGF-ß1 production *in vivo* both in spinal cord tissues and in plasma from Lys[Z(NO$_2$)]-pyrrolidide-treated mice as compared to mice treated with PBS or Lys[Z(NO$_2$)]-OH.

Furthermore, we demonstrated that Lys[Z(NO$_2$)]-pyrrolidide increases the secretion of latent TGF-ß1 by antigen-stimulated lymph node cell populations. We extended these findings, demonstrating for the first time that anti-TGF-ß1 can also block the effect of inhibition of DP IV activity on antigen-specific T cell proliferation. Our *in vitro* data suggested that the cell types induced to secrete latent TGF-ß by Lys[Z(NO$_2$)]-pyrrolidide treatment include T cells. One can assume that TGF-ß1 secretion is increased both by the encephalitogenic T cells initiating the infiltrate and by T cells that are attracted and activated during the later stages of lesion formation in a bystander fashion. Not surprisingly, macrophages and microglia, in addition to a majority of T cells, appear to produce TGF-ß1 in acute EAE lesions. Whether those or other cell types are susceptible to regulation of DP IV remains to be investigated. Taken together, our data suggest that this therapeutic effect may be mediated at least in part by upregulation of the immunosuppressive cytokine TGF-ß1 *in situ* and the inhibition of T cell effector functions.

In conclusion, these findings from *in vitro* and *in vivo* studies demonstrate that DP IV activity plays an important role in the activation of autoreactive T cells. Inhibition of DP IV and DP IV-like activity and possibly other ectopeptidases *in vivo* provides a new approach to down modulate tissue-specific autoimmunity in the CNS. These results may have important implications for the treatment of human diseases with a putative autoimmune pathogenesis. At present, major research efforts are directed at the investigation of DP IV and DP IV-like enzymes as potentially powerful and safe pharmacological targets.

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Chapter 7

Role and Regulation of Proteases in Human Glioma

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1. INTRODUCTION

The invasive and destructive characteristics of malignant neoplasms in the central nervous system are of great clinical importance, as they contribute to a poor prognosis. Patients diagnosed with glioblastomas have a mean survival of only 8-12 months following surgery, chemotherapy or radiation therapy. The infiltrative growth pattern of gliomas contrasts with that of brain metastases, which most often grow with self-defined borders to the adjacent neural tissue. Whereas, the unregulated proliferation of primary brain tumor cells within the skull is a major factor in the prognosis of afflicted patients, the ability of neoplastic cells to migrate and invade surrounding normal brain tissue presents a major hurdle to successful therapeutic intervention. Brain tumor invasion is a complex phenomenon, which involves the interaction between tumor cells, normal stromal cells and the extracellular matrix (ECM). Proteolytic modification of the ECM leads to tumor cell migration into the surrounding tissue (Edwards *et al* 1998; Quaranta 2000; Werb *et al* 1999). The ECM degradation processes involve a highly organized interplay between proteases and their cellular binding sites as well as specific substrates and internalization receptors. Proteases have been known to be critically involved in a number of steps in tumor progression, such as tumor growth, invasion, migration and metastasis (Koblinski *et al* 2000). Members of the cysteine proteinase, serine proteinase and metalloproteinase superfamilies play functional roles in the degradation of ECM proteins (Rooprai *et al* 1997).

2. CATHEPSINS

Cathepsins are a group of cysteine proteases predominantly located in the lysozymes. There are 11 cysteine cathepsins present in the human genome (B, C, F, L, K, V, S, X/Z, H, W and O), each with different expression patterns, levels and specificities, all of which contribute to their varying physiological roles. While non of them are completely specific, some such as cathepsins B, L, and H are very abundant and most are highly active but differently stable at neutral pH—collectively making these proteases potentially harmful if transposed outside of their normal endosomal/lysosomal localization (Turk *et al* 2001). Cathepsins are implicated in tumor angiogenesis, apoptosis and inflammatory and immune responses (Turk *et al* 2004) via their involvement either directly in the degradation of ECM components, such as laminin, fibronectin, and collagen, or through the modulation of protease-sensitive regulatory networks involving other proteases (i.e. uPA) as well as non-proteases (i.e. annexin II) found at the cellular surface of cancer cells (Koblinski et al 2000; Roshy et al 2003) (Figure 1).

Cathepsin B has been the most investigated of the lysosomal enzymes and several studies indicated that the activity of cathepsin B is an order of magnitude higher in glioma tissue than in matched normal brain tissue, and these levels also strongly correlated with clinical invasion as assessed by MRI (Rempel *et al* 1994). Cathepsin B promotes tumor progression by direct proteolytic activity of the ECM components or by activating other proteases (Kobayashi *et al* 1992). Our previous work has demonstrated that cathepsin B activity and 25-26 kDa mature enzyme proteins were the highest in glioblastoma, lower in anaplastic astrocytomas and the lowest in low-grade gliomas and normal brain tissue (Sivaparvathi *et al* 1995).

The involvement of cathepsins in regulation of angiogenesis reveals yet another distinct role for cathepsins in tumor progression (Joyce *et al* 2004). Increased abundance and intensity of cathepsin B staining was observed in 79 glioma tissue samples as compared with 5 normal brain tissue samples (Mikkelsen *et al* 1995). This study also demonstrated intense cathepsin B staining at the tumor margin and in endothelial proliferation in high grade tumors, especially in the region of tumor infiltration into adjacent normal brain, suggesting that cathepsin B is functionally significant in the process of tumor invasion and angiogenesis during glioma progression.

In biopsies of human gliomas, cathepsin B levels correlated with pathological evidence of invasion into normal brain tissue and with survival rate of patients (Strojnik *et al* 1999). Significantly higher expression of cathepsin B correlating with clinical symptoms was demonstrated in studies involving 100 primary brain tumor patients, including 73 malignant and 27 benign tumors of different histologies. This study also demonstrated that

intense cathepsin B staining in endothelial cells was a significant prognostic marker for glioblastoma multiforme (Demchik *et al* 1999).

We have previously reported that cathepsin L is also significantly increased in high-grade astrocytomas when compared with low-grade astrocytomas and normal brain. Further, specific cathepsin L antibodies significantly lowered glioblastoma cell invasion (Sivaparvathi *et al* 1996c). Cathepsin L expression was also shown to be elevated in anaplastic astrocytomas and glioblastoma cell lines when compared to benign astrocytoma cell lines (Lah *et al* 2000). Stable transfection of the glioblastoma cell line ITPT-98 with Cat-L cDNA in the antisense orientation downregulated intracellular cathepsin L expression and significantly diminished *in vitro* invasion into Matrigel as compared with non-transfected and sense cat-L cDNA-transfected cells (Levicar *et al* 2003).

Levels of cathepsin H protein have been found to be significantly higher in glioblastomas and anaplastic astrocytomas as compared to low-grade gliomas and normal brain. The cathepsin H antibody also inhibited the invasion of glioblastoma cells through Matrigel (Sivaparvathi *et al* 1996b). Cathepsin S is also highly expressed in astrocytic tumor cells as compared to normal astrocytes as determined by immunohistochemical analysis (Gibson *et al* 2000) and has been shown to play a role in the invasive process (Flannery *et al* 2003). Further, intracellular cathepsin S activity has been demonstrated in astrocytoma cultures and biopsy specimens. An active form of extracellular cathepsin S enzyme was expressed 5-fold higher in gliomas, suggesting that the secreted cathepsin S may play a role in high-grade astrocytoma invasion (Gibson *et al* 2000).

In relation to meningiomas, cathepsins have been proposed as a marker for malignancy for the more aggressive type of these tumors. Cathepsins B and L expression was studied in 67 benign and 21 atypical meningiomas (Strojnik *et al* 2001). This study indicated that a high level of cathepsin B protein serves as a diagnostic marker for invasive types of benign meningiomas, distinguishing between histomorphologically benign but invasive tumors from the histomorphologically but clearly benign tumors.

Previous studies have demonstrated that cathepsins play a role in apoptosis of cultured cells (Shibata *et al* 1998; Isahara *et al* 1999). The anti-apoptotic activity of cathepsin B and cathepsin L was reported using cysteine cathepsin inhibitor Z-Phe-Gly-NHO-Bz, which induces rapid apoptotic death in glioblastoma cells (Zhu *et al* 2000). Cathepsin L affects expression and the rate of caspase-3 activation after induction of apoptosis, suggesting that cathepsin L possibly interferes with an apoptotic cascade upstream of the caspase-3 as demonstrated in a glioblastoma cell line (Levicar *et al* 2003). Cathepsins B and D are involved in the H_2O_2-induced apoptosis in cultured astrocytes; cathepsin D acts as a death-inducing factor upstream of caspase-3 and caspase-independent apoptosis is regulated antagonistically by cathepsins

B and D (Takuma *et al* 2003). Prolonged inhibition of the lysosomal proteolytic pathway is incompatible with cell survival, leading to apoptosis of neuroblastoma cells. Treatment with E-64 or CA074Me (two specific inhibitors of cathepsin B) or with pepstatin A (a specific inhibitor of cathepsin D) was cytotoxic for 2 neuroblastoma cell lines having differing degrees of malignancy. Since inhibition of either cathepsin B or D affected cell viability, the authors hypothesized that in cultured neuroblastoma cells, cathepsin B and cathepsin D are sequentially involved in the degradation of pro-apoptotic factors and that the inhibition of either enzyme leads to accumulation of substrates that eventually impact on the caspase cascade (Castino *et al* 2002).

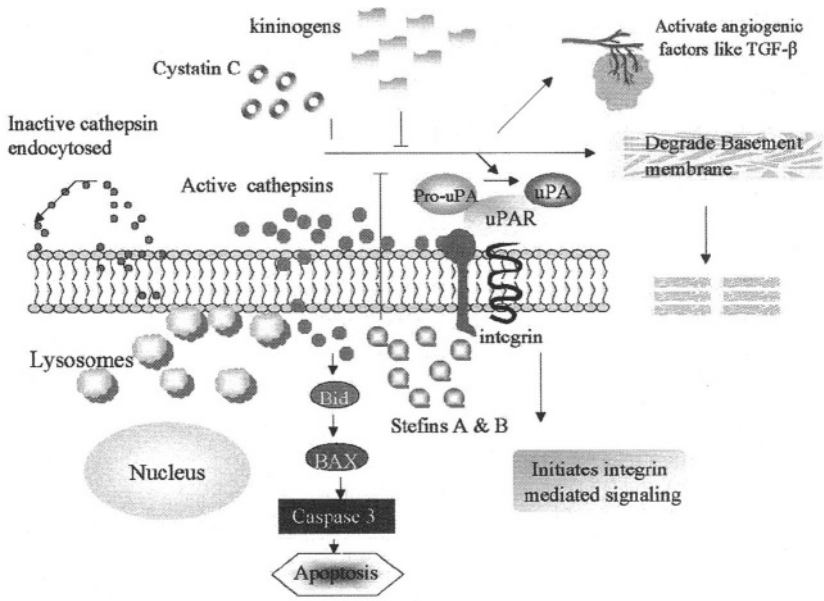

Figure 1: Function of cathepsins in cancer progression. Secretion of cathepsins facilitates invasion by direct action on basement membrane and indirectly by the cleavage and processing of growth and angiogenic factors and their receptors leading to angiogenesis. Cathepsin was also shown to play a role in apoptosis. Endogenous cysteine protease inhibitors inhibit their activity. Stefins A and B are localized intracellularly while cystatin C and kininogens are secretory proteins.

Cysteine protease inhibitors (CPI) of the cystatin family are important endogenous proteins, which protect extracellular structures from abnormal proteolysis from cysteine proteases. Inhibitors capable of blocking both intra and extracellular fractions of cathepsin B were the most effective in reducing

the invasive potential of tumor cells (Premzl *et al* 2003). They form tight, equimolar non-covalent complexes with the cysteine proteases to protect cells from the harmful effects of continuous proteolysis. We have previously reported high cysteine protease inhibitory activity in glioma cells and culture medium. Low levels of cysteine protease inhibitory activity were observed in high-grade gliomas (anaplastic and glioblastoma) as compared to low-grade and benign tumors. Similarly CP inhibitory activity was high in benign and atypical meningiomas as compared to invasive meningiomas (Sivaparvathi *et al* 1996a).

Three types of CPI inhibitors have been identified: stefins (Mr ~11kD), cystatins (Mr~13) and kininogens (Mr~60 and 100 kDa) (Calkins *et al* 1995). The major intracellular or endogenous cysteine proteinase inhibitors are stefins A and B. Cystatin C is an extracellular molecule that is synthesized within the human brain including choroid plexus, cerebral and cerebellar neurons and astrocytes (Lignelid *et al* 1997). We have demonstrated that cystatin C levels were significantly higher in low-grade and anaplastic astrocytoma cell lines as compared to glioblastoma cells (Konduri *et al* 2002).

The synthetic cathepsin B inhibitor K11017 blocked glioblastoma migration and substantially reduced glioma cell invasion in a Matrigel assay and reduced the infiltration of glioma spheroids into normal brain aggregates (Demchik *et al* 1999). An *in vitro* invasion assay has demonstrated that the invasiveness of glioblastoma cells was significantly impaired by cysteine protease inhibitor E-64 (Konduri *et al* 2001) and by intracellular and extracellular inhibition of cathepsin B by its selective inhibitors, Ca074Me and Ca074 respectively (Lah *et al* 2000). Glioblastoma cells expressing antisense cathepsin B cDNA exhibited significant reductions in cathepsin B mRNA and protein and invasiveness in Matrigel and spheroid models *in vitro.* Intracerebral injection of SNB19 stable antisense transfectants resulted in reduced tumor formation in nude mice (Mohanam *et al* 2001).

Blockade of cathepsin B expression in human glioblastoma cells is also associated with suppression of angiogenesis. Antisense cathepsin B stable clones demonstrated a marked reduction in capillary network formation by endothelial cell *in vitro* and microvasculature development *in vivo* dorsal air sac assay as compared to vector-transfected and parental controls (Yanamandra *et al* 2004). Stable transfection of cystatin C gene in the sense orientation reduced the invasiveness of glioblastoma cell line *in vitro* and did not form tumors in nude mice *in vivo* (Konduri *et al* 2002). The bicistronic antisense construct for uPAR and cathepsin B (Ad-uPAR-Cath B) infected cells revealed a marked reduction in migration, invasion, angiogenesis and tumor growth in both *in vitro* and *in vivo* models (Gondi *et al* 2004; Lakka *et al* 2004). Further, our recent studies demonstrated that bicistronic siRNA construct against cathepsin B and MMP-9 regressed completely pre-established intracerebral tumors in nude mice (Lakka *et al* 2004). These

results strongly support a role for cathepsin B in the invasiveness of human glioblastoma cells and suggest that an antisense cathepsin B strategy may prove useful in cancer therapy.

3. UROKINASE PLASMINOGEN ACTIVATOR AND ITS RECEPTOR

The urokinase plasminogen activator (uPA) system consists of the serine protease uPA, its glycolipid-anchored receptor, uPAR, and its two serpin inhibitors, plasminogen activator inhibitor-1 (PAI-1) and plasminogen activator inhibitor-2 (PAI-2). Urokinase plasminogen activator was first identified in urine and later in human plasma, seminal fluid, and certain cancers (Dano *et al* 1985). It is a single polypeptide chain (sc-uPA) consisting of 411 amino acids and the triad of His^{204}, Asp^{255} and Ser^{356} dictates its serine proteinase activity. uPAR is a glycosyl-phosphatidylinositol-anchored glycoprotein on the surface of various cell types that serves to bind the urokinase plasminogen activator and localize activation reactions in the proteolytic cascade system of plasminogen activation. Through its own proteolytic function, plasmin degrades a range of extracellular basement membrane components and activates other proteases including the matrix metalloproteinases. The role of uPA and uPAR in tumor progression is schematically represented in Figure 2. uPAR is a three-domain molecule. Each domain is numbered 1 to 3 from the amino-terminus to the carboxy-terminus. Domain 1 is generally the only domain involved in (pro)-uPA binding but domain 3 is also involved in providing the uPA binding site.

Overexpression of uPA or uPAR is a feature of malignancy and is correlated with brain tumor progression and invasion. uPA levels/activity and PAI-1 antigen levels were significantly elevated in malignant brain tumors (malignant meningiomas, high-grade gliomas, and metastatic tumors) and acoustic schwanomas but are present in significantly lower levels in benign meningiomas, low-grade gliomas and normal brain (Arai *et al* 1998). We have previously shown that brain tumors, and specifically gliomas, overexpress components of the uPA/uPAR system, as well as a correlation between tumor grade and expression levels (Yamamoto *et al* 1994a,c; Mohanam *et al* 1999). Notably, tumor grade and patient survival are correlated with uPA expression (Hsu *et al* 1995).

Independent of catalytic activity, uPAR is also involved in cell signaling, interactions with integrins, cell motility, adhesion, invasion and angiogenesis. uPAR plays a role in the migration of a variety of cell types and evidence is accumulating that uPAR-dependent migration is mediated through integrins. Several signaling pathways, including MEK-ERK (mitogen-activated protein

kinase-extracellular signal-regulated kinase) and Jak-STAT (Janus kinase-signal transducer and activator of transcription), have been implicated in uPAR-mediated cell migration and adhesion. These effects, which involve cytoskeletal components and cytosolic and transmembrane kinases, were shown to be associated with uPAR interactions with various molecules such as vitronectin, several members of the integrin family, caveolin and G protein-coupled receptors (Blasi *et al* 2002; Resnati *et al* 2002). Antisense-mediated downregulation of uPAR in a human glioma cell line altered cell morphology, cell diffusion and cytoskeletal organization and upregulated $\alpha 3\beta 1$ integrin expression (Chintala *et al* 1997). uPAR is now known to interact directly with ECM components including vitronectin (VN) on various cell types (Kanse *et al* 1996; Waltz *et al* 1997). Association of uPAR with VN is enhanced by ligand occupancy of uPAR (Kanse *et al* 1996; Waltz *et al* 1994) cells and the intact receptor is required for the VN-uPAR interaction to occur (Hoyer-Hansen *et al* 1997). Glioblastoma cells stably transfected with antisense uPAR cDNA undergo apoptosis when plated onto fibronectin or vitronectin-coated plates *in vitro* or when injected intracerebrally *in vivo* (Kin *et al* 2000). Recently, we demonstrated that migration and adhesion of glioblastoma cells on VN-coated plates was uPA and uPAR-dependent (Gondi *et al* 2003). Decreases in the amount of cell-bound uPA and disruption of actin cytoskeleton formation and cell migration in stably transfecting human glioblastoma cells with antisense uPA was mediated by the phosphatidylinositol 3-kinase (PI3k) and Akt signaling pathways (Chandrasekar *et al* 2003).

Several growth factors (bFGF, VEGF, HGF) sequestered by the ECM (in addition to TGF-β) can also be activated or released from binding sites as part of this cascade and feedback into the uPA system, resulting in an increase in uPA and uPAR expression (Mandriota *et al* 1995; Pepper *et al* 1992, 1993). The addition of basic fibroblast growth factor (bFGF) or transforming growth factor-α (TGF-α) increased the invasiveness of human gliomas cell lines as well as uPA and uPAR mRNA levels. The growth factor-induced invasiveness can be reversed with uPAR-specific antibodies (Mori *et al* 2000). In a collagen lattice assay, TGF-β1 induced collagen lattice contraction of T98G glioma cells via enhanced expression of the subunit α2-integrin expression in a concentration-dependent manner. An anti-α2 antibody, P1-E6, and antisense phosphorthioate oligonucleotides against α2 integrin have been shown to inhibit these effects of TGF-β1 (Miyake *et al* 2000). TGF-β1 and TGF-β2 promote migration, induce αv and β3 integrin mRNA expression, and enhance the cell surface expression of αvβ3 in a concentration-dependent manner (Platten *et al* 2000).

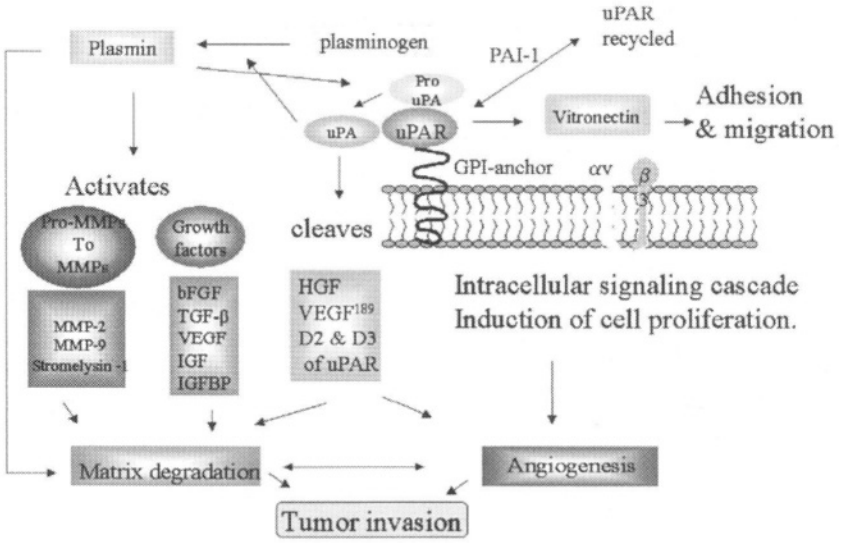

Figure 2: Role of uPA/uPAR pathway in tumor progression. uPA in association with uPAR activates plasminogen to plasmin. Plasmin activates uPA and other matrix metalloproteases and cleaves fibrin, laminin and fibronectin resulting matrix destruction. Degradation of basement membrane releases and activates growth factors such as bFGF, TGF-b, VEGF, IGFBP, which are involved in tumor invasion and angiogenesis. uPAR, in association with integrin molecules, transduces signals for cell migration, morphogenesis and vessel formation. uPA activity is inhibited by its endogenous inhibitor, PAI-1, which is also involved in recycling uPAR.

Expression of uPA and its receptor have also been observed during tumor angiogenesis (Mazar *et al* 1999). In particular, the uPA system seems to be important for the migration and invasion of endothelial cells during angiogenesis. Angiogenesis involves several processes, including proteolysis and remodeling of the basement membrane, endothelial cell activation, proliferation, migration and tissue infiltration from pre-existing blood vessels. *In vitro* experiments have shown the importance of pro-angiogenic factors, such as bFGF and VEGF, in inducing endothelial cells to migrate (Park *et al* 1993; Ribatti *et al* 1999). These same growth factors are released by the proteolytic action of the uPA system in cancer. A peptide derived from the connecting peptide region of uPA inhibits the migration of microvascular endothelial cells and GBM tumor progression in combination with an agent that inhibits endothelial cell proliferation [cisplatin (CDDP)] (Mishima *et al* 2000).

uPA can be inactivated irreversibly by reaction with any one of several protein inhibitors including PAI-1 (which is abundant within the ECM), PAI-2 (which is induced in lymphocytes and macrophages), PAI-3 (protein C inhibitor), α2-macroglobulin and protease nexin (also found in ECM).

Paradoxically, high tumor levels of the uPA inhibitor plasminogen activator inhibitor-I (PAI-I) are associated with a poor prognosis, and as such, high levels of PAI-1 have been correlated with poor prognosis in cancer patients (Muracciole *et al* 2002). PAI-1 mRNA is abundantly expressed in glioblastomas and anaplastic astrocytomas but weakly expressed in low-grade astrocytomas or normal brain, suggesting that high expression of PAI-1 is associated with the malignant progression of astrocytic tumors. The distribution of PAI-1 mRNA expression has been found to be particularly abundant around areas of vascular proliferation and in remnant tumor cells surrounding necrotic foci and could be possibly associated with intratumoral necrosis in glioblastomas (Yamamoto *et al* 1994b). Localization of PAI-1 in proliferating vessels of intracranial tumors (glioblastoma multiforme, 5 anaplastic gliomas, 4 malignant meningiomas, and 9 metastatic tumors) suggests that PAI-1 may be involved in angiogenesis (Kono *et al* 1994). In addition to recruiting, focusing, and enhancing the activation of plasminogen by uPA locally on cell surfaces, uPAR also participates in the regulation of this activity through internalization of the uPA-PAI complexes. Ternary complexes of uPAR-uPA-PAI are rapidly endocytosed through recognition and uptake by the lipoprotein receptor-related protein (LRP, α2-macroglobulin receptor). The uPA and PAI components are degraded while uPAR is recycled to the cell surface (Rao *et al* 1995). As demonstrated by RT-PCR analysis, LRP mRNA was frequently expressed in glioblastomas and anaplastic astrocytomas as compared with low-grade astrocytomas and was well-correlated with uPAR expression. Altered LRP expression might contribute to the stimulation of cell-surface proteolytic activity that, in turn, facilitates the invasiveness of glioblastomas *in vivo* (Yamamoto *et al* 1998). PAI-1 shares a mutually exclusive binding site for VN (in the somatomedin B domain) with uPAR and is capable of displacing uPAR from VN (Deng *et al* 1996). Andreasen et al., reported that PAI-1, independent of its role as a proteinase inhibitor, inhibits cell migration by competing for vitronectin binding to integrins, while the interference of PAI-1 with binding of vitronectin to the urokinase receptor may play a secondary role (Andreasen *et al* 1997). The effect of adenoviral-mediated transfer of the PAI-1 gene in regulating the *in vitro* invasiveness of D54MG glioma cells into Matrigel and into fetal rat brain aggregates was studied by (Hjortland *et al* 2003). The results show that PAI-1 overexpression can inhibit glioma cell motility and invasion through extracellular matrix (ECM) components, such as laminin and collagen, but does not inhibit tumor cell invasion in a three-dimensional invasion assay that simulates normal brain tissue with varying ECM and interstitial composition. The different results obtained in the two invasion assays reflect the complex biological effects of the uPA/PAI-1 system.

Elevated levels of PAI-1 have also been reported in primary tumors of advanced neuroblastomas (Sugiura *et al* 1999). Further, PAI-1 inhibited

endothelial tube formation on Matrigel in the presence of vitronectin, but had a stimulatory effect in the presence of fibronectin suggesting that PAI-1 acts as a positive switch for angiogenesis by promoting endothelial cell migration away from their vitronectin-containing perivascular space toward fibronectin-rich tumor tissue (Isogai *et al* 2001). PAI-I seems to be multifunctional as it is expressed by multiple cell types and has multiple molecular interactions. The potential utilization of PAI-I as a target for anti-cancer therapy depends on further mapping of these functions.

Several *in vivo* studies have indicated that modulation of uPAR expression or blockade of uPAR with catalytically inactive uPA fragments leads to reductions in tumor growth and tumor angiogenesis, and an increase in tumor dormancy in a variety of tumor types (Tressler *et al* 1999). Strategies that target uPA or its receptor with the aim of disrupting the interaction between the two or the ligand-independent actions of uPAR include antisense technology, monoclonal antibodies, cytotoxic antibiotics and synthetic inhibitors of uPA. These studies have shown a reduction in the invasive capacity gliomas. Downregulation of uPAR expression using antisense and gene therapy approaches has resulted in increased survival in animal models. The antisense stable uPAR glioblastoma clones resulted in an inability of the cells to generate tumors when transplanted into nude mice (Go *et al* 1997). The infection of glioblastoma cells with antisense uPAR adenoviral vectors in cultures resulted in tumor growth *in vivo* (Mohan *et al* 1999). Adenovirus-mediated downregulation of antisense bicistronic constructs of uPA and uPAR expression inhibited glioma cell migration, invasion and tumor-induced capillary formation (Gondi *et al* 2003). In another study, stably transfected glioma cells expressing the ATF domain did not form tumors in nude mice (Mohanam *et al* 2002). Treatment of U87MG gliomas implanted orthotopically in mice with single species-specific or combination of uPAR ligands resulted in significant decreases in tumor size, which translated to increases in survival time, particularly when the murine-specific ligand was included. Reduced tumor sizes were correlated with a decrease in tumor cell proliferation and mean vessel density and an increase in tumor cell apoptosis.

The authors suggest that the major role of the uPAR system in brain tumor progression is in the stromal compartment, particularly in neovascularization, which is a hallmark of invasive brain tumors (Bu *et al* 2004). Among the integrins, integrin $\alpha v\beta 3$ has been identified to play a particular role in gliomas (Uhm *et al* 1999). Both the $\alpha v\beta 3$ integrin and its ligand, vitronectin, are specifically expressed at the advancing margin of high-grade gliomas (Gladson *et al* 1991). Inhibition of $\alpha v\beta 3$ integrin ligation reduces glioma cell migration (Paulus *et al* 1994). Adenovirus-mediated suppression of uPAR and restoration of INK4 activity decreased $\alpha v\beta 3$ integrin levels in glioblastoma cells and resulted in decreased cell adhesion, migration, proliferation and survival (Adachi *et al* 2001).

A recombinant fusion protein (DTAT) consisting of the non internalizing amino-terminal fragment (ATF) of urokinase-type plasminogen activator (uPA) for binding, the catalytic portion of diphtheria toxin (DT) for killing, and the translocation enhancing region (TER) of DT for internalization caused the regression of small, subcutaneous uPAR-expressing tumors with minimal toxicity to critical organs. This hybrid protein was highly effective against tumor cells cultured from glioblastoma multiforme patients (Rustamzadeh *et al* 2003).

Peptides based on the growth factor domain of uPA, which mediates the binding of uPA to uPAR, have been found to be potent inhibitors of metastasis and tumor growth (Guo *et al* 2000). Suramin inhibited the binding of uPA to U937 cells in a non competitive manner (Behrendt *et al* 1993).

4. MATRIX METALLOPROTEINASES

Matrix metalloproteinases (MMPs) constitute a family of zinc-containing enzymes with more than 20 members identified to date. They are divided into four groups such as interstitial collagenases, stromelysins, gelatinases and membrane type metalloproteinases (MT-MMPs) based on protein structure (Romanic *et al* 1994). Unlike MMPs, MT-MMPs are membrane-bound, and studies have shown that they are activated either intracellularly or on the cell surface (Yana *et al* 2000). Another group of proteolytic metalloproteases is the ADAMs family, which consists of at least 33 different enzymes (Primakoff *et al* 2000). More recently, a new group of metalloproteases called ADAMs has been identified. Like the ADAMs, these enzymes contain a disintegrin and metalloprotease domain. However, instead of a transmembrane domain, the ADAMs express one or several thrombospondin 1-like repeats (Tang 2001). Although their physiological functions are not yet completely understood, nineteen different members have been identified (Cal *et al* 2002).

MMPs are secreted as pro-MMPs and then activated following sequential cleavage steps (Murphy *et al* 1999; Shapiro *et al* 1995). Removal of the signal peptide and pro-peptide domains or a change in configuration activates the enzymes. MMP expression and proteolytic activity are tightly regulated at three stages: gene transcription, proenzyme activation and activity of natural inhibitors (tissue inhibitors of metalloproteinase known as TIMPs). The balance between production, activation and inhibition prevents excessive proteolysis or inhibition. Several factors like cytokines, growth factors, phorbol esters; cell-cell and cell-matrix interactions are thought to control MMP expression (Westermarck *et al* 1999). Most MMPs are secreted as inactive zymogens, which may be proteolytically activated by different

proteinases such as other MMPs, plasmin, trypsin, chymotrypsin and cathepsins. Several cell types produce MMPs in the CNS by microglia, astrocytes, oligodendrocytes and neurons (Gottschall *et al* 1996; Maeda *et al* 1996; Uhm *et al* 1998). MMP-2 and MMP-9 are secreted by microglia and astrocytes as active forms (Muir *et al* 2002).

The expression of several forms of metalloprotease inhibitors by fetal astrocytes and glioma cell lines has been reported (Apodaca *et al* 1990; Rucklidge *et al* 1990). MMPs have been shown to regulate tumor cell invasion through their interactions with extracellular matrix components including cell matrix embedded growth factors and cell adhesion molecules (Kruger *et al* 2000; Itoh *et al* 1998). Furthermore, a metalloprotease secreted by the rat glioma cell line BT5C in serum-free medium was observed to be capable of degrading fetal rat brain aggregates (Bjerkvig *et al* 1986; Lund-Johansen *et al* 1991). Significantly high levels of MMP-2 (gelatinase A) and MMP-9 (gelatinase B) enzymatic activity, protein and mRNA levels have been observed in glioblastoma tissue samples when compared to control brain tissue samples (Apodaca *et al* 1990; Nakano *et al* 1993; Nakagawa *et al* 1994, 1996; Uhm *et al* 1996; Yamamoto *et al* 1996; Forsyth *et al* 1998, 1999; Raithatha *et al* 2000; Sawaya *et al* 1996; Rao *et al* 1993; Rao *et al* 1996). MMP-2 has been observed to be localized in malignant glioma cells and blood vessels along with MT1-MMP, whereas MMP-9 was strongly expressed in blood vessels at proliferating margins (Nakagawa *et al* 1994) as well as in the tumor cells (Raithatha *et al* 2000). Furthermore, glioblastoma cells injected intracranially into nude mice resulted in significantly increased levels of MMP-2 and MMP-9 during the progression of tumors (Sawaya *et al* 1998). In addition, astroglioma cells constitutively express high levels of MMP-2 mRNA, protein and bioactivity as assessed by ribonuclease protection assay, immunoblotting and zymography assay respectively (Qin *et al* 1998).

From the subfamily of collagenases, MMP-1 has been found in gliomas (Nakagawa *et al* 1994) and MMP-13 in childhood astrocytomas (Bodey *et al* 2000a) and medulloblastomas (Bodey *et al* 2000b). MMP-3, a stromelysin, has been shown to be expressed in higher levels in glioblastomas as compared to controls (Vince *et al* 1999). MMP-3 is expressed during the insidious invasiveness of astrocytoma either directly through degradation of specific matrix macromolecules, or indirectly via activation of other MMPs that might elicit an enhanced scope of ECM digestion (Mercapide *et al* 2003). MMP-7, 10 and 11 were strongly expressed by glial tumor cells, showing a significantly stronger immunoreaction in neoplastic astrocytes as compared to oligodendroglial tumor cells (Thorns *et al* 2003). Membrane-type MMPs (MT1-MMP, MT2-MMP, MT3-MMP and MT4-MMP) have shown proteolytic activity towards several ECM molecules including fibronectin, vitronectin, laminin, tenascin, proteoglycans and collagen (d'Ortho *et al*

1997; Ohuchi *et al* 1997). Several studies reported the evidence of MT-MMP expression in glioma cells lines. MT1-MMP was overexpressed in D54, LG11, T98 and U87MG cells as compared to A173, Hs683, U251 MG and U373 (Hur *et al* 2000). High levels of MT1-MMP and MT3-MMP mRNA were detected in U87MG whereas MT2-MMP was expressed in low levels (Shofuda *et al* 1998). MT1-MMP mRNA was absent or barely detectable in normal brain and was overexpressed in high-grade gliomas. Further, its expression was shown to correlate with the expression and activation of MMP-2 (Yamamoto *et al* 1996). MT2-MMP was shown to increase accordingly with the histological grade of malignancy, whereas MT3-MMP was variably expressed in low levels in brain tumor samples (Kachra *et al* 1999; Nakada *et al* 1999). Although MT5-MMP transcripts were not detected in normal brain, studies have demonstrated that it is overexpressed in a number of glioblastomas, astrocytomas and anaplastic astrocytomas (Llano *et al* 1999). ADAM8 (CD156) is present in neurons and oligodendrocytes and has been implicated in cell adhesion in neurodegenerative disorders (Satoh *et al* 2000).

Growth factors, such as endothelial growth factor (EGF), basic fibroblast growth factor (b-FGF), transforming growth factor (TGF-β1 and β2) and vascular endothelial growth factor (VEGF) and transcriptional factors have been shown to up regulate MMP-2 and MMP-9 in gliomas. This effect was most prominent with TGF-β1 and β-2 (Rooprai *et al* 2000; Choe *et al* 2002). MMP-2 is constitutively present in the brain at low basal levels. Basal gene transcription is mediated via normal cytokine signaling events, which cause basal levels of transcriptional activation at SP1, ASP3 and AP-2 binding sites (Qin *et al* 1999).

We have previously shown that MMP-9 production is induced by cytoskeletal changes involving protein kinase C activation mediated by NF-κβ (Chintala *et al* 1998). Another study demonstrated that vanadate and phenyl arsenic oxide inhibited migration and invasion of glioma cells by their effects on the cytoskeleton and inhibition of MMP-9 expression (Chintala *et al* 1999). Factors that have been reported to induce MT1-MMP expression in glioma cell lines include EGFR activation (VanMeter *et al* 2001), EMMPRIN (CD147) (Sameshima *et al* 2000), HGF/SF stimulation (Hamasuna *et al* 1999) PMA (Park *et al* 2000), and possibly the β-amyloid peptide (Deb *et al* 1999). Matrilysin (MMP-7) was also reported to be overexpressed in gliomas. However, this protease is generally overexpressed in comparison to other MMPs in the brain (Pagenstecher *et al* 1997). High basal levels of MMP-7 could increase the expression of other MMPs involved in tumor progression in gliomas since MMP-7 was shown to activate MMP-9 by a proteolytic interaction which dissociates the inhibitory pro-MMP-9/TIMP complex (von Bredow *et al* 1998). TGF-β1 stimulated the expression of matrylisin in two human glioma cell lines and activated MMP-9 in C6 rat

glioma cells (Nakano *et al* 1993). Recombinant TGF-β2 additions to U87MG and Ln-229 glioma cell lines resulted in a marked increase in MMP-2 expression and suppression of TIMP-2 protein. Further, MT1-MMP-mediated activation of pro-MMP-2, may be an important factor during glioma invasion (Yamamoto *et al* 1996; Uhm *et al* 1996). Protein kinase C (PKC) also plays an important role in the regulation of glioma MT1-MMP mRNA expression, MMP-2 activation and invasion (da Rocha *et al* 2000).

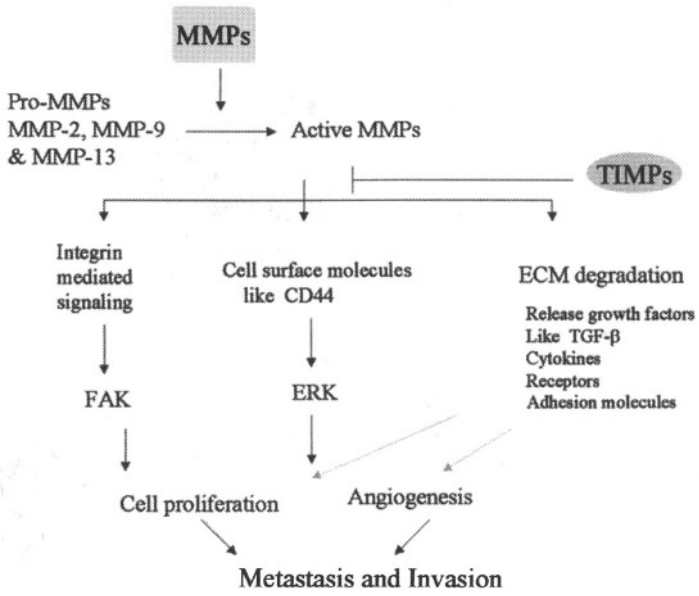

Figure 3: Role of MMP in tumor growth. MMPs promote invasion by degrading extracellular membranes directly and also by activating other MMPs. In particular MT1-MMP was shown to activate MMP-2, and MMP-9 was further activated by the activated MMP-2. MMPs also play additional roles in association with several cell surface molecules, such as integrin αv3; CD44, TGF-β and TNF further modulating the activities to promote cell proliferation, invasion and angiogenesis. Their endogenous inhibitors, TIMPs, inhibit their activity.

The mitogen-activated kinase/extracellular signal-regulated kinase (MEK/ERK) signaling pathway is essential for MMP-9 upregulation in astrocytes after PMA [PKC induction and TNF-α (cytokine) stimulation]. It has been reported that SNB19 cells transfected with dominant negative JNK, MEKK and ERK1 expression vectors decreased MMP-9 expression as well as promoter activity (Lakka *et al* 2000). The mt-ERK stable SNB 19 cells showed decreased levels of MMP-9 and less invasiveness as compared to parental and vector-transfected stable clones (Lakka *et al* 2002).

MMPs mediate cell-surface-receptor cleavage and release, cytokine and chemokine activation and inactivation, and the release of apoptotic ligands (Egeblad *et al* 2002). These cellular processes are all involved in promoting aspects of tumor growth, such as cell proliferation, adhesion and dispersion, migration, differentiation, angiogenesis, apoptosis and host defense evasion (Figure 3). β1 integrin is highly expressed in invasive gliomas, and in vitro invasion of β1-integrin expressing glioma cells can be blocked with the exogenous application of specific antisera (Rooprai *et al* 2000). We have previously observed that stimulation of intracellular β1 integrin can increase the activation of MMP-2 and the invasiveness of glioma cells *in vitro* (Chintala *et al* 1996). In addition to cleaving structural ECM components, MMPs and the related proteinases, such as the ADAMs (a disintegrin and metalloproteinase), participate in the release of cell-membrane-bound precursor forms of many growth factors, including transforming growth factor-α (TGF-α) (Peschon *et al* 1998).

Glioma cells transfected with MT1-MMP cDNA constructs displayed increased cell surface expression of MT1 -MMP and TIMP-2 and resulted in increased migration in spheroid outgrowth assays (Hotary *et al* 2000) as well as remodeling of extracellular matrix *in vitro* (Deryugina *et al* 1997). Studies have demonstrated that cytokines, tumor necrosis factor-α and interferon-γ inhibit MMP-2 expression in glioma cells, resulting in decreased invasion (Qin *et al* 1998). We have demonstrated that glioblastoma cells expressing antisense MMP-9 exhibited decreased migration and invasion in vitro and did not form tumors when injected intracranially in nude mice (Kondraganti *et al* 2000). Intracranial injections of glioblastoma cells (SNB19) infected with an adenovirus expressing antisense MMP-9 did not produce tumors in nude mice (Lakka *et al* 2002). A bicistronic Ad-construct with antisense uPAR and MMP-9 had more effect in regard to inhibition of invasion, angiogenesis and tumor growth *in vivo* (Lakka *et al* 2003). Our recent studies demonstrated that siRNA bicistronic construct for cathepsin B and MMP-9 completely repressed pre-established intracranial tumors (Lakka *et al* 2004). Stable transfection of PTEN (Phosphatase and tensin homologue) reduced MMP-9 secretion caused by hyaluronic acid-induced phosphorylation of focal adhesion kinase and ERK1/ERK2 signaling (Park *et al* 2002). Glioblastomas with EGFR VIII amplification demonstrated the highest levels of MMP-9 (Choe *et al* 2002). It has been reported that hypoxic GBM cells *in vitro* secrete high levels of pro- and activated MMP-2 (Bart *et al* 2004; Bart & Van Meir, 2004).

Studies utilizing native MMP inhibitors, such as the TIMPs, have demonstrated that downregulation of both TIMP-1 and TIMP-2 contributes significantly to the invasive potential of gliomas (Mohanam *et al* 1995; Nakano *et al* 1995; Lampert *et al* 1998). Introduction of TIMP-1 cDNA into an invasive astrocytoma cell line reduced its invasive potential (Matsuzawa *et*

al 1996). Also, synthetic MMP inhibitors Batimastat Marimastat, AG3340 BE16627B, SI-27 effectively reduce glioma invasion *in vitro* (Tonn *et al* 1999; Naito *et al* 1994). It has been reported that post-surgical administration of PEX or PF-4/CTF significantly reduced the incidence human malignant glioma recurrences for a significant period of time (Bello *et al* 2002). Recent studies demonstrated that testican 2 may contribute to glioma invasion by inactivating other testican family members and MT-MMPs (Nakada *et al* 2003). Chlorotoxin inhibits glioma cell invasion by inhibiting MMP-2 enzymatic activity of MMP-2 and causes a reduction in the surface expression of MMP-2 (Deshane *et al* 2003). Pro-peptide cleavage of gelatinase A to its active form appears to be a critical step in invasion and metastasis in gliomas with the potential of initiating further proteolytic cascades via cleavage of the pro-forms of collagenase 3 and MMP-9 (Fridman *et al* 1995).

5. CONCLUSIONS

The major challenge presented by glioblastomas is the tendency of the malignant cells to invade the adjacent normal brain tissue, and extensive search has focused on understanding the invasive behavior of these glioma cells in both *in vitro* and *in vivo.* Invasion of glioma cells is a complex process involving several molecules, cell-cell and cell-ECM interactions. The main focus of this chapter is an overview of these three classes of proteases during the progression and regression of these tumors *in vivo.* Based on the above observations, the net proteolytic, and therefore the invasive potential of a given tumor cell might depend on the interplay between enzymes. Several studies indicated that inhibitors, small peptides and antibodies specific for these molecules, signalling pathway molecules and growth factors inhibit the invasive behavior in both *in vitro* and *in vivo* models. Further, anti-sense and sense stable clones of Ad-constructs for various proteases, protease inhibitors, growth factors and integrins inhibited the invasive behavior of human glioma cells in both *in vitro* and *in vivo* models. Using micro-array technology, it should be possible to characterize in detail the different gene clusters that regulate the invasive phenotype of malignant gliomas and also to determine to what extent such clusters might be affected by new therapeutic strategies. For translation application a combination therapy targeting more than one of the interacting proteolytic agents might be required for effective anti-angiogenic and anti-invasive therapies. Our recent Ad-bicistronic constructs or bicistronic siRNA constructs clearly demonstrated more effective methods in which to inhibit invasion, angiogenesis and tumor growth. Ultimately, further investigation

and an understanding of the interaction of these molecules at the molecular level should have an important effect on the future development of new, target-selective treatments.

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Chapter 8

Proteases and Prion Diseases

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1. INTRODUCTION

In this chapter we shall review the physiological role of the prion protein (PrP^{C}) and its metabolism, with special reference to proteases, as well as to the physiopathological role of abnormal prion protein (termed PrP^{Sc}) in various disorders known as prion diseases or transmissible spongiform encephalopaties (TSEs) (Collinge, 2001). In humans TSEs can be classified as sporadic, familial and acquired (or infectious). The majority prion disease in human occurs as sporadic Creutzfeldt-Jakob disease (sCJD); the familial TSEs include hereditary CJD, Gertsmann-Sträussler-Scheinker disease (GSS) and fatal familial insomnia (FFI), and the acquired include the new variant (vCJD), iatrogenic CJD and kuru (Hetz and Soto, 2003; Kim *et al* 2004; Aguzzi, 2004). Prions also cause diseases in other species, termed bovine spongiform encephalopathy in cattle, scrapie in sheep and goat (Kim *et al* 2004). Clinically, these are fatal disorders, characterized by progressive cognitive and neurological impairment with dementia and motor dysfunction (Johnson and Gibbs, 1998). Neuropathological findings include spongiform degeneration of the brain and sometimes deposition of amyloid plaques, abnormal growth of astrocytes and microglial cells (Prusiner, 1998; Hetz and Soto, 2003).

Sporadic CJD disease accounts for approximately 85-90% of all CJD cases, with a worldwide incidence of one per million inhabitants (Hetz and

Soto, 2003), except for Slovakia, Chile, and Lybian and Tunisian-born Jews living in Israel, where an incidence of 2 per million inhabitants is reported. (Kettlun *et al* 2003a). The disease susceptibility and phenotypic expression are influenced by polymorphism at codon 129 that encodes methionine (M) or valine (V) (Parchi *et al* 1999; Notari *et al* 2004).

Familial CJD, in which there are two or more individuals in a given family affected with the illness, accounts for 5-10% of CJD cases. The substitution of glutamic acid (E) for lysine (K) in the prion protein gene *(PRNP)* located in the short arm of chromosome 20, is the most frequently occurring mutation (Goldfarb *et al* 1991). The cerebral cortical PrP deposits in genetic CJD do not differ from sCJD, but in most of the cases including the two alleles with codon 129M/M, or M/V, but not V/V, exhibit an additional PrP immunostaining in the cerebellum suggesting that this selective vulnerability of cerebellum might be peculiar to some genetic CJD (Jarious *et al* 2003). GSS and FFI are other prion induced TSEs associated with some characteristic mutations in *PRNP* (Kovács *et al* 2002).

The infectious vCJD, commonly known as "mad-cow disease", was first reported in the United Kingdom in 1996 in association with an epidemic of BSE, which historically significantly contributed to an increasing interest in prion research (Will *et al* 1996). Some rare iatrogenic CJD cases have been reported after corneal transplants from unknown infected individuals or from the use of non-suitable sterilized surgical instruments. Kuru is a particular infectious form of CJD restricted to New Guinea tribes, transmitted during endocannibalistic feasts (Mead *et al* 2003).

The diagnosis of CJD is suspected clinically, electroencephalogram (EEG) and by magnetic resonance imaging (MR imaging), additionally, the increase of protein 14-3-3 in the cerebrospinal fluid (CSF) provides good diagnostic tool (with a good predictive values for sCJD). Nevertheless, up to now diagnosis can only be confirmed by histopathological changes of brain (Green 2002; Demaerel *et al* 2003).

The electrophoretic profile after treatment with proteinase K (PK) of PrP^{Sc} appears to be characteristic for various clinicopathological CJD subtypes (Parchi *et al* 1999, 2000). The concept of prion strains evolved from the findings of distinct versions of prion diseases which differ at the symptomatic and biochemical level, even if the PrP gene is identical (Aguzzi 2004). The combination of the codon 129 genotype (M/V) with the type of PrP^{Sc} correlates fairly well with six distinct phenotypes in sCJD (Parchi *et al* 1999; Pan *et al* 2001).

2. PRION PROTEIN

2.1 Physiological Roles of Prion Protein

Defining the physiological role of PrP^C is relevant for understanding the prion disease, since the abnormal the protein may fail to perform the physiological function when is converted to PrP^{Sc} isoform (Harris 1999). Several physiological roles have been attributed to PrP^C, including protection against oxidative stress by its superoxide dismutase (SOD) activity, participation in copper ion uptake, cell adhesion and differentiation, membrane excitability, synaptic transmission, signalling, cell survival by preventing apoptotic neuronal cell death and mitochondrial dysfunction (Ellis *et al* 2002; Lasmézas 2003; Prado *et al* 2004).

PrP^C is associated with the plasma membrane of synapses and also with synaptic vesicles (Lasmézas 2003), where the signalling function can be mediated through its interaction with protein growth factor receptor-bound protein 2 (Grb2) which is known to link signals coming from extracellular and/or transmembrane receptors to intracellular signalling molecules (Prado *et al* 2004).

An important function of PrP^C is its involvement in sequestering copper ions in a redox-inactive state (Cui *et al* 2003; Lasmézas, 2003; Millhauser, 2004). PrP^C modulates copper content at the cellular level, and therefore modification of synaptic copper homeostasis could play a determinant role in the neuropathology of prion diseases (Rachidi *et al* 2003; Cui *et al* 2003; Brown 2003; Rossi *et al* 2004). Copper is a redox transition element, that under conditions of oxidative stress mediates damage of proteins through ROS, causing direct damage of nucleic acids, proteins, lipids, as well as apoptosis (Turnbull *et al* 2003). PrP^C has a Cu^{2+}-dependent SOD activity, which is significantly reduced in CJD brain purified prion protein (Brown *et al* 2001; Brown 2002). Analysis of purified prion protein from brains of scrapie-infected mice showed a reduction in copper binding to the protein and a proportional decrease in antioxidant activity, occurring early in the course of the disease (Thackray *et al* 2002).

2.2 Characteristics of Prion Proteins: Normal and Pathologic

PrP^C, normal prion protein, encoded by a gene located on chromosome 20 in humans, is a 33-35 kDa glycosyl phosphatidylinositol (GPI)-anchored cell surface glycoprotein that is strongly expressed in the central nervous system (CNS) (neurons and glia of the brain and spinal cord) and also in several

other cell types (Prusiner 1998; Harris 1999). Cleavage of the N-terminal signal peptide and processing in the C-terminal due to the GPI-group renders a mature product of 208 amino acids (Figure 1). This structure includes a N-terminal domain with a set of octapeptide repeats related to copper ion-binding, a central hydrophobic domain, a C-terminal containing the two glycosylation sites, and an internal disulphide bond. The glycosylation sites include the presence of high mannose type N-glycans (Ermonval *et al* 2003).

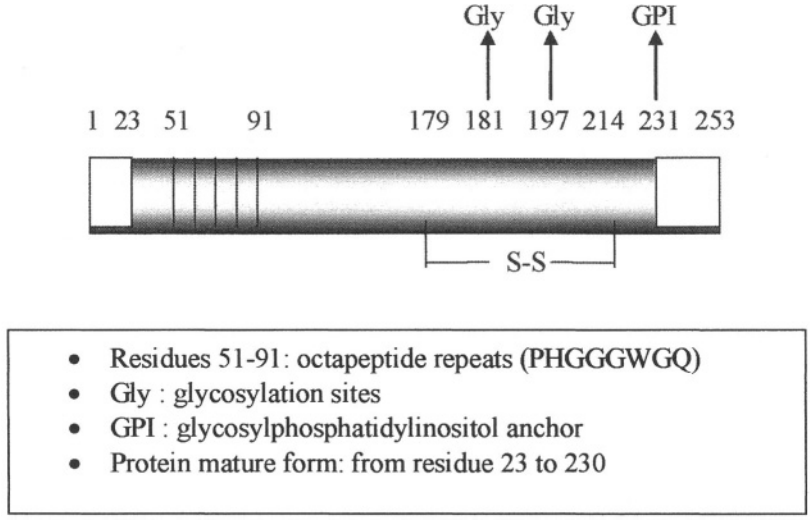

Figure 1: Schematic depiction of the primary structure and structural features of human prion protein (NCBI AAS80162, prion protein *Homo sapiens*).

Electrophoretic analysis in sodium dodecyl sulphate (SDS/PAGE) reveals a heterogeneous population of PrP^{C}, with a minor band at 25 kDa representing the non-glycosylated form and two other bands between 29-30 and 33-35 kDa corresponding to mono-, and diglycosylated forms, respectively (Ermonval *et al* 2003). PrP^{C} binds up to five copper atoms with a remarkable degree of selectivity, via a glycine-proline rich octapeptide repeat region (PHGGGWGQ), at sites upstream this region, and in the C-terminal region (Garnett and Viles, 2003; Millhauser, 2004). Copper ions are coordinated by imidazole ring of histidine, and deprotonated amides from glycine with similar affinity to the brain milieu. This copper ion binding site shares a common domain with the SOD activity (Cui *et al* 2003; Millhauser 2004).

Prion diseases are associated with a pathogenic conformational transition of the PrP^{C} cellular protein to the infectivity-form PrP^{Sc}, being the main

transition from an α-helix to a β-sheet structure. Normal PrP contains 42% of α-helix and only 3% of β-sheet, while PrP^{Sc} is composed of 30% α-helix and 43% β-sheet, as deduced from circular dichroism measurements (Hetz and Soto, 2003; Nishina *et al* 2004). In contrast with PrP^{C}, PrP^{Sc} is an insoluble, proteinase K-resistant oligomer of PrP that accumulates in the brain during prion infection (Prusiner 1998). Conversion of PrP^{C} to the proteinase K-resistant form PrP^{Sc} is produced as a transition within the central hydrophobic and C-terminal domains between residues 90 and 125 (Jiménez-Huete *et al* 1998).

PrP^{Sc} formation seems to be a multistep process which might be influenced by several factors (Caughey 2003; Ermonval *et al* 2003): (A) the initial PrP^{C} conformational state, where post-translational modifications (or lack of them) could play an important role in its transformation to PrP^{Sc}, (B) the membrane environment, suggested from evidences that PrP^{Sc} generation occurs at the cell surface or in endosomal compartments, and (C) specific interactions with proteins and metal ions, such as the binding of copper ions to PrP^{C} that results in conformational changes in the unstructured N-terminal region, promoting the formation of β-sheet structure (Wong *et al* 2004; Rossi *et al* 2004).

The possible relation between copper ion binding and prion disease has been studied using recombinant PrP variants with some of the classical point mutations reported in familial CJD (E200K), GSS (F198S), and FFI (D178N). Although these variants partially showed abnormalism in the copper ion binding, neither destabilization of PrP^{C} nor higher stability of PrP^{Sc} seems to be general mechanisms underlying spontaneous generation of this abnormal protein in patients with inherited TSE (Cereghetti *et al* 2003).

The conversion process leading to PrP^{Sc} has not been completely elucidated, but it appears to occur in membrane microdomains enriched in sphingolipids and cholesterol, known as lipid rafts (Martins *et al* 2002; Caughey 2003). A direct contact between uninfected cells and prion-infected surfaces seems to be required for prion propagation. However, the *in vitro* conversion of PrP^{C} to the abnormal protein using a detergent-solubilised preparation, suggests that the membrane attachment is not required for the conversion of protease-sensitive to the protease-resistant form (Nishina *et al* 2004). Conversion between purified PrP isoforms can be stimulated by heparansulphate proteoglycan (HSPG) through binding via the N-terminus in a Cu^{2+}-dependent way (Pan *et al* 2002).

A recent study of our group in CJD patients showed a large increase of HSPG in CSF (Cartier *et al* 2004). HSPG has been described tightly bound to synaptic basal lamina, therefore the increased level could be probably related to synaptic diminution associated with grey matter spongiosis and neuronal loss (VanSaun and Werle, 2000; Brown 2001; Cartier *et al* 2004). Binding of PrP^{C} to HSPG in addition of stimulating PrP^{Sc} conversion, mediates its internalisation or endocytosis (Pan *et al* 2002; Hetz and Soto, 2003). Hence

the higher HSPG levels in the CSF could reflect higher levels in the brain extracellular matrix thus facilitating the endocytosis of prion protein.

A modification occurring only in PrP^{Sc} but not in PrP^{C}, from animal and human tissues, is the formation of advanced glycosylation end products (AGEs), through modification of some lysines (residues 23, 24 and 27 in human) and arginine (37 in human) (Choi *et al* 2004). Two possible roles of AGE in the formation of PrP^{Sc} are proposed. One role could be the facilitation of the conversion, and a second one could be providing protection to PrP^{Sc} from cellular degradation.

2.3 Trafficking and Postranslational Processing

Plasma membrane PrP^{C} can be either endocytosed, recycled, and degraded or can bind PK resistant PrP and thus be converted into PrP^{Sc} forming aggregates (Marella *et al* 2002). Like other membrane proteins, PrP is synthetised in the rough endoplasmic reticulum (ER) and transits the Golgi on its way to the cell surface (Harris 2003). During its biosynthesis in the ER, PrP^{C} is subjected to several post-translational modifications (see Figure 1), including cleavage of the N-terminal signal peptide, addition of N-linked oligosaccharide chains (and syalic acid in humans and hamsters), formation of a single disulphide bond, and the attachment of the GPI anchor following the cleavage of the C-terminal hydrophobic peptide (Harris 2003; Ermonval *et al* 2003; Li *et al* 2003).

Microtubule-associated trafficking of fluorescent PrP^{C} in neuronal cells shows the participation of different N-terminal domains for the anterograde transport towards the plasma membrane, and for the retrograde trafficking or endocytosis (Hachiya *et al* 2004). Both PrP^{C} and PrP^{Sc} are recognized by the ER quality control machinery; and as a result misfolded protein in the ER are transported backwards to the cytoplasm, where they are degraded by the proteasome (Ma and Lindquist, 2002).

PrP^{C} has the potential of adopting three distinct topological forms during import into the ER (Heske *et al* 2004). The GPI-anchored version that predominates under physiological conditions (as a 33-35 kDa protein), and two pathogenic forms with an unusual transmembrane topology e.g., either the C-terminus or the N-terminus facing the ER lumen. Studies on neuronal cells showed impairment of the ER import of two pathogenic PrP mutants linked to inherited prion diseases in human, such as W145Stop and Q160Stop, while mutations in the putative transmembrane domain, like A117V, increase the formation of the pathogenic transmembrane topology as reported in scrapie-infected mice (Heske *et al* 2004). Other studies with recombinant prion protein with codon E200K mutation results in various changes on the metabolism of human neuroblastoma such as the assembly of

abnormal glycans in the site flanking the mutation, increased formation of truncated fragments, a partial insolubility and elevated resistance to digestion with PK (Capellari *et al* 2000).

PrP^{C} internalisation or endocytosis from the plasma membrane occurs via clathrin-coated pits in a copper ion-stimulated process (Harris 2003; Prado *et al* 2004). It has been proposed that PrP^{C} internalisation may hinder PrP^{Sc} interaction with this molecule, thereby decreasing the rate of prion disease propagation (Hijazi *et al* 2003; Prado *et al* 2004). Deletions or additions of octapeptide repeats, and some punctual mutations in PrP associated with familial prion diseases prevent this copper-mediated endocytosis (Prado *et al* 2004).

Experimental evidences suggest that the retrograde transport toward the ER of the prion protein plays a significant role in PrP^{Sc} conversion (Béranger *et al* 2002). ER retention seems to be a common feature in familial prion diseases (human disease-associated mutations: P102L, D178N/M129 and F198S/M129V) (Kovács *et al* 2002; Harris 2003). Retention of abnormal proteins in the ER triggers stress response that could explain the pathogenic effects of the mutant PrP^{Sc} (Ivanova *et al* 2001; Ma and Lindquist, 2002; Drisaldi *et al* 2003).

PrP^{C} can move from one cell to another in a regulated process protein kinase C-dependent, that may have implications for the pathogenesis of the prion disease, because PrP^{Sc} acquired by ingestion infection has to reach the CNS(Liu *et al* 2002).

3. METABOLISM OF PRION PROTEIN

PrP^{C} may be present as distinct isoforms depending not only on its topology (GPI-anchored, transmembrane or soluble), and degree of glycosylation (non-, mono-, and diglycosylated), but also due to proteolytic processing (full length and truncated) (Ermonval *et al* 2003). Final degradation of PrP^{C} after endocytosis and removal of the misfolded PrP^{Sc} occurs through the ubiquitin-proteasome system (Yedidia *et al* 2001; Kang *et al* 2004). In the metabolism of a membrane-bound protein, cleavage from the anchored membrane allows its secretion, and appears to be an important regulatory mechanisms (Kang *et al* 2004). If at the plasma membrane level, PrP^{C} interaction with PrP^{Sc} results in *de novo* formation of PrP^{Sc} aggregates, a reduction in the level of the cell surface PrP^{C}, through enhanced endocytosis or release of the protein from the membrane, would reduce the production of the PrP^{Sc} form.

As part of its metabolism, PrP^{C} undergoes two types of cleavages resulting in the so-called "shedding" (Figure 2), one with the participation

of phospholipases and the second one by different proteases (Parkin *et al* 2004; Yadavalli *et al* 2004).

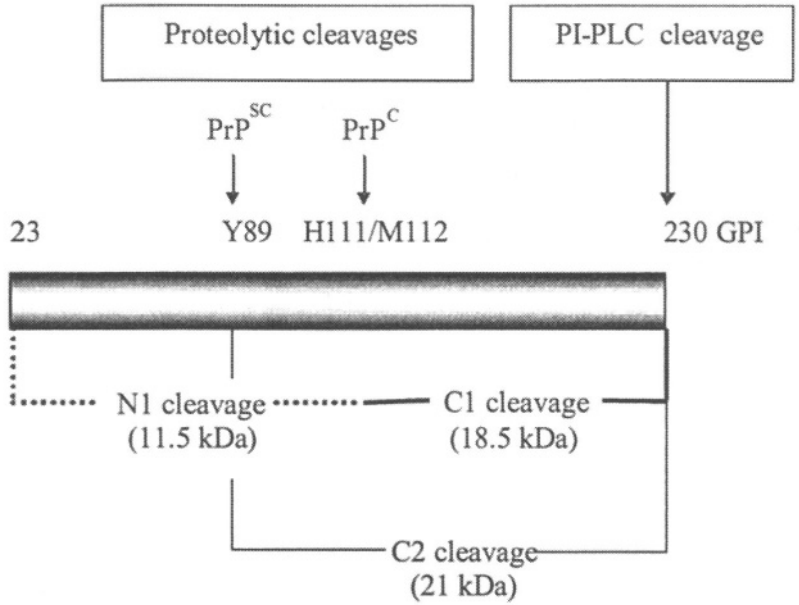

Figure 2: Schematic representation of human PrP^{C} and PrP^{Sc} hydrolytic processing. Endoproteolytic processing by calpain or ADAMs (sheddases or secretases); phospholipase processing by PI-PLC (phosphatidylinositol-specific phospholipase C).

3.1 Trafficking and Postranslational Processing

Neurodegenerative diseases, associated with protein misfolding events and accumulation of these protein aggregates, can overwhelm the required ubiquitin-proteasome system for the removal of the damaged proteins (Kang *et al* 2004). A marked increase of ubiquitin CSF levels in CJD (Manaka *et al* 1992) and an up-regulation of the two major lysosomal cysteine proteases, cathepsin B and L, in scrapie-infected neuronal cells has been reported (Zhang *et al* 2003). This group also observed that PrP^{Sc} ubiquitination is a late event phenomenon occurring after the formation of protease-resistant PrP^{Sc}. Therefore, cytosolic accumulation of misfolded aggregated prion protein could produce neurotoxicity (Hooper 2003).

3.2 Phospholipase-Mediated Shedding

Cleavage of the GPI anchor by PI-PLC releases the resultant polypeptide chain into the extracellular medium as a soluble full length protein (see Figure 2). Albeit in small portion, this form has been detected in human CSF and blood (Parizek *et al* 2001). This phospholipase-mediated shedding is induced by treatment with lipid-raft-disrupting agents such as the antibiotic filipin which both limits endocytosis and reduces the amount of membrane-bound prion protein (Marella *et al* 2002; Parkin *et al* 2004). Studies on PrP^{Sc} isolated from infected brain and recombinant mutated forms (glycosylated and expressed on the cell surface) show to be resistant to PI-PLC release from the membrane, probably due to physical inaccessibility to the phospholipase (Li *et al* 2003).

3.3 Proteolytic Processing

Presence of truncated forms of PrP^{C} under normal conditions, in addition to the full-length protein, indicates a proteolytic cleavage (Chen *et al* 1995). An additional N-terminal truncated fragment detergent-insoluble and resistant to proteases has been found in the brain of subjects with prion diseases (Chen *et al* 1995). The production of a 18.5 kDa C-terminal fragment (referred as C1) from PrP^{C} is due to proteolytic cleavage at amino acids 111/112 (see Figure 2) (Parkin *et al* 2004).

Metalloproteases known as ADAMs (a desintegrin and metalloproteinase domain) have been implicated in the normal metabolic proteolysis of prion protein (Parkin *et al* 2004). This cleavage breaks the potentially pathogenic PrP region, and may be a crucial step in its metabolism precluding the accumulation of pathogenic fragments (Yadavalli *et al* 2004). On the other hand, the calpain family of proteolytic enzymes seems to be involved in the formation of a PK-resistant PrP fragment, produced in infected brain apparently as a result of cleavage at the same location that PK cleaves PrP^{Sc} *in vitro* (following amino acid residue 89) (Yadavalli *et al* 2004). This latter cleavage produces a larger C-terminal fragment, referred as C2, with the same apparent molecular mass as nonglycosylated PrP27-30.

PrP^{C} N-terminal fragment functions as a putative targeting element and, as mentioned before, is essential for both PrP^{C} transport to plasma membrane and modulation of endocytosis (Hachiya *et al* 2004). Cleavage of this N-terminal portion occurs within an endocytic compartment of the cell, although cholesterol-rich domains of the plasma membrane are also proposed to be involved. Another type of PrP^{C} cleavage observed occurs at a site-specific cleavage of the octapeptide repeat region of the N-terminal portion on exposure to ROS (Wong *et al* 2000; McMahon *et al* 2001). This cleavage,

yielding a 28.5 kDa protein, is both copper- and pH-dependent and is inhibited by the presence of other divalent metal ions. These proteolytic cleavages could initiate or facilitate further proteolysis of the molecule within the cell or on the cell surface (McMahon *et al* 2001).

3.3.1 Secretases or Sheddases in the CNS

Recently discovered ADAMs and ADAMTs (a desintegrin and metalloproteinase trombospondin domain) enzymes generally called adamalysins, belong to the group of zinc metalloproteinases known as secretases or shedasses (Primakoff and Myles, 2000; Blobel 2000; Rosenberg 2002). Encompassing at least 30 members, they play important roles on the membrane cell surface acting as sheddases releasing growth factors, death receptors, and death-inducing ligands. They are regulated by four endogenous metalloproteinase inhibitors (tissue inhibitors of metalloproteinases, TIMPs) with inhibitory capacity on both ADAMs and the family of matrix metalloproteinases termed matrixins (Rosenberg 2002).

ADAMs contain a cytoplasmic tail, a signal peptide, a prodomain, and several other domains such as cysteine-rich, metalloproteinase, desintegrin, and epidermal growth factor (Primakoff and Myles, 2000). At least 17 ADAMs family members are widely expressed in the adult CNS, with some differential localization in astroglia, endothelial cells, oligodendrocytes and cortical neurons (Kärkkäinen *et al* 2000; Bernstein *et al* 2004). These proteases have, in addition of the proteolytic activity, adhesive functions and can be involved in cell fusion events (Primakoff and Myles, 2000; Novak 2004). ADAM 10 and 17 hydrolyse PrP^C, and they are probably responsible for the *in vivo* cleavage between residues 111 and 112 on human prion protein (Vincent *et al* 2001; Plamont *et al* 2003).

Studies on neuroblastoma cells have shown that the shedding of the prion protein may proceed by at least two distinct enzymatic mechanisms e.g., action of phospholipases C and/or D, and through a phorbol ester-stimulated process involving the action of zinc metalloproteinases (Parkin *et al* 2004). Lower copper ion concentrations, compared with levels known to stimulate PrP^C endocytosis, also promote protein shedding, which in turn is inhibited by hydroxamate inhibitors of metalloproteinases, suggesting the involvement of these proteases in this process. It has been proposed that the decrease in PrP shedding at higher copper ion concentration may be due to a competition between shedding and endocytosis processes of the protein (Parkin *et al* 2004). Conversely, enhanced shedding in the presence of copper ions may be attributed to the induction of conformational changes that facilitates proteolytic cleavage by the zinc metalloproteinase.

3.3.2 Calpain-Dependent Endoproteolytic Cleavage of Prion Protein

The calpain family, at least 12 mammalian enzymes, includes ubiquitous isoforms of Ca^{2+}-activated cysteine proteases known to modify the properties of substrate proteins by cleavage at a limited number of specific sites (Huang and Wang, 2001). A regulatory function has been proposed for this specific processing of some proteins, which is different to the ultimate digestion by the lysosomal proteases or the proteasome. Calpain proteolysis is involved in several physiological cellular functions as well as in a number of neurodegenerative diseases (Yadavalli *et al* 2004). Calpain activity is tightly regulated *in vivo* by Ca^{2+} and by the specific intracellular protein inhibitor calpastatin. Two major calpain ubiquous isoforms are known: calpain I (μ-calpain) that requires micromolar levels of Ca^{2+}, and calpain II (m-calpain) with a millimolar Ca^{2+}-requirement (Morford *et al* 2002). Calpain is largely, but not exclusively, located in the cytosol and the presence of calpain II has been found in T-cell lipid rafts (Morford *et al* 2002).

Demonstration of a role for calpain II in PrP proteolysis was obtained using pharmacological and genetical approaches in a model of scrapie-infected cells. Inhibitors of lysosomal proteases, caspases and proteasome did not affect C2 production. Cleavage of PrP^{Sc} to produce C2 occurs immediately distal to the five octapeptide repeats, frequently expanded in familial cases of CJD. Since Ca^{2+} modulates calpain activity, the observation that treatment of neuroblastoma cells with the neurotoxic PrP106-126 peptide lead to a rapid rise in intracellular Ca^{2+} with a concomitant elevation in calpain activity, suggest that scrapie infection could induce abnormalities in Ca^{2+} homeostasis (O'Donovan *et al* 2001; Yadavalli *et al* 2004).

4. MATRIX METALLOPROTEINASES AND TIMPS IN CSF

The differential diagnosis of dementia can be difficult in the early stages of the disease. Since the 1960s, when the concept of brain-specific proteins was proposed, great methodological advances in CSF protein determination for their potential usefulness as diagnostic tool for CNS disorders were achieved (Green 2002). This approach is based on the fact that approximately 20% of the CSF proteins are produced within the CNS and may be considered brain-derived. The emergence of vCJD in the 1990s has greatly increased the interest in the clinical diagnosis of CJD through specific molecular markers, and the use of a brain-specific protein, protein 14-3-3, is widely considered a valuable diagnostic test.

Molecular changes in the extracellular environment resulting from CNS lesions are accompanied by rapid activation of neuroglial and non-neural cells secreting matrix metalloproteinases (MMPs) as well as several components of the extracellular matrix (ECM) (Nagase and Woessner, 1999; Crocker *et al* 2004). Overproduction of MMPs could produce abnormal ECM proteolysis resulting in progressive and persistent tissue damage, a process observed in several neurological diseases (Yong *et al* 2001). A preliminary study of various patient populations revealed the presence of an additional proteolytic MMP (MMP-9) in two CJD patients (Valenzuela *et al* 1999). This finding prompted us to investigate the MMPs and TIMPs profiles in a larger number of patients.

4.1 Characteristics of MMPs

MMPs constitute a 28 plus-member family of Ca^{2+}-activated, proteolytic (zinc endopeptidases) enzymes involved in ECM remodelling; they are capable of degrading *in vitro* and *in vivo* a variety of ECM protein components e.g., interstitial and basement membrane collagens, proteoglycans, fibronectin and laminin (Sternlicht and Werb, 2001; Vise and Nagasse, 2003). Collectively called matrixins, MMPs belong to a larger subgroup of cell membrane-bound and secretable proteinases including collagenases, gelatinases and stromelysins. MMPs show some substrate specificity, and in general they are expected to cleave peptide bonds placed before a residue with a hydrophobic side chain e.g., Leu, Ile, Met, Phe, or Tyr (Visse and Nagase, 2003).

Under physiological conditions MMPs activity is finely regulated at different levels: (A) transcriptionally by growth factors, cytokines and hormones, (B) by proteolytic cleavage of the inactive proenzyme or zymogen precursor, (C) by interaction with TIMPs which inhibit MMPs and ADAMs through noncovalent associations in a 1:1 stoichiometry reaction, and (D) by interaction with specific ECM components (Gardner and Ghorpade, 2003; Visse and Nagase, 2003).

Recent evidence suggests an association between MMPs over-production (mainly MMP-9) and some neurodegenerative (amyotrophic lateral sclerosis), and inflammatory diseases (meningitis, multiple sclerosis) (Yong *et al* 2001; Van den Steen *et al* 2002). The adequate balance between MMP activity and TIMP levels, a family of at least four secreted proteins with different degree of glycosylation, appears to be essential for preventing above conditions (Sternlicht and Werb, 2001; Gardner and Ghorpade, 2003; Crocker *et al* 2004).

4.2 Studies of MMPs and TIMPs in CSF Samples

CSF MMPs (MMP-2, MMP-3 and MMP-9) were determined in 16 subjects with clinical CJD diagnosis using zymographic analysis (Kettlun *et al* 2003a). The zymographic analysis consisted in SDS/PAGE copolymerised with gelatin as substrate, that allows the detection of MMP-9 and MMP-2 in their proenzyme and active forms (Kettlun *et al* 2003a). MMP-9, in the proenzyme or latent form, was detected in samples from 12 out of the 16 patients studied but absent in the control group. Instead, proMMP-2 (72 kDa) was observed in all samples both from controls and CJD subjects, however MMP-2 active form (68 kDa) was detected in only 12 of 16 CJD patients and in 2 control samples (Figure 3). Higher frequencies of proMMP-9 and active MMP-2 were statistically significant compared with the control group (chi square test for all cases $p < 0.001$).

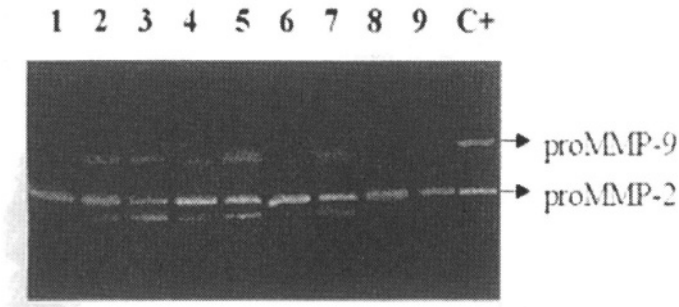

Figure 3: Zymography in SDS/PAGE under non-reducing conditions of CSF samples of control, and various CJD patients, using gelatin as substrate. Lanes 1-7 CJD patients, lanes 8-9 normal subjects, lane C+: a positive control of proMMP-2 and -9.

Presence of proMMP-9 in the CSF is generally considered a non-specific marker for various CNS diseases (Yong *et al* 2001; Kettlun *et al* 2003a,b). However, a recent publication indicates no elevation in MMP-9 in Alzheimer' disease (Adair *et al* 2004). Therefore, the zymography could be an important analysis for the differentiation of CJD from Alzheimer's disease patients, the most common condition mimicking CJD, sharing some clinical and neuropathological features (Kapaki *et al* 2001).

MMP-3 levels determined by immunological methods in CSF of CJD patients did not show any statistical difference with the control group. These results differ from our findings in other neurological pathologies produced by different types of infectious diseases (HIV, tropical spastic paraparesis, neurocisticercosis, viral meningitis), where we observed a large over-expression of MMP-3 (Kettlun *et al* 2003a,b).

Parallel to MMP determinations, we measured levels of the four TIMPs in CSF of CJD patients and compared with them control subjects. These determinations, also done by immunological methods, showed an over-expression of TIMP-1 and TIMP-2, which is different from our results on various infection pathologies, where also TIMP-3 and -4 showed a statistically significant increase (Kettlun *et al* 2003a,b).

Astrocytes are intimately involved in the mechanisms of neural injury and CNS repair, therefore a central role for astrocytic TIMP-1 expression has been proposed to counterbalance the observed MMP-9 upregulation in these conditions (Gardner and Ghorpade, 2003). CJD associated astrogliosis could account for the secretion of both MMPs and TIMPs, suggesting that both the overexpression pattern of TIMPs and the lack of increase of MMP-3 mark differences between CJD and various infectious and classic inflammatory diseases. Although prion diseases are no inflammatory disorders, growing evidences show induced microglia recruitment at sites of amyloid prion deposit, that could also be involved in the disease dissemination (Marella and Chabry, 2004). *In vivo* accumulation of activated microglial cells is concomitant with the PrP^{Sc} deposition and precedes neuronal death. The presence of activated microglia is required for neurotoxicity.

5. ACTIVATION OF CASPASES BY PrP^{Sc}

Whereas accumulated abnormal prion protein in the cytoplasm is highly cytotoxic and could produce neuronal death by apoptosis, cytosolic PrP^{C} protects human primary neurons against Bax-mediated cell death (Drisaldi *et al* 2003; Hetz *et al* 2003; Roucou *et al* 2003)). Nanomolar concentrations of purified mouse scrapie PrP^{Sc} induce apoptosis in neuronal cell in a process mediated via caspase-12 activation, a member of a large family of cysteine proteases known as the central executioner molecules of apoptosis (Hetz *et al* 2003). It has been proposed that an initial PrP^{Sc} interaction with some unknown cell surface receptor(s) triggers a signal to the ER resulting in the release of intra-ER Ca^{2+}; this ER-stress also could trigger the up-regulation of certain chaperone proteins with neuroprotective function. Other reports have suggested an association between prion peptide PrP106-126 induction of caspase-3, -6 and –8 and a rise in intracellular calcium through release of mitochondrial calcium stores (O'Donovan *et al* 2001; Sisó *et al* 2002). This perturbation in Ca^{2+} homeostasis could produce a concomitant activation of calpain. The neuronal cell death induced by this peptide is mediated by p38 MAP kinase (Corsaro *et al* 2003).

In mitochondria from scrapie-infected brains a significant decrease in Mn-superoxide dismutase and cytochrome *c* oxidase, along with an elevation of

several oxidative stress markers has been observed (Kim *et al* 2004). These results suggest that mitochondrial damage and increased oxidative stress may be involved in the pathogenesis of prion diseases (O' Donovan *et al* 2001). Studies on a neuronal cell line suggest that PrP^{C} may play a central role as an anti-apoptotic protein through caspase—dependent pathways in mitochondria, therefore the alteration of PrP^{C} and consequent reduction of its anti-apoptotic capacity may be one of the pathogenic mechanism of prion diseases.

6. MOLECULAR CLASSIFICATION OF PRION DISEASES

As previously noted, the concept of prion strains evolved from the observation of distinct versions of prion diseases which present biochemical differences in spite of an identical PrP gene. (Kovács *et al* 2000; Aguzzi 2004). Particular domains appear to be relevant to the conformational change that distinguishes PrP^{Sc} from PrP^{C}, with the production of distinct proteolytic fragments with different susceptibility to conversion (Jiménez-Huete *et al* 1998). The main knowledge of the proteolytic processing of the abnormal prion protein is limited to the non-physiological proteolysis by PK (Notari *et al* 2004). However, this approach allowed the differentiation of prion diseases based on the molecular mass and degree of glycosylation of the PK-resistant PrP cores (Figure 4) (Parchi *et al* 2000).

In human prion diseases, two major types of PrP^{Sc}, type 1 and 2, can be distinguished based on the difference in electrophoretic migration of the PK-resistant core fragment (Parchi *et al* 2000). The various prion strains differ in the relative ratio of the glycoforms with none, one or two N-linked glycans (Pan *et al* 2001; Ermonval *et al* 2003). Also, alterations of PrP glycosylation have been observed in prion diseases. (Ermonval *et al* 2003). In the point mutational case of E200K aberrant N-glycans at Asn^{197} are linked, and an increased production of truncated fragments with impaired transport of non-glycosylated isoforms was detected. Other classical mutation in the familial diseases, D178N, reduced the level of N-glycosylation affecting PrP^{C} stability either directly or trough trafficking alteration.

After treatment with peptide N-glycosidase PrP^{Sc} type 1 samples migrate in SDS/PAGE at 21 kDa, and PrP^{Sc} type 2 at 19 kDa (Parchi *et al* 2000). Sequencing studies demonstrated that these differences in size result from the cleavage at different sites which likely reflects the distinct conformation or different ligand interaction of the two types. Heterogeneities observed from the sequencing studies are explained by the existence, in addition of the two primary cleavage sites (at residue 82 and 97 for type 1 and type 2 PrP^{Sc}, respectively, see Figure 4) numerous secondary cleavages distributed along

the region spanning residues 74-102. Secondary cleavages are related to the PrP^{Sc} type and the genotype at codon 129. Codon 129 polymorphism (M or V residues) seems to regulate the size of the variable region and probably the extent of the β-sheet transformation in PrP^{Sc} (Parchi *et al* 2000). These data point out the involvement of the prion protein conformation in the effect of the PrP genotype on the phenotypic expression of the disease.

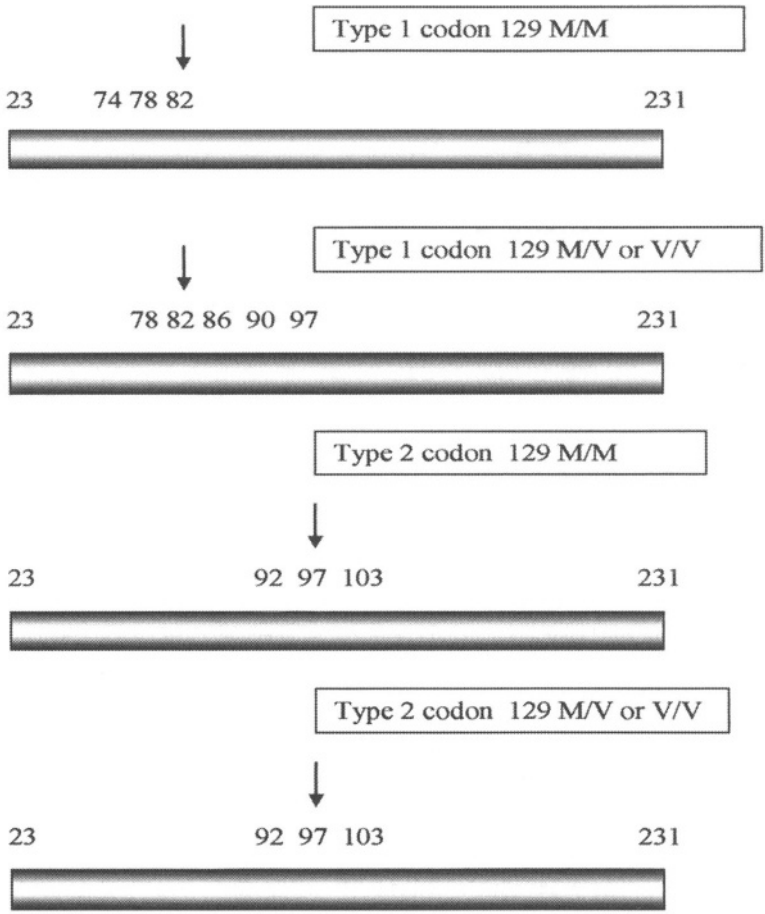

Figure 4: Schematic representation of the different types of human PrP^{Sc} related to the PK cleavage patterns.

A study with a large number of patients (300 subjects) related the biochemical classification based on PK-peptide profile with the neurological characteristics of the patients (Parchi *et al* 1999). The 70% of the cases clustered in classic CJD phenotype type 1 with at least one methionine allele at codon 129, show among the neuropathological features prominent involvement of occipital cortex and "synaptic type" PrP staining, and among the clinical features rapidly dementia, early and prominent myoclonus and typical electroencephalogram (EEG). The 25% of the cases were associated with type 2 with valine homozygosity or heterozygosity at codon 129, and the neuropathological features comprised a prominent subcortical involvement, including brain stem nuclei in neocortex, spongiosis is limited to deep layers and PrP staining showed kuru-plaque like, and clinically they showed ataxia at onset, late dementia and no typical EEG in most of the cases. From the remaining patients, the 9% include a group of type 2, with similar features as the last mentioned but with the presence of amyloid-kuru plaques in the cerebellum, more consistent plaque-like and focal PrP deposits, and among the clinical features include ataxia. The rest of the cases include variants of type 2 and a single case a variant of type 1.

An additional phenotype comprising methionine homozygous and type 2, was revealed by two-dimensional gel electrophoresis (Pan *et al* 2001). This last electrophoretic PrP^{Sc} pattern is characterized by the presence of three rather than one set of glycans linked to the full-length prion protein, and a relatively larger amount of the full-length protein.

In some studies, PrP^{Sc} type 1 from codon 129 MM subjects was further distinguished into two subtypes, that show indistinguishable fragment sizes only when PK digestion is performed in the presence of EDTA, therefore representing two distinct conformations acquired by the protein in the presence of metal ions such as copper and zinc (Notari *et al* 2004). Recently two novel PK-resistant, but much smaller C-terminal fragments of PrP in brain of subjects with sCJD have been identified (Zou *et al* 2003). These fragments have the N-terminal starting at residues 162/167 and 157/156, respectively, they derived from both glycosylated and nonglycosylated forms.

It is known that the hydrophobic domain spanning amino acids 112-136 form a critical site in the prion protein at which the protein increase the β-sheet conformation (Cui *et al* 2003).This region also spans the site of normal metabolic cleavage of PrP^{c}, and its deletion inhibits conversion of the protein to PrP^{Sc} in infected cells. Conversion of this site to one that forms β-sheet facilitates the aggregation of the protein prevent cleavage of the protein together with the abolishment of the SOD activity. In addition, the interaction between this site on PrP^{C} and PrP^{Sc} or peptides such us PrP106-126 also inhibits the antioxidant activity of PrP^{C}, or causes conformational change of the protein (Cui *et al* 2003).

The partial resistance of PrP^{Sc} to PK-digestion is probably a result of the greater aggregation and a higher content of β-sheet structure compared with PrP^{C}. Studies of truncation of the N-terminal flexible region showed the importance of several residues for the efficient formation of the protease-resistant forms (Lawson *et al* 2004). The kinetic analysis of proteasomal digestion of two different alleles of prion protein shows that even small changes in the protein sequence can affect the overall efficiency of proteasomal processing and could be related with the allelic variations and the pathogenicity of prion proteins (Tenzer *et al* 2004).

7. CONCLUSIONS

PrP^{C} appears to play a significant role in diverse physiological functions such as copper ion uptake, cellular oxidative processes and regulation of cell death or survival. Conversion to an inactive form, PrP^{Sc} accounts for at least some of the pathophysiological manifestations of prion disease. Turnover of this GPI-anchored protein involves classical exocytic and endocytic trafficcking, resulting in accumulation of PrP^{Sc} in the cytoplasm. Although abnormal prion protein can be degraded by the lysosomal/proteasome system, elevated levels can overwhelm the ubiquitin conjugating system required for the initiation of its final removal.

Prion protein undergoes extensive metabolic processing including glycosylation and hydrolytic cleavage of both lipolytic and proteolytic types. Proteolytic split by ADAMs and calpains accounts for the occurrence of different peptide fragments isolated from brain-tissue of controls and CJD patients. *PRNP* mutations identified in inherited prion diseases explain some of the differences observed. In the case of sCJD codon 129 polymorphism of *PRNP* plays a role in the different isoforms of the prion protein detected, showing a tight relation with the clinicopathological characteristics. The apoptotic effect of PrP^{Sc} is associated with an increased level of caspases, and a reduction of anti-apoptotic PrP^{C} *PRNP*.

Relative prion protein sensitivity towards proteinase K-treatment has been an important issue when describing the various sCJD subtypes. Analysis of CSF proteins shows an upregulation of MMP-9 and two of its inhibitors TIMP-1 and -2, which could represent a characteristic pattern of this disease. Progress in the characterization of prion disease subtypes bears important implications in terms of molecular medicine, but furthermore, it may have important implications in terms of clinical neurology, epidemiology and public health.

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Chapter 9

Neuropeptide Processing

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1. INTRODUCTION

The past decades have seen an increasing number of identified neuroactive peptides reported in the scientific literature. These compounds constitute a large family of biologically active molecules that participate in the transmission or modulation of signals in the central nervous system (CNS) (Kastin *et al* 1996). They are shown to be involved in a broad range of neurological functions including those related to memory, mood, pain, stress, reward, intake of food and neuroendocrinological as well as neuro-immunological regulation. The neuropeptides are released from nerve terminals of peptidergic neurons and the mechanisms of their action includes their binding to and activation of pre- or postsynaptically located receptors imbedded in the nerve cell membrane. A general pathway for the neuropeptide biosynthesis starts at the ribosomes in the nerve cell bodies, where the messenger RNA is translated into an amino acid sequence. Neuropeptides are mostly derived from large, biologically inert protein precursors (prepropeptides). The neuropeptide precursors are subsequently cleaved in a series of proteolytic steps in a sequence-specific and tissue-specific fashion to deliver smaller bioactive peptides. Frequently, the conversion of pre-propeptides to active peptides is associated with additional modifications such as amidation and truncation of the released sequence may also occur. Following secretion and interaction with its receptor the active peptide is inactivated by enzymatic degradation. In some cases the released active peptide is converted to fragments with retained biological activity and in other cases the new fragments can exhibit a very different biological profile from

that of the parent peptide. The present knowledge about the functional relevance of most neuropeptides is still not fully clarified and there is a real need to further explore this field. This chapter is dealing with neuropeptide processing, conversion and inactivation. A particular emphasis will be directed to enzymes and pathways involving limited proteolysis generating bioactive fragments, which may counteract or modulate the response of the parent peptide.

2. BIOSYNTHESIS AND PROCESSING OF NEUROPEPTIDES

The biosynthesis of the neuropeptide precursor protein (prepropeptides) occurs at the ribosomes located on the endoplasmic reticulum in the cell body of the peptide-producing neuron (Burger 1988). The precursor protein, usually 100-250 amino acid residues in length, comprises of an N-terminal signal sequence adjacent to a variable stretch of a so far unknown function. This stretch is followed by a peptide-containing region, which often includes several copies of active sequences. Following synthesis the precursor protein is lead by the signal peptide and subsequently packaged into secretory vesicles and subjected to a series of proteolytic steps before the biologically active peptide is finally generated. The first cleavage results in removal of the signal peptide and is followed by core glycosylation, glucose trimming, and a suitable folding of the propeptide. Further modifications of the carbohydrate content take place within the Golgi compartment, wherein the initial release of intermediate-sized peptides also occurs. Other modification steps, such as sulfation (Bonetto *et al* 1999) and phosphorylation, may also occur at this stage.

The release of smaller-sized precursor fragments, containing the active peptide units, is accomplished by proteases acting at the C-terminal of paired basic amino acid residues, or sometimes at single arginine sites (Canaff *et al* 1999; Foulon *et al* 1996; Seidah and Chretien, 1999). A large number of enzymes participating in this endoproteolytic processing have been characterised. They share a catalytic domain with structural homology to that of the well-known protease *subtilisin.* Subtilisin has been classified as a bacterial and calcium dependent serine proteinase. Due to their similarities with the bacterial subtilase most of the propeptide cleaving enzymes so far identified are believed to belong to the enzyme family of subtilases. Presently, at least seven mammalian subtilases capable of hydrolysing peptide bonds at single or pairs of basic amino acid residues have been described (Seidah and Chretien, 1999). The subtilases are generally recognised as precursor convertases (PC) and the individual members of this enzyme family are

denoted as PC1, PC2, PACE (also known as furin), PACE4, PC4, PC5 and PC7.

Examples of other proteases capable of releasing peptide fragments containing bioactive units are an aspartic protease (PCE) acting as a primary proopiomelanocortin (POMC)-converting enzyme (Loh and Cawley, 1995) and a protease belonging to a new class of subtilisin-kexin-like convertases (SKI-1) (Nagase *et al* 1995). For long it has been a debate about the specificity of the processing convertases. The question whether a given peptide precursor is cleaved by processing enzymes, which are common to all or unique to the particular neuropeptide system has been discussed by many investigators. At present it seems that a large number of neuropeptide precursors are processed intracellularly by a redundant set of convertases. However, it should be noted that studies describing precursor selectivity among processing enzymes have been reported. For instance, a recently described processing convertase, prohormone thiol protease (PTP), was found to exhibit high specificity for proenkephalin (proENK) (Hook *et al* 1996).

The completion of proteolyses, including amino- and carboxyterminal trimming as well as terminal modifications of acetylation (Jornvall 1975; Loh 1987) and amidation (Katopodis *et al* 1991), takes place in the immature and mature secretory granules. The secretory granules containing the active peptides are stored near the nerve endings and following activation of the particular neuron the granule is fused with the plasma membrane and the peptide secreted into the synaptic cleft.

The structure of at least three different opioid peptide precursor (propeptides) proteins (figure1), proopiomelanocortin (POMC), ProENK and prodynorphin (ProDYN) have been elucidated (Cooper *et al* 1996). Each propeptide include several active peptide units in their structure. These are released during processing to finally yield the mature peptides. ProENK may give rise to at least seven enkephalin-containing sequences, while ProDYN includes dynorphin A, dynorphin B and α-/β-neo-endorphin within its structure. From POMC β-endorphin and acetylated or truncated forms thereof, may be released. The opioid-like prepropeptide *proorphanin* is a recently discovered precursor protein, which gives rise to nociceptin/orphanin (Meunier *et al* 1995). Nociceptin/orphanin is a neuropeptide that exerts antiopioid effects under some circumstances but under other conditions it seems to provide opioid effects. Regarding the tachykinin family of neuropeptides (Hokfelt *et al* 1994) at least three genetically distinct precursor proteins have been described. They may give rise to several neuroactive products sharing a common C-terminal sequence. Moreover, proteases capable of releasing bioactive fragments from various neuropeptides are also known.

Several studies have shown that limited hydrolysis of neuroactive peptides may lead to the formation of fragments with retained or different biological

activity (Hallberg and Nyberg, 2003). This type of biotransformation results from the action of more or less specific endoproteases, named convertases. A number of proteinases capable of releasing bioactive fragments from their substrates have been identified in various CNS tissues (Nyberg and Terenius, 1991) but also in cerebrospinal fluid (CSF) (Persson *et al* 1995). An example of this biotransformation is seen for the opioid peptide dynorphin A that is converted to **Leu-enkephalin-Arg^6** and subsequently to Leu-enkephalin (Persson *et al* 1995; Silberring *et al* 1992a). Both these fragments remain biological active, while their receptor activation profile compared to the parent peptide, dynorphin A, is changed. Similarly, the tachykinin peptide substance P is converted by an endoprotease (SPE) to a bioactive N-terminal heptapeptide fragment Substance P(1-7) (Persson *et al* 1995). The heptapeptide mimics some but opposes other effects seen for the intact undecapeptide (Hallberg and Nyberg, 2003).

Another interesting pathway for the formation of neuroactive peptides is represented by a group of peptides acting on opioid receptors. These peptides, even denoted atypical opioid peptides, are formed by partial hydrolysis of some known functional proteins. A common structural property of these peptides is the N-terminal dipeptide sequence Tyr-Pro, which is essential for their opioid activity, but also for their relatively high resistance towards metabolic degradation. Among these peptides are the **β-casomorphins** released from the milk protein **β-casein** (Nyberg *et al* 1989), the cytochrophins from mitochondrial cytochrome b (Brantl *et al* 1985) and the hemorphins from the blood-protein hemoglobin (Nyberg *et al* 1997). These peptides are endogenously found in quite high amounts but their agonistic actions on opioid receptors are comparatively weak. However, recently two opioid tetra-peptides, endomorphin 1 and endomorphin 2 (Horvath 2000; Zadina *et al* 1997), with similar structure to hemorphin and **β-casomorphin,** respectively, which exhibit a very high potency to stimulate the **μ-opioid** receptor, were discovered. The precursor protein giving rise to the endomorphins has, however, not yet been identified.

The process for neuropeptide inactivation also involves several proteases. Most enzyme activities that hydrolyse neuropeptides acts by an exopeptidase action. This group of enzymes includes several aminopeptidases (Taylor 1993), carboxypeptidases (Fricker and Snyder, 1983), as well as dipeptidyl-amino and carboxypeptidases (Roques *et al* 1993). A common route for neuropeptide inactivation is accomplished by sequential release of amino acids by aminopeptidase actions. For instance, the enkephalins are shown to drop their opioid activity after removal of the N-terminal tyrosine residue and the substance P action related to its N-terminal sequence is lost by the action of dipeptidyl-aminopeptidase IV (Watanabe *et al* 1993). In addition, several other enzymatic pathways for neuropeptide degradation have also been described. One example of these is the inactivation of enkephalins by neutral

endopeptidase (NEP), which hydrolysis the pentapeptides by both endo- end exopeptidase actions (Roques *et al* 1993). An additional example is represented by inactivation of bradykinin, which is due to hydrolysis by a variety of endo- and exopeptidases (Skidgel 1992).

3. NEUROPEPTIDE CONVERSION TO FRAGMENTS WITH RETAINED OR MODIFIED BIOLOGICAL ACTIVITY

Neuropeptides are defined as chains of amino acids of limited length released from neurons in a calcium-dependent manner to have an effect on target cells. In this section neuropeptides and their active and inactive fragments, all of which are abundant in the CNS, are discussed. The peptides addressed in this section will be calcitonin gene-related peptide, tachykinins, opioid peptides, nociceptin/orphanin FQ, angiotensins, bradykinins and neuropeptide Y. The amino acid sequences of selected neuropeptides and bioactive fragments are shown in table 1. These peptides and their role as substrates in enzymatic processing and conversion will be highlighted as well as mechanisms of their inactivation. Unlike classic transmitters, termination of the physiological action of neuropeptides is not via specific synaptic reuptake mechanisms, but rather by degradation mediated by extracellular proteases anchored in the cell membrane.

3.1 Calcitonin-Gene Related Peptide

The calcitonin family consists of several different peptides including calcitonin, amylin, adrenomedullin and two forms of calcitonin gene-related peptide (CGRP). Furthermore, intermedin has been suggested to represent a novel member of the calcitonin family although sequence homology between the peptides are restricted to the mature peptide and not to the prepropolypeptides (Roh *et al* 2004). CGRP, mediating its action via the G-protein-coupled receptor subtypes CGRP1 and CGRP2, has probably attracted most interest in recent years. It is derived from the same primary gene transcript as calcitonin (Amara *et al* 1982; Rosenfeld *et al* 1981; Rosenfeld *et al* 1983). The peptide is a potent vasodilator (Brain *et al* 1985; Girgis *et al* 1985; Struthers *et al* 1986) and mediator of neurogenic inflammation (Nohr *et al* 1999; Weihe *et al* 1995) and is widely distributed in the CNS and in the cardiovascular system (Edvinsson *et al* 1987; Inagaki *et al* 1986; Mulderry *et al* 1985). Nerves containing CGRP are closely associated with blood vessels and the most pronounced effect of the peptide is vasodilation. This vasodilatory effect and the observed levels of CGRP in

connection to migraine headache attacks initiated programs aimed at finding receptor antagonists as new agents against migraine and pain (Doggrell 2001; Edvinsson 2003).

Table 1: Amino acid sequences of selected neuropeptides and bioactive fragments

Peptides	Amino acid sequence
α -CGRP (hu)	Ala-Cys-Asp-Thr-Ala-Thr-Cys-Val-Thr-His-Arg-Leu-Ala-Gly-Leu-Leu-Ser-Arg-Ser-Gly-Gly-Val-Val-Lys-Asn-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-Lys-Ala-Phe-NH_2
β -CGRP (hu)	Ala-Cys-Asn-Thr-Ala-Thr-Cys-Val-Thr-His-Arg-Leu-Ala-Gly-Leu-Leu-Ser-Arg-Ser-Gly-Gly-Met-Val-Lys-Ser-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-Lys-Ala-Phe-NH_2
CGRP(8-37) (hu)	Val-Thr-His-Arg-Leu-Ala-Gly-Leu-Leu-Ser-Arg-Ser-Gly-Gly-Val-Val-Lys-Asn-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-Lys-Ala-Phe-NH_2
Substance P	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
Substance P(1-7)	Arg-Pro-Lys-Pro-Gln-Gln-Phe
Neurokinin A	His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2
Neurokinin B	Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH_2
Hemokinin 1(hu)	Thr-Gly-Lys-Ala-Ser-Gln-Phe-Phe-Gly-Leu-Met-NH_2
Leu-enkephalin	Tyr-Gly-Gly-Phe-Leu
Met-enkephalin	Tyr-Gly-Gly-Phe-Met
Dynorphin A	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln
Dynorphin B	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr
β-Endorphin (hu)	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu
Endomorphin-1	Tyr-Pro-Trp-Phe-NH_2
Endomorphin-2	Tyr-Pro-Phe-Phe-NH_2
α-Neoendorphin	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys
Nociceptin/orphanin FQ	Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln
Angiotensin I	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu
Angiotensin II	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe
Angiotensin III	Arg-Val-Tyr-Ile-His-Pro-Phe
Angiotensin IV	Val-Tyr-Ile-His-Pro-Phe
Angiotensin(1-7)	Asp-Arg-Val-Tyr-Ile-His-Pro
Kallidin	Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
Bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
des-Arg^{10}-kallidin	Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe
des-Arg^{9}-bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe
Neuropeptide Y	Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Met-Ala-Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-NH_2

hu = human

The 37 amino acid long peptide with a characteristic six amino acid residue ring system made by a disulfide bridge between Cys^2-Cys^7 exists in an α and a β form, which in humans only differ in three amino acid residues. In the N-terminal the disulfide bridge is crucial for the agonistic activity of CGRP, and removal of two amino acids in the C-terminal sequence reduces its biological activity (Zaidi *et al* 1990). Cleavage of the Cys^7–Val^8 bond

leads to the formation of an antagonist, CGRP(8-37) (Chiba *et al* 1989; Mimeault *et al* 1992). This CGRP fragment is an antagonist with high affinity to the CGRP1 receptor but do not affect the CGRP2 receptor to the same extent (Rovero *et al* 1992). Not only CGRP(8-37) have been shown to exert antagonistic properties but also CGRP(19-37). Furthermore also CGRP(23-37) exhibits an ability, although weak, to antagonize α-CGRP (Rovero *et al* 1992). Interestingly, the fragment CGRP(19-37) has also been reported as a major metabolite in the CNS (Le Greves *et al* 1997; Sakurada *et al* 1991). The inactivation of CGRP is not thoroughly investigated although NEP and SPE is known to generate essentially inactive fragments such as CGRP(17-37) and CGRP(1-16) (Le Greves *et al* 1989; Mentlein and Roos 1996; Sakurada *et al* 1991). Administration of a neutral endopeptidase inhibitor in humans has also been reported to increase plasma levels of CGRP in humans (McDowell *et al* 1997). CGRP fragments generated by neutral endopeptidase are further degraded by aminopeptidase N (Ludwig *et al* 1996; Mentlein and Roos 1996). While the C-terminal fragment CGRP(8-37) is acting as an antagonist, N-terminal fragments have shown agonistic activities. Thus, CGRP(1-12), CGRP(1-15) and CGRP(1-22) have all been reported to exhibit similar activities as their parent peptide, α-CGRP, although the most effective fragment CGRP(1-15) was approximately 100 times less potent (Maggi et *al* 1990).

3.2 Tachykinins

The tachykinin family encompasses substance P (SP), neurokinin A and neurokinin B as the most well known members. These neuropeptides are widely distributed within the CNS but are also present in peripheral tissues. The peptides, also referred as to the neurokinins (NK) prefer the following receptors; NK1 (SP), NK2 (neurokinin A) and NK3 (neurokinin B). New putative tachykinins are hemokinin and endokinins (Kurtz *et al* 2002; Page *et al* 2003; Zhang *et al* 2000). Hemokinin, encoded from the PPT-C gene exhibit a similar biological effect as SP with regard to binding and activation of the NK receptors, and primarily the NK1 receptor subtype (Kurtz *et al* 2002). SP, neurokinin A, neurokinin B and hemokinin share the same C-terminal sequence Phe-X-Gly-Leu-Met-NH2, where X is either Phe as in SP and human hemokinin (Tyr in mouse hemokinin) or Val as in neurokinin A and neurokinin B.

The undecapeptide SP (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2), with two basic side chain residues in the N-terminal (Chang *et al* 1971), has most often been associated with its putative role in pain transmission (Harrison and Geppetti 2001; Severini *et al* 2002; Zubrzycka and Janecka, 2000). SP is present in the primary sensory afferent fibers, in

the dorsal root ganglia (DRG) as well as in the dorsal horn of the spinal cord. In the primary afferent fibers, the unmyelinated C-fibers use SP as a neuromodulatory substance. Antagonists to the tachykinin receptors exhibit an analgesic effect in mice during tail withdrawal tests (Lembeck *et al* 1981), which provides additional strong support for the involvement of the tachykinins in pain transmission and pain modulation. Notably, studies on the role of SP and its receptor in pain processing have suggested that the undecapeptide does not mediate pain through the NK1 receptor (Garces *et al* 1993). Moreover, parallel pathways mediating pain sensation or reflex activity complicate analysis.

The tachykinins, as exemplified above with SP are important not only in pain processing but also for the induction and progression of inflammatory response (Barnes 1986; Barnes *et al* 1991; Lembeck and Holzer, 1979; Levine *et al* 1986; Mantyh *et al* 1988). Furthermore, beside its involvement in pain and inflammation, high levels of substance P are also associated with aggression and SP and its NK1 receptor were early associated with depression (Rimon *et al* 1984). Studies with small organic receptor ligands as research tools have provided strong support for this hypothesis (Argyropoulos and Nutt, 2000; Kramer *et al* 1998). It is not surprising that tachykinin receptor antagonists have emerged as potential drugs for the treatment, not exclusively of primary pain and inflammatory diseases, such as asthma, arthritis, psoriasis and inflammatory bowel diseases, but also for other disorders related to the CNS, e.g. depression, anxiety, schizophrenia, migraine and emesis (Kramer *et al* 1998; Lowe *et al* 1993; Michaud *et al* 1998; Navari *et al* 1999).

SP originates from at least three distinct gene transcripts. These are α-, β- and γ-preprotachykinin. The peptide is released from these precursors by proteolytic processing before its C-terminal residue is modified by an amidating enzyme (Eipper *et al* 1992). The two prolines in the N-terminal segment (Pro^2 and Pro^4) and the amidated C-terminal contribute to the stability of the peptide. There are several different proteases engaged in the degradation of SP in the CNS and CSF. The proteolytic degradation of SP has been shown to deliver at least six lysine-containing metabolites SP(1-4), SP(1-6), SP(1-7), SP(1-9), SP(2-11) and SP(3-11) (Freed *et al* 2001a; Sakurada *et al* 2004). The SP(1-4), SP(1-7), SP(1-9) and SP(3-11) peptides were identified in the striatum, where a microdialysis technique was used in the analytic procedure (Freed *et al* 2001b). Furthermore, it was recently reported that in the mouse spinal cord, the major SP metabolites are SP(1-6), SP(1-7), SP(1-9), SP(8-9), SP(10-11) and phenylalanine (Sakurada *et al* 2004). The formation of the above mentioned fragments are attributed to the activity of some major enzymes. These are angiotensin converting enzyme (ACE), neutral endopeptidase (NEP), and substance P endopeptidase (SPE). The post-proline cleaving enzyme or prolyl endopeptidase cleaves SP between

the Pro^4-Gln^5 residues. Post proline dipeptidyl aminopeptidase successively removes the dipeptides Arg^1-Pro^2 and Lys^3-Pro^4 from SP. ACE cleaves SP at Phe^8-Gly^9 and Gly^9-Leu^{10} (Skidgel *et al* 1984) as the major cleaving sites. Furthermore, ACE is able to serve as a peptidyl dipeptidase, releasing dipeptides from the remaining N-terminal fragment, and is thus able to generate e.g. SP(1-7) (Skidgel *et al* 1984; Yokosawa *et al* 1983). The endopeptidase NEP primarily hydrolyzes SP at the Gln^6-Phe^7, Phe^7-Phe^8 and Gly^9-Leu^{10} bonds (Matsas *et al* 1983; Skidgel *et al* 1984).

The N-terminal of SP, SP(1-7) has received particular interest. This N-terminal fragment has been shown to be a major metabolite of substance P in the rat (Persson *et al* 1995; Sakurada *et al* 1985; Zhou *et al* 1998). SPE, ACE as well as NEP are all capable of producing SP(1-7) from SP. A SPE-like activity has been encountered in the CSF (Persson *et al* 1995) and in spinal cord tissue (Karlsson *et al* 1997), but is also demonstrated in various other areas of the brain (Zhou *et al* 2001).

As in the case with SP, the C-terminal fragment, SP(6-11) produced anxiogenic effects when injected to the dorsal periaqueductal gray (PAG) in rats. This effect is mediated through the NK1 receptor. In contrary, the N-terminal fragment SP(1-7) did elicit the opposite reaction resulting in a less anxiogenic effect. The latter effect was reported not to be mediated through the NK1 receptor (De Araujo *et al* 2001). Apparently the C-terminal but not the N-terminal sequence of SP mimics the effect of the parent neuropeptide, suggesting that the level of enzymatic processing in various brain regions and the character of the proteolytic enzymes involved are of importance for observed physiological responses. Several actions and functions have been attributed to SP(1-7) but a receptor for this fragment is not yet identified. It is possible that the heptapeptide SP(1-7) may exert its effect by acting on accessory sites present on other neuropeptide receptors.

3.3 Opioid Peptides

The enkephalins, the first opioid peptides to be discovered (Hughes *et al* 1975), are pentapeptides with a tyrosine residue in their N-terminal sequence (Tyr-Gly-Gly-Phe-X; X= Leu or Met). Recently, peptides with even shorter structures with opioid activity have been identified, e.g. the endomorphins (Zadina *et al* 1997). The enkephalin sequence resides in the majority of the opioid peptides, including β-endorphin, dynorphin A and B and α-neoendorphin, all appearing to represent C-terminal extensions of the pentapeptide entities.

The opioid peptides exert modulatory actions in pain processing (Wagner and Chauvkin, 1995) and have been attributed to a variety of behavioural processes, such as reward, dependency, sedation, and stress response. In the

brain, the opioid peptides (β-endorphin, dynorphins and enkephalins) are widely distributed, whereas in the spinal cord dynorphins are present mainly in interneurons. Spinal enkephalins are found mainly in long descending pathways from midbrain to the dorsal horn (Cooper *et al* 1996). Opioid peptides are also produced in non-neuronal cells, such as endocrine cells and cells of the immune system. The enkephalins activate mainly the δ-opioid receptors, while the dynorphins exhib it preferences for κ-opioid receptors. It seems that β-endorphin is less selective versus receptor targets and can produce a response through all three receptors, with some preference for the μ opioid receptor.

The opioid peptides are often converted into fragments with retained or modified biological activity. For example dynorphin A(1-17), derived from preprodynorphin, degrades *in vivo* into dynorphin A(1-8) (Seizinger *et al* 1984). Both are agonists of the opioid receptors with selectivity for the κ receptor type (Chavkin *et al* 1982; Corbett *et al* 1982). Another typical example is the dynorphin conversion to Leu-enkephalin. The latter peptide can be released from dynorphin A, dynorphin B and α-neoendorphin. The enkephalin sequence is frequently directly released from its precursor (Camargo *et al* 1987) but in many cases the hexapeptide Leu-enkephalin-Arg^6 is first formed. Several enzymes are capable of generating the enkephalin hexapeptide from the dynorphins (Chesneau *et al* 1994; Nyberg *et al* 1985; Nyberg and Silberring, 1990; Silberring *et al* 1992a; Silberring and Nyberg, 1989). The extended enkephalin unit Leu-enkephalin-Arg^6 is further converted to Leu-enkephalin by carboxypeptidase action (Flicker and Snyder, 1983). The release of Leu-enkephalin from dynorphin reflects a conversion of a κ-agonist to a product acting on μ/δ-opioid receptors. In brain areas where κ signals oppose those of μ/δ-opioid receptors, this type of conversion may be of relevance, e.g. in the brain reward system, where κ agonists produce dysphoria, while stimulation of μ/δ-opioid receptors results in euphoria (Koob 1996).

Dynorphin conversion leading to modified bioactivity constitutes another example, where removal of the N-terminal tyrosine from dynorphin A yields a peptide product with retained biological activity but lack of the opioid effect of the parent peptide (Laughlin *et al* 1997). This fragment was reported to interact with specific sites located on the NMDA receptor. More recently, the biotransformation of dynorphin A in striatum of freely moving rats after direct infusion of the peptide was investigated. As deduced from microdialysis-mass spectrometry peptides consistent with enzymatic cleavage of e.g. the Arg^7-Ile^8 bond to provide dynorphin A(1-7) and dynorphin A(8-17) was reported (Reed *et al* 2003). Several different enzymes are involved in the enzymatic processing of the opioid peptides and as an example dynorphin A is a substrate for carboxypeptidase E with Ile^8-Arg^9 as cleavage site (Day *et al* 1998), for a thiol-dependent dynorphin A converting enzyme with Arg^6-Arg^7

as major cleavage site (Silberring *et al* 1992a), and for a thiol-sensitive metalloprotease, called dynorphin A-17 processing enzyme with Ile^{8}-Arg^{9} as cleavage site (Berman *et al* 1999).

3.4 Nociceptin / Orphanin FQ

Nociceptin (Meunier *et al* 1995) or frequently also denoted orphanin FQ (Reinscheid *et al* 1995) is a 17 amino acid residue long peptide with actions in the CNS. The peptide is associated with processes with relevance for e.g. pain (Flores *et al* 2001; Meunier 1997), learning and memory (Manabe *et al* 1998; Sandin *et al* 1997) as well as stress and anxiety (Griebel *et al* 1999; Reinscheid and Civelli, 2002). Nociceptin shows homology with dynorphin A, although nociceptin does not bind to the κ- opioid receptor, nor does it interact with the μ- and δ-opioid receptors. The peptide has been characterized as the endogenous ligand for the naloxone insensitive opioid-receptor-like orphan receptor-1 (ORL_1). Both the ORL_1 receptor and nociceptin are abundant in the CNS, particularly in the limbic system regions and in several areas known to be involved in pain perception (Neal *et al* 1999).

Like all neuropeptides nociceptin is metabolized by more or less specific proteinases. In mouse brain slices aminopeptidase N cleaves the Phe^{1}-Gly^{2} bond delivering phenylalanine and nociceptin(2-17), whereas endopeptidase 24.15 acts by cleaving nociceptin at the Ala^{7}-Arg^{8}, at the Ala^{11}-Arg^{12} and at the Arg^{12}-Lys^{13} bonds (Montiel *et al* 1997). NEP was reported not to be involved in the metabolism. However, contrary in mouse spinal cord synaptic membranes endopeptidase 24.15 is not involved but instead the neutral endopeptidase delivers nociceptin(1-13), nociceptin(14-17) and phenylalanine as the major metabolites from nociceptin (Sakurada *et al* 2002). Furthermore, small amounts of nociceptin(1-7), (1-9), (1-10), (1-11), (2-9), (2-11), (2-13), (2-17) and (13-17) were traced in the synaptic membranes. In rat hippocampus nociceptin is first metabolized to nociceptin(1-13) and (14-17), whereafter the nociceptin(1-13) fragment is further degraded to nociceptin(1-9) and nociceptin(10-13) (Sandin *et al* 1999). In human plasma, nociceptin(2-17) was found to be the major metabolite. This metabolite was subsequently transformed to nociceptin(3-17), (4-17) and (5-17) (Yu *et al* 1996). Thus, the metabolism pattern of nociceptin seems to be highly tissue specific. In conformity with other peptides discussed in this chapter some of the nociceptin fragments are inactive while others retain biological activity. As an example the N-terminal nociceptin(1-7), (1-9) and (1-13) fragments are reported to modulate nociceptin-induced scratching, biting and licking behaviour in mice (Sakurada *et al* 2000). Some peptide fragments derived from nociceptin degradation retain a similar biological profile as the parent

peptide whereas others exhibit opposite or diverse effects as compared to the nociceptin. Thus, frequently effects attributed to nociceptin may well originate from the release of its bioactive fragments. As an example, since nociceptin is known to produce both antinociception and hyperalgesia, it was suggested that the antinociceptive activity arise after the conversion of the parent peptide. In fact, this speculation was supported by studies showing that *i.c.v.* injection of the nociceptin fragments (1-7) and (1-11) elicited antinociception without giving rise to hyperalgesia (Rossi *et al* 1997). Moreover, studies on nociceptin C-terminal fragments, such as nociceptin (13-17), was found to induce a nociceptive response in mice spinal cord, whereas N-terminal fragments of the heptadecapeptide had no effect (Chen *et al* 2002; Inoue *et al* 2001). It appears that effects of N-terminal nociceptin fragments like (1-7) and (1-11) are mediated through receptors not recognized by the parent compound. There seems to be convincing evidence suggesting that the enzymatic processing of neuropeptides generating fragments modulating the action of the parent peptide also is applicable for the nociceptin/orphanin FQ system of neuroactive peptides.

3.5 Angiotensins

The potent vasoconstrictor angiotensin II (Ang II, Arg-Asp-Val-Tyr-Ile-His-Pro-Phe) is most often associated with the regulation of blood pressure and salt and fluid homeostasis (Hall *et al* 1990) and is the peptide within of the renin-angiotensin system that has attracted great attention during the last decades. Angiotensin exerts it action via two receptors denoted AT1 and AT2. The hypertensive effect of Ang II has mainly been attributed to activation of the AT1 receptor but recently considerable interest has also been devoted to the AT2 receptor as a mediator of blood pressure regulation (Ichiki *et al* 1995; Masaki *et al* 1998) as well as sodium carbonate secretion from the intestinal tract (Johansson *et al* 2001). Activation of the AT2 receptor affects also neuronal cell differentiation and nerve regeneration (Laflamme *et al* 1996; Lucius *et al* 1998; Meffert *et al* 1996). Both the AT1 and AT2 receptors have been cloned and characterized as G-protein-coupled receptors (Mukoyama *et al* 1993; Murphy *et al* 1991). It is now clear that not only angiotensin II but also some of its metabolites are present in the CNS, where the peptides act as neuropeptides and elicit pronounced effects on the brain function. The AT1 receptor is expressed in certain brain nuclei, such as the hypothalamus and the pituitary. Some known effects of Ang II on pituitary hormones, such as prolactin, result from stimulation of the AT1 receptor (Becu-Villalobos *et al* 1994; Dornelles and Franci, 1998; Moreau *et al* 1994). The AT2 receptor is identified in distinct brain regions, including the hypothalamus and the midbrain, and has been suggested to be involved in

growth development and exploratory behaviour (Ichiki *et al* 1995; Tebbs *et al* 1999). Interestingly, several peripheral effects mediated through the AT2 receptor oppose those of the AT1 receptor possibly implicating a similar balance in the CNS (Carey *et al* 2000; de Gasparo *et al* 2000).

Angiotensin II is formed from angiotensin I, an essentially inactive peptide derived from circulating angiotensinogen. The proteolytic cleavage of angiotensin I is mediated by the metalloproteinase ACE, an established target for drug therapy, e.g. captopril. As in the cases of the tachykinins and the opioid peptides, metabolism of Ang II results in the formation of fragments with retained but modified biological activity. For example sequential removal of single amino acid residues from the N-terminal of the peptide results in Ang III (Ang II(2-8)) and Ang IV (Ang II(3-8)), respectively. These peptides play important roles as neuropeptide fragments in the CNS (Blair-West *et al* 2001; Cesari *et al* 2002; Lee *et al* 2001; Wright *et al* 1993). Ang IV seems to be of particular relevance in the CNS and has been attributed to distinct action, e.g. on cerebral blood flow and memory function (Hall *et al* 1995; Pederson *et al* 2001; Wright and Harding, 1997). Furthermore, it has been shown by experiments with an angiotensinogen knock-out mouse model that the hexapeptide Ang IV can prevent neuronal cells from apoptosis (Kakinuma *et al* 1997).

Ang IV may be formed by the action of aminopeptidases acting on Ang II but also by aminopeptidases acting on Ang I prior to the conversion to Ang II (Ardaillou and Chansel, 1997). The hexapeptide binds to and activates the so-called AT4 receptor. Interestingly, it was suggested that the AT4 receptor could represent a membrane-bound aminopeptidase (Albiston *et al* 2001). It was suggested that the peptide exerts its effects by inhibition of this aminopeptidase. Whatever the mechanism of action would be for Ang IV it is obvious that the appearance of this peptide represents an important example of enzymatic conversion of active peptides to a fragment with different biological action.

Processing of both the decapeptide Ang I and the octapeptide Ang II to deliver Ang(1-7) are also important metabolic pathways (Chappell *et al* 1990; Greene *et al* 1982). Ang(1-7) is in fact the major product from Ang I in NG108-15 cells and can be formed directly by deletion of the tripeptide Phe-His-Leu from Ang I (Chappell *et al* 1990). Prolyl endopeptidase is reported to be responsible for almost 50% of the Ang(1-7) generation in these above-mentioned cells. However, in rat, NEP seems to be the major enzyme generating Ang(1-7) from Ang I (Yamamoto *et al* 1992). Furthermore, Ang(1-7) may also be generated from Ang II by prolyl endopeptidase and prolyl-carboxypeptidase (Koida and Walter, 1976; Orlowski *et al* 1979; Welches *et al* 1991; Yang *et al* 1968). The bioactive Ang(1-7) fragment has been detected in several areas of the rat brain, e.g. hypothalamus and amygdala, in approximately the same amounts as Ang I and Ang II (Chappell

et al 1989). Aminopeptidase A can delete the **Asp^1-Arg^2** residues from both Ang I and Ang II (Ahmad and Ward, 1990; Mentlein and Roos, 1996; Robertson *et al* 1992; Ryan 1989) and aminopeptidase N can remove **Asp^1-Arg^2** from Ang I and in addition **Arg^2-Val^3** from Ang III (Mentlein and Roos, 1996; Vaghy *et al* 1995). Furthermore it was recently suggested that arginine-aminopeptidase can process angiotensin peptides in myocardium (Petrov *et al* 2004).

Recently, a new angiotensin-converting enzyme-related carboxypeptidase named ACE2 from a human heart failure ventricle cDNA library. The metalloprotease catalytic domains of ACE2 and ACE are 42% identical. The ACE2 transcripts are found in heart, kidney and testis of the human tissues examined and differs in this regard from the more ubiquitous ACE (Donoghue *et al* 2000). Recombinant ACE2 catalyzes the proteolytic cleavage of Ang I to give Ang(1-9). Thus the C-terminal leucine residue was deleted. The enzyme, that is not inhibited by classical ACE inhibitors such as captopril and lisinopril, can also cleave des-Arg bradykinin and neurotensin but not bradykinin (Donoghue *et al* 2000). The enzyme also converts Ang II to Ang(1-7). ACE2 probably plays an essential role in the local renin-angiotensin system of the heart and kidney. The Ang(1-9) metabolite is also observed in atrial homogenate preparations. Notably, cathepsin A (deamidase) was here reported to be responsible for the transformation of Ang I to the Ang(1-9) fragment (Jackman *et al* 2002). Both Ang(1-9) and Ang(1-7) are bioactive. For example, the two peptides potentiated NO liberation by bradykinin from endothelial cells and at higher concentrations Ang(1-9) was significantly more active than Ang(1-7) (Jackman *et al* 2002).

3.6 Bradykinins

The kinins, bradykinin and kallidin are active components in many different physiological and pathological conditions, including inflammation, pain and vascular processes. These peptides play important roles in the CNS following injury, infection and inflammation (Couture *et al* 2001). The two major kinin receptors, the **B_1** and the **B_2** receptors (Hall 1992; Regoli and Barabe, 1980) have been cloned and sequenced in a variety of species (Hess *et al* 1992, 2001; McEachern *et al* 1991; Menke *et al* 1994).

In tissue, bradykinin and kallidin are produced by enzymatic processing of low-molecular-weight kininogen by kallikreins. Thus, the kallikreins, that are endopeptidases of the trypsin type, have functions similar to that of renin in the renin-angiotensin system. Under normal conditions these kallikreins are adopting an inactive form but are activated by a variety of mechanism following e.g. injury. Bradykinin and kallidin as well as kallikrein have all

been identified in the CNS where bradykinin is most abundant in the hypothalamus and the pituitary (Campbell *et al* 1993; Chao *et al* 1987; Kariya *et al* 1985). Kallidin, is processed by an aminopeptidase to yield bradykinin via the **Lys^{1}-Arg^{2}** bond cleavage. Notably, in plasma high-molecular-weight kininogen, in combination with other factors e.g. factor XII, is the precursor of bradykinin. Both kallidin and bradykinin are further degraded primarily by carboxypeptidase N and ACE (Regoli and Barabe 1980). Furthermore, both endopeptidase 24.15 and NEP are involved in this degradation process (Rosenbaum *et al* 1995). A combined proteolytic processing of kallidin and bradykinin in human heart membranes by aminopeptidase M and NEP provide the inactive bradykinin(1-7) (Kokkonen *et al* 1999). In the rat liver the aminopeptidase P pathway constitutes the major route for bradykinin degradation but in the rat lung the bradykinin metabolism is primarily attributed to the aminopeptidase P and ACE pathways. Bradykinin(1-5) is formed to a large extent both in liver and lung (Griswold *et al* 1996). While proteolytic cleavage most often delivers inactive metabolites e.g. as in the case of the **Pro^{7}-Phe^{8}** bond cleavage to provide bradykinin(1-7), arginyl-carboxypeptidase mediated removal of the arginine residue at the C-terminal part of both kallidin and bradykinin deliver active metabolites. Thus, bradykinin(1-8), more frequently named **des-Arg^{9}-bradykinin,** exhibits higher affinity for the **B_1** receptor than bradykinin itself (Menke *et al* 1994). The effect is even more pronounced with **des-Arg^{10}-kallidin** displaying a nanomolar **B_1** receptor affinity, implicating a powerful reinforcing effect of the N-terminal Lys. However, the binding affinities of **des-Arg^{10}-kallidin** and **des-Arg^{9}-bradykinin** to the **B_2** receptor subtype are considerably lower. Thus, even with regard to bradykinin, peptide processing may lead to both bioactive and inactive smaller peptide fragments.

3.7 Neuropeptide Y

Neuropeptide Y (NPY) is a member of the pancreatic polypeptide family, which involves the structurally related peptides, peptide YY (PYY) and pancreatic polypeptide (PP). NPY, consisting of 36 amino acid residues is involved in many physiological systems and is widely distributed in both the central and peripheral nervous system (Parker *et al* 2002; Redrobe *et al* 2002; Silva *et al* 2002; Tatemoto *et al* 1982). The effects of NPY are mediated of a series of G-protein-coupled receptors sharing only modest primary sequence homology (30-50%) (Michel *et al* 1998; Silva *et al* 2002). The role of NPY in body weight regulation has attracted a particular interest. A pathophysiological profile similar to that in human obesity is observed after chronic central administration of NPY to normal rats (Vettor *et al* 1994;

Zarjevski *et al* 1993). The feeding response is to be mediated by Y1 and/or the Y5 receptor subtypes expressed in the hypothalamus. Fasting leads to an increased level of NPY in brain regions associated with energy homeostasis e. g. the paraventricular nucleus and the arcuate nucleus (Sahu *et al* 1988). Notably, peripheral administration of PYY(3-36) to humans significantly reduced appetite and food intake (Batterham *et al* 2002). This and other studies (Sainsbury *et al* 2002) suggest an involvement also of the Y2 receptor in energy homeostasis. NPY receptor modulation has also been connected with anxiety and depression (Kask *et al* 2002; Silva *et al* 2002), pain (Taiwo and Taylor 2002), alcohol dependence (Thiele *et al* 2002; Thorsell *et al* 2002) and bone formation (Baldock *et al* 2002).

Cleavage of the 98 amino acid residue prepro-NPY sequence (Minth *et al* 1984) yields pro-NPY consisting of a 69 amino acid residue sequence. This sequence contains a) the 36 residues that subsequently will form NPY, b) Gly-Lys-Arg, i.e. a three-peptide unit required for the cleavage and amidation and the prohormone, and c) a 30 amino acid sequence that will form the C-flanking peptide of NPY, often denoted CPON which is liberated from the prohormone by a convertase. Released NPY is further degraded by proteolytic enzymes. For example NPY is a substrate for NEP which may cleave the **Tyr^{20}-Tyr^{21}** and the **Leu^{30}-Ile^{31}** bonds consistent with the known specificity of the enzyme (Medeiros Mdos and Turner, 1996). NPY was also investigated in hippocampal synaptosomes to reveal potential cleavage sites. As in the previous example the processing of NPY resulted in the formation of NPY(1-30) and NPY(31-36). However, this transformation was blocked by pepstatin suggesting an involvement of an aspartic protease (Stenfors *et al* 1997). Enzymatic removal of **Tyr^{1}-Pro^{2}** in the N-terminal by dipeptidyl-aminopeptidase IV yields NPY(3-36). Notably, this special transformation results in a fragment inactive at the Y1 receptor subtype but not at the Y2 receptor (Mentlein *et al* 1993). This observation implicates that the enzymatic processing of the parent NPY, leading to fragments receptor subtype selectivity, might have an important regulatory function. Considering that not only dipeptidyl peptidase IV like enzymes but also specific endopeptidases such as meprin and neprilysin-like enzymes as well as post-arginine hydrolysing enzymes and aminopeptidase P, etc are involved in NPY processing, it is tempting to suggest that selective inhibition by protease inhibitors in the future might constitute an alternative therapy strategy as complement to NPY receptor antagonists.

4. SOME CLINICAL AND FUNCTIONAL ASPECTS ON NEUROPEPTIDE PROCESSING CONVERTING ENZYMES

The regulation of neuropeptide signalling may not only depend on the rate of transcription, rate of biosynthesis or rate of release. It also appears that the regulatory mechanisms in peptide systems involve alterations in the enzyme activity responsible for processing, conversion and inactivation. Therefore, studies of enzymes involved in these processes with regard to functional aspects have received attention. For instance, research on the gene transcripts for precursor cleaving enzymes PC1 or PC2 have been carried out using animal models for examination of inflammatory responses. In immune cells of the blood and inflamed rat paw tissue the opioid peptide **β-endorphin** and its precursor (POMC) were found to co-exist with PC1, PC2 and carboxypeptidase E (Mousa *et al* 2004). Similar models were also used to study the activity of neuropeptide convertases in acute or chronic inflammation (Persson *et al* 1992a; Persson *et al* 1992b). A number of studies have focused on studies of neuropeptide converting or degrading enzymes in CSF collected from patients with various neurological disorders (for review, see (Persson *et al* 1995). In the following we will review some studies focused on functional aspects of neuropeptide converting enzymes. Among these we have chosen ACE, DCE, NEP, SPE and aminopeptidase, which belong to the best studies convertases in experimental animal models and in human, as well.

The mean levels of ACE were examined in fluid CSF samples from a group of patients with dementia of the Alzheimer's type, a group of comparably demented patients with Parkinson's disease, and a neurologically healthy elderly control group. Both pathological groups exhibited a significant decrease in the mean activity levels of ACE activity. Moreover, independent of CSF protein concentration, ACE activity exhibited a positive correlation with AChE activity within the control and Parkinson's disease groups, whereas a statistically significant correlation for these CSF hydrolases was not observed within the Alzheimer's disease (AD) group. Thus, the CSF profiles for patients with mild dementias associated with AD or Parkinson's disease differed by at least two neurochemical criteria. The CSF level of ACE was also measured in two patients considered to have neurosarcoidosis, three patients with possible neurosarcoidosis and in 38 control patients suffering from prolapsed intervertebral discs. It was found that both of the two neurosarcoidosis patients exhibit elevated levels, while the possible neurosarcoidosis patients had values similar to the control patients (Jones *et al* 1991). The authors suggested that the CSF-ACE values may be of use in some patients as a diagnostic test for neurosarcoidosis and provide a reference range of normal controls. In an additional study CSF ACE was determined in

acute and chronic in-patients with schizophrenic psychoses diagnosed according to DSM-IV. Results indicated that ACE correlated significantly with length of schizophrenic psychosis. Also a positive significant correlation between CSF ACE and duration of current psychotic episode as well as duration of current hospitalization was observed. However, serum ACE did not correlate with any clinical variable. The elevation of CSF ACE seemed to be confined to a subgroup of chronic patients with few positive symptoms. Elevated CSF ACE was suggested to reflect an increased solubilization of ACE from cell membranes in the CNS or constitute an increased expression of the ACE gene in response to some stimuli. The ACE gene has also been examined with regard to Alzheimer disease. Polymorphism in the ACE gene has been considered as a risk factor for Alzheimer's disease (AD) independently of the risk conferred by the apolipoprotein E sigma4 allele (APOEsigma4) in diverse populations. A meta-analysis of published data on a common Alu indel polymorphism in ACE was performed which indicated highly significant association of the insertion allele with AD. Further studies revealed strong evidence of association in case-control models and also in a model examining the influence of variation in ACE upon CSF levels of amyloid beta42 peptide (Abeta42). The most significant evidence for association with AD was found for a single nucleotide polymorphism (SNP), A-262T, located in the ACE promoter (Kehoe *et al* 2003). This observation was suggested to support a model whereby decreased ACE activity might influence AD susceptibility by a mechanism involving beta-amyloid metabolism. However, another study (Camelo *et al* 2004) that analysed the distribution of genotypes and alleles of the insertion/deletion (I/D) polymorphisms of ACE in AD patients and normal controls did not demonstrate any association between the I/D polymorphisms of ACE with AD. Using logistic regression and multiple correlation analysis (MCA), it was confirmed that the main risk factor associated and consistently grouped with AD patients in this population is APOE4, but this association was not observed with alleles and genotypes of ACE and A2M. The association with ACE to various pathological conditions as described above may involve peptides that are degraded or products that are released by this enzyme. As mentioned in section 3, there are several neuroactive peptides in addition to the angiotensins, which are affected by ACE action.

Other peptidases, which have been frequently studied in neurological and psychiatric disorders, are the DCE and the SPE (Persson *et al* 1995). The activity of DCE was found to be affected in CSF of several animal models used to study acute and chronic inflammation. It was thus found that in polyarthritic rats the CSF activity of DCE was significantly decreased during the acute phase of inflammation (Persson *et al* 1992a) but restored to that of controls during the chronic phase of arthritis. In monoarthritic rats DCE was also affected. Thus the enzyme activity was significantly decreased 15 days

after the intraarticular injection. Despite the degree of arthritis that was sustained equally at four weeks after inoculation, DCE was back to control levels at that time. A similar observation was made for SPE both in monoarthritic and polyarthritic rats (Persson *et al* 1992a; Persson *et al* 1992b). It was concluded that during acute inflammation both in the collagen-induced polyarthritic form and from a single joint is sufficient to elicit changes in the two convertase activities, and that these effects disappear at a later stage, although the arthritis persists. In an additional study focused on DCE in spinal cord tissue in adjuvant-induced arthritis in rats a similar phenomenon was seen (Silberring *et al* 1992b). The observed decrease in DCE and SPE activity would be compatible with increased level of dynorphin and SP, respectively, as reported in previous studies. In opioid tolerant rats the activity of both DCE and SPE was found to be increased (Persson *et al* 1989). In the case of SPE, this enzyme activity was also enhanced in several brain regions, including the ventral tegmental area (VTA) and spinal cord, in rats during opioid withdrawal (Zhou *et al* 2001). A consequence of increased level of the SPE activity would be increased levels of the SP fragment SP(1-7), which in fact was observed earlier in rat VTA and spinal cord following naloxone precipitated opiate withdrawal (Zhou *et al* 1998). Accordingly, it seems that both DCE and SPE have a role in the regulation of peptide level during pain condition as well as in opioid dependence. Interestingly, SPE metabolize both CGRP and SP, two functionally but not structurally related peptides. The two peptides, which co-exist in many sensory neurons, act like competitors and CGRP has in fact been shown to enhance and prolong SP-induced effects (Cridland and Henry, 1988; Mao *et al* 1992). This may reflect that SP remains available and active for a longer period of time based on the inhibitory effect of CGRP on SP degradation (Le Greves *et al* 1985). A similar functional mechanism on enzyme level has also been described for dynorphin and nociceptin/orphanin FQ (Terenius *et al* 2000).

In human studies the activity of DCE and SPE have been probed in CSF from patients during various pain conditions. For instance, at term pregnancy the level of DCE was significantly lower than in non-pregnant controls (Lyrenas *et al* 1988). Furthermore, a CSF fraction of prodynorphin-derived Leu-enkephalin-Arg^6-containing polypeptides was significantly increased in samples from pregnant women. This indicates that a reduced activity of the opioid peptide-converting enzyme might contribute to an increased resistance to pain at term pregnancy. Moreover, at term pregnancy the activity level of SPE was negatively correlated with an increase in the level of the undecapeptide SP (Liu *et al* 1997). In studies of DCE and SPE in CSF from chronic pain disorders no correlation between pain scores and enzyme activity was found. Thus, self-reported pain experience and affective covariates (anxiety, depression, hostility, somatization) of pain, and myelography data were not found to be related to the enzyme activity levels (Hyyppa *et al*

1990). Also, the activity levels of the enzymes measured in this study had no predictive value for the long-term outcome of rehabilitation and therapy at a five-year follow-up of the patients.

A few studies on NEP and aminopeptidases in human body fluids from neurological patients have been reported. One study measured NEP activity in serum from non-smoking healthy individuals and in CSF from patients without neurological or inflammatory diseases. In studies of serum NEP activity (sNEP) of 25 males and 25 females, aged 20 to 65 years, no significant difference in sNEP activity between the sexes was detected. However, there was a significant positive correlation between sNEP and age. Probing the NEP activity in the CSF (cNEP) no significant difference in activity between the sexes was recorded. Three patients with severe body pain showed cNEP activity dissimilar from those of patients with other disorders (Muraki *et al* 1996). Enzymes of importance for enkephalin degradation or inactivation include NEP but also aminopeptidases. Enkephalin degrading enzyme activity in CSF was measured in three patients suffering from pain (Hasumi *et al* 1989). As compared with controls the levels of Leu-enkephalin was decreased, whereas those of enkephalin degrading enzymes were increased in all the patients. After subjected to treatment, two patients were fortunately cured of pain, and their two values returned to normal. The third patient could not recover from severe pain, and the recorded values remained abnormal. It was suggested that Leu-enkephalin and enkephalin degrading enzyme in CSF may have a function role in regulation of pain sensitivity.

Taken together it seems evident that in several cases the convertase activity is related to alterations in behaviour or to some physiological functions or dysfunctions. These observations stress the importance of convertases as regulators in several peptidergic systems.

5. CONCLUSIONS

Current and progressive studies on neuropeptide processing suggest that this is a growing and rapid expanding area of research that should attract many investigators. Enzymatic processing of neuropeptide precursors seems to represent an important step in the action of neuroactive peptides. Of particular interest, as highlighted in this article, is the biotransformation accomplished by the conversion reactions. Thus, one active neuropeptide may be converted to a peptide product with retained or changed biological activity profile. In this chapter a particular attention has been directed to enzymatic reactions, where convertases transform the agonistic action of a certain compound to become antagonistic. This kind of biotransformation is suggested to represent a modulatory pathway in several neuropeptide systems that so far has not yet been widely acknowledge.

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Chapter 10

Proteases and Neuronal Plasticity

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1. INTRODUCTION

Multi-cellular organisms possess the ability to form extracellular matrix (ECM) permitting a tissue mosaic that is configured by cell-to-cell and cell-to-matrix adherence. This yields organized groups of cells encased in ECM molecules that include collagens, proteoglycans and glycoproteins. These molecules interact with each other and they also activate signal transduction pathways via cell membrane receptors. In this way cell functions including proliferation, migration, and morphological differentiation can be accomplished. Many of these ECM molecules are now thought to be involved in maintaining and changing the synaptic architecture presumed to underlie the processes of neuronal plasticity, learning and memory. Modern interest in exploring the neurobiology of learning and memory began with Tanzi (1893), Müller and Pilzecker (1900), Cajal (1894, 1928) and Sherrington (1906), and continued with Hebb (1949) and Lashley (1950). Work completed by these early researchers, and those who followed, led to the "consolidation theory of memory" suggesting that important experiences initiate a sequence of neural events that eventually result in consolidation of the experiences into stable "memory traces" or "engrams". Such engrams are thought to be initially fragile and easily disrupted, however, over time become fixed in form and location such that important experiences are stored and available for retrieval at a later time (Dudai 1996). The Hebb-Konorski (1948) hypothesis suggests that learning and memory are dependent upon synaptic plasticity and is widely held today (Bliss and Collingridge, 1993; Martin *et al* 2000; Tsien 2000;

Lynch 2004). The necessary steps involved in the neural modifications that permit the formation and long-term storage of the engram are not well defined but appear to begin with extracellular signals that ultimately impact nuclear function working through intracellular intermediates. This cell signaling process likely initiates the specific gene expression necessary for synthesis of molecules important to reconfiguration of connections among neurons that underlie the phenomenon of memory.

Neuronal plasticity appears to involve activity driven changes in the efficacy of synaptic transmission. These changes may produce long-term reconfiguration of synaptic connections leading to strengthening or weakening of these connections (Benington and Frank, 2003; Dityatev and Schachner, 2003). The neuronal plasticity that underlies the phenomenon of memory appears to undergo an ongoing pattern of engram maintenance punctuated by updating and reconsolidation. During the past several years considerable interest has been devoted to identifying those ECM and related molecules responsible for reconfiguring and storing these memory traces. These research efforts have identified a number of potentially important components, and in turn, are forcing reevaluation of the current theory of memory consolidation and retrieval.

The present chapter initially describes components of the ECM including matrix metalloproteinases (MMPs), tissue inhibitors of MMPs (TIMPs), and cell adhesion molecules (CAMs). The ECM consists of many glycoproteins that provide attachment sites for cells to adhere. MMPs and TIMPs are responsible for the degradation and preservation, respectively, of the ECM. CAMs control cell-to-cell adhesion during development by influencing neurite outgrowth, neural migration and adhesion, synaptogenesis, and intracellular signaling. CAMs also regulate cell-to-ECM adhesions. This process is very important to the phenomena of long-term potentiation (LTP), long-term depression (LTD), learning and memory (Wright *et al* 2002a,b; Dityatev and Schachner, 2003; Lamprecht and LeDoux, 2004). The next section describes the role of LTP in neuronal plasticity with emphasis on the roles of Ca^{2+}, cytoskeletal and adhesion molecules, and proteases. LTP has been generally accepted as a building block of memory consolidation, and as such appears to be critical to the formation and maintenance of memory traces. We then discuss the potential roles of CAMs, calpains, tissue plasminogen activator (tPA), and MMPs in memory consolidation. These molecules appear to contribute to memory consolidation and reconsolidation as mechanisms designed to modify and update existing memory traces. This notion is in keeping with the theme that memories, once consolidated, are dynamic and continue to be modified via neuronal plasticity. It is becoming clear that dysfunctional memory diseases target many of the molecules that are critical to normal memory formation.

2. EXTRACELLULAR MATRIX MOLECULES

Neuronal plasticity requires ongoing changes in cell morphology and cell-to-cell interactions. Proteolytic modifications of proteins are exquisitely suited to serve a plasticity function, and thus extracellular matrix molecules provide the scaffolding to which cells adhere. The ECM is predominantly composed of glycoproteins, proteoglycans, and glycosaminoglycans that offer physical support, regulate ionic and nutritional homeostasis of surrounding cells, and possess ligands that interact with cell surface receptors that promote signaling events that guide many functions including cellular proliferation, motility, differentiation, neuritic outgrowth, growth cone targeting, synaptic stabilization, and apoptosis (Bignami *et al* 1993; Juliano and Haskell, 1993; Adams and Watt, 1997; Ruegg 2001). The ECM accounts for up to 20 percent of total CNS volume (Bignami *et al* 1993) and largely consists of the proteins fibronectin, laminin, vitronectin, thrombospondin, tenascin, and collagen IV (Rutka *et al* 1988; Reichardt and Tomaselli, 1991; Venstrom and Reichardt, 1993; Goldbrunner *et al* 1998). Extracellular matrix molecules are well suited to contribute to the plasticity that accompanies memory consolidation given that ECM molecules form a network that supports neurons and glia, and is intimately involved in a wide range of signaling concerned with cellular proliferation, growth, movement, synapse stabilization, and apoptosis.

2.1 Matrix Metalloproteinases

Matrix metalloproteinases (Table 1) are a family of proteolytic enzymes important to the maintenance and restructuring of the ECM (Berkedal-Hansen 1995; Kahari and Saarialha-Kere, 1997). Matrix metalloproteases have been prominently implicated in the physiological processes of angiogenesis, blastocyst implantation, ovulation, cardiac arrhythmnias, and wound healing (Ennis and Matrisian, 1994; Kahari and Saarialho-Kere, 1997; Goldbrunner *et al* 1998; Yong *et al* 1998; Goette *et al* 2003). The ability of MMPs to degrade the protein constituents of the ECM is tightly controlled and is accomplished by three mechanisms: 1) regulation of gene transcription; 2) modulation of pro-enzyme activation; and 3) presence and abundance of tissue inhibitors of metalloproteinases. The majority of MMPs are nonconstituitively expressed, however gene transcription may be induced by such stimuli as growth factors, oncogene products, phorbol esters, as well as cell-to-ECM and cell-to-cell interactions. These stimuli typically result in the activation of various transcription factors including members of the c-fos and c-jun proto-oncogene families, which in turn, contribute to the formation of homo- and hetero-dymeric forms of activator protein-1 (AP-1) transcription

factors. AP-1 binds to specific DNA sequences that are present in the promoters of most MMP genes. The activation of MMP genes is dependent on the combined effects of AP-1 proteins and other transcription factors (Mann and Spinale, 1998).

Table 1: Matrix metalloproteinases and tissue inhibitors of metalloproteinases.

Group	Members	Abbreviation	M.W. (kDa)
Collagenases:	Fibroblast collagenase	MMP-1	54
	Neutrophil collagenase	MMP-8	53
	Collagenase-3	MMP-13	54
	Collagenase-4	λCol 4	57
Gelatinases:	Gelatinase A	MMP-2	73.8
	Gelatinase B	MMP-9	78
Membrane- Type:	Membrane-type 1-MMP	MMP-14	63
	Membrane-type 2-MMP	MMP-15	64
	Membrane-type 3-MMP	MMP-16	64
	Membrane-type 4-MMP	MMP-17	72
Stromelysins:	Stromelysin-1	MMP-3	54
	Stromelysin-2	MMP-10	55
	Stromelysin-3	MMP-11	55
	Macrophage metalloelastase	MMP-12	54
	Matrilysin	MMP-7	30
Others:	Enamelysin	MMP-20	54
	Xenopus collagenase	MMP-18	55
	?	MMP-19	?
TIMPs:		TIMP-1	28
		TIMP-2	21
		TIMP-3	24
		TIMP-4	22

Adapted from Yong *et al* (1998), Kahari and Saarialho-Kere (1999), Murphy *et al* (1999), Rivera and Khrestchatisky (2000), Wright *et al* (2002b), and Wright and Harding (2004).

The MMPs are initially present as inactive proMMP zymogenes with the zinc atom of the catalytic domain bound to the cysteine residue of the pro-peptide region. This cysteine-zinc bond is disrupted by activation factors, a "cysteine switch", that exposes the catalytic site. This results in an intermediate form of MMP that cleaves the pro-peptide region by autocatalysis, thus permitting full enzymatic activity (Van Wart and Birkedal-Hansen, 1990). MMP activation factors are typically proteases that include plasmin, thrombin, kallikrein, and the plasminogen activators tPA and urokinase-type plasminogen activator (uPA), as well as other MMPs. Plasmin is a serine protease derived from the inactive euglobulin plasminogen

via tPA and uPA, and is a major participant in MMP activation (Turgeon and Houenou, 1997). Although much is known about the roles these proteases play in coagulation, less is understood concerning their involvement in neuronal cell development. Recent evidence points to their influence over neurite retraction and death, and modulation of apoptosis.

Thrombin is especially active in regulating neurite retraction and synaptic elimination that accompanies nervous system development. In addition, several studies have indicated that thrombin possesses neural protective activity (eg,Yoshida and Shiosaka, 1999). Vaughn *et al* (1995) have reported that thrombin protects neurons and astrocytes from hypoglycemic and oxidative stress-induced injury. MMPs are also activated by other MMPs. For example, MMP-2, MMP-3, and membrane type MMPs (MT-MMPs) are known to activate other MMPs such as MMP-1 and MMP-9. Membrane type MMPs are also activated by the removal of an inhibitory pro-peptide, however, this is accomplished by the serine proteinase furin (Yong *et al* 1998). These characteristics of MMPs make them an important target for experimental investigation in the areas of memory consolidation and retrieval. An understanding of how MMPs contribute to these events should also facilitate our understanding of the potential contribution of proteases to cognitive processing.

2.2 Caspases Tissue Inhibitors of Metalloproteinases

Tissue inhibitors of metalloproteinases are a family of secreted glycoproteins that presently include four members, TIMP-1-4. The proteolytic activity of MMPs is carefully regulated by TIMPs designed to inhibit the active forms of MMPs by forming tight non-covalent complexes with them (Bode *et al* 1999; Jeng *et al* 2001). Following proteolytic injury, ECM remodeling occurs accompanied by elevations in TIMPs presumably to inhibit further action by MMPs. Pathology may result with an imbalance between appropriate levels of MMPs to facilitate remodeling, and their inhibition by TIMPs (Lukes *et al* 1999). Such imbalances have been implicated in disease states including cancer, arthritis, atherosclerosis, multiple sclerosis, Alzheimer's disease, Guillain-Barre syndrome, ischemia, infections, and others (Lukes *et al* 1999; Rivera and Khrestchatisky, 2000; Jeng *et al* 2001). Thus, the maintenance of an appropriate balance among ECM and TIMPs molecules is imperative.

2.3 Cell Adhesion Molecules

Interactions among cells and ECM molecules are dependent upon several types of CAMs. These molecules are cell surface macromolecules that dictate

cell-to-cell and cell-to-ECM contacts by mediating the processes of adhesion, migration, neurite outgrowth, fasciculation, synaptogenesis, and intracellular signaling (Fields and Itoh, 1996; Schachner 1997). The extracellular domains of CAMs are targets for proteinase activity, while their intracellular domains interact with cytoskeletal proteins. CAMs are functionally separated into Ca^{2+}-dependent (integrins and cadherins) and Ca^{2+}-independent (immunoglobulins and selectins) classifications. Integrins, cadherins, syndecans, and neural cell adhesion molecules (NCAMs, classified within the immunoglobulin family of CAMs), are of special importance to the topic of neuronal plasticity.

The integrin receptors are widely distributed dimeric transmembrane proteins with an extracellular portion that interacts with ECM molecules and cell surface proteins, and an intracellular portion that makes contact with actin cytoskeleton via intermediate proteins such as α-actinin, talin, tensin, and vinculin. Thus, the binding of a ligand to an integrin receptor can result in a link between the ECM and the actin cytoskeleton through these intermediate proteins. Such intermediate proteins, in turn, trigger intracellular signaling pathways that mediate changes in cellular shape, motility, growth, gene regulation, and apoptosis (Frisch and Ruoslahti, 1997).

Cadherins are important adhesion molecules at the synaptic junction (Barth *et al* 1997; Kohmura *et al* 1998; Takeichi 1990). These molecules serve as homophilic adhesive molecules with cell adhesion preferences for other cells that express the identical cadherin family member (Nose 1988). The adhesion complex of N-cadherin has been carefully examined (Shapiro *et al* 1995). Each cadherin dimer on one cell can make adhesion contact with two cadherins on a second cell. These contacts between the extracellular domains of different molecules are repeated to form a zipper-like ribbon structure that interlocks the two cells.

Neural cell adhesion molecules are very important members of the immunoglobulin superfamily of CAMs. Within the CNS three primary forms of NCAMs are expressed by neurons and glial cells. NCAM-120 (120 kDa) is anchored to the cell membrane via phosphatidyl inositol, while NCAM-140 and NCAM-180 are transmembrane spanning (Bock 1987). All three of these NCAMs have been implicated in neurite extension and axon guidance during development of the nervous system (Linneman and Bock, 1989; Baldwin *et al* 1996). This appears to be accomplished by mediating Ca^{2+}-independent homophilic (NCAM-to-NCAM) cell-to-cell adhesions (Moran and Bock 1988). NCAMs also participate in heterophilic interactions with other CAMs including L1 (an immunoglobulin CAM) (Burden-Gulley *et al* 1997), the FGF receptor (Williams *et al* 1994), and TAG-1/axonin-1 (Milev *et al* 1996). NCAMs also make contact with ECM proteins such as collagen (Probstmeier *et al* 1989), agrin (Storms *et al* 1996), and chondroitin sulphate proteoglycans (Grumet *et al* 1993). In addition to influencing adhesion properties of cells,

NCAM binding triggers intracellular signaling pathways that ultimately increase Ca^{2+} influx via voltage-dependent calcium channels (VDCCs, Williams *et al* 1994, 1995). Thus, it appears that integrins are very important for cell-to-ECM substrate adhesion, while cadherins and NCAMs are involved with cell-to-cell adhesion (Goldbrunner *et al* 1998). Each of these CAMs appears to contribute to neuronal plasticity and memory formation.

It now appears that the long lasting changes in excitability precipitated by the phenomenon of LTP may serve as a necessary prelude to memory consolidation. LTP is recognized as a form of plasticity induced by electrical or chemical stimulation of synaptic input pathways (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999; Xu-Friedman and Regehr, 2003). It is also illustrative of "Hebbian plasticity" based on the premise that activation of pre- and post-synaptic neurons produces long lasting changes in synaptic efficacy, thus resulting in associative links between these cells. This alteration in synaptic efficacy appears to be dependent upon Ca^{2+} influx into the post-synaptic neuron via N-methyl-D-aspartate (NMDA) receptors and L-type VDCCs (Grover and Teyler, 1990; Miyakawa *et al* 1992; Magee and Johnston, 1995, 1997; Sabatini *et al* 2001; Bauer *et al* 2002). Elevations in intracellular Ca^{2+} result in the rearrangement of cytoskeletal proteins at the synapse yielding reconfigured synaptic structuring (Matus 2000). Thus, the underlying mechanisms responsible for memory consolidation appear to include rapidly induced synaptic changes via cytoskeletal molecules accompanied by structural changes due to gene expression and protein synthesis (Lamprecht and LeDoux, 2004). The next section summarizes information concerning the relationship among proteases, neuronal plasticity, and LTP.

3. LONG-TERM POTENTIATION (LTP)

Long-term potentiation was first observed in the rabbit hippocampal slice by Bliss and colleagues (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973). Short electrical stimulations of afferent fibers of the dentate gyrus resulted in enhanced excitatory post-synaptic potentials evoked in the dentate gyrus. LTP is thought to serve as the basic physiological mechanism underlying memory storage by many investigators (Lynch *et al* 1991; Morris *et al* 1991; Eichenbaum and Otto, 1992). However, other interpretations favor LTP as an arousal/attention mechanism (Shors and Matzel, 1997). Subsequent investigations established that LTP could be generated in several locations including each of the tri-synaptic circuits of the hippocampus and the dentate gyrus (Larson and Lynch, 1968; Diamond *et al* 1988; Huang *et al* 1996). Once established the strength of LTP diminishes over hours or days if

further stimulation is withheld (Barnes 1979; Racine *et al* 1983). Hippocampal LTP is, in part, dependent upon functioning NMDA receptors (Collingridge *et al* 1983; Morris *et al* 1986; Zhang and Levy, 1992) given that NMDA receptor antagonists prevent LTP and interfere with memory tasks mediated by the hippocampus (Morris *et al* 1986, 1990; Robinson *et al* 1989).

3.1 Phases of LTP

The basic phases of LTP include "induction" characterized by post-synaptic Ca^{2+} influx via NMDA receptors and VDCC that lasts for several minutes. Interference with several protein kinases that mediate this phase blocks induction and also memory consolidation (Mayford *et al* 1995; Chen and Tonegawa, 1997; Dineley *et al* 2001). Thus, the "expression" phase appears to be sustained by kinase activity. Repeated trains of electrical stimulation of the Shaffer-collaterals produce a longer lasting late phase LTP referred to as the "maintenance" phase, that is associated with increases in cAMP (Chetkovich *et al* 1991; Frey *et al* 1993), protein synthesis, and gene expression (Sweatt 1999). This phase is also thought to be dependent upon the NMDA receptor by many, but not all, investigators (Teyler and DiScenna 1987), and appears to be secondary to the activation of Ca^{2+} via Ca^{2+}-sensitive adenylyl cyclase (Poser and Storm, 2001). The increase in cAMP is associated with PKA activation and CREB phosphorylation, and increased expression of a CRE-lacz reporter gene (Impey *et al* 1996). In support of these observations CREB knock-out mice exhibited long-term memory dysfunctions accompanied by deficits in the expression phase of LTP (Bourtchuladze *et al* 1994). Further, Huang and Kandel (1994) have prepared a R(AB) transgenic mouse designed to possess reduced PKA activity in the hippocampus. This R (AB) transgenic mouse functioned such that hippocampal PKA activity was reduced by approximately 50%. These animals evidenced normal early stage LTP in the CA_1 field of the hippocampus as compared with wild-type mice. However, the maintenance phase of LTP was significantly impaired (Abel and Kandel, 1998).

NMDA-independent LTP has also been reported (reviewed in (Shors and Matzel, 1997; Morgan and Teyler, 1999). Along these lines Teyler and colleagues (Morgan and Teyler, 1999; Teyler 1999, 2001; Borroni *et al* 2000) have proposed two forms of LTP. One form is NMDA-dependent as described above, while the second form relies uponVDCC and can be blocked with VDCC blockers such as verapamil. Both types of LTP are presumed to occur during high frequency tetanus-induced LTP (100-200-Hz), while NMDA-dependent LTP occurs at a lower frequency of tetanization (eg. 25-Hz) than VDCC-dependent LTP. It is further argued that the activation of the

VDCC-LTP system is necessary for the mediation of memory for long durations, i.e. over several days (Borroni *et al* 2000).

Aniksztien and Ben-Ari (1991) used the K^+ channel blocker, tetraethylammonium (TEA), to induce LTP that is also dependent on VDCC. While both TEA-LTP and 200 Hz VDCC-LTP increase cytoplasmic Ca^{2+} levels, several of the signal transduction pathways are active in both NMDA receptor-dependent and -independent forms of LTP (Ramakers *et al* 2000). At present the signaling cascades involved in VDCC-LTP are not fully understood (Kanterewicz *et al* 2000). Like NMDA-LTP, it appears that TEA-LTP is blocked by serine/threonine kinase inhibitors (Huber *et al* 1995), but that 200 Hz-induced LTP remains unaffected (Grover and Teyler, 1990, 1995). Additionally, tyrosine kinase inhibitors block 200 Hz-induced LTP. Tyrosine kinases have been implicated in the activation of the Src family of kinases and MAP kinases, that phosphorylate VDCCs and regulate NMDA receptors (Lu *et al* 1998; Yu *et al* 2001).

NMDA receptors and VDCCs are not the only mechanisms that permit an elevation in intracellular Ca^{2+}. Internal stores of Ca^{2+} exist primarily in the endoplasmic reticulum and are released through IP_3 and ryanodine receptors (Obenaus *et al* 1989; Harvey and Collingridge 1992; Fagni *et al* 2000). Ironically it is Ca^{2+} influx that triggers the release of internal Ca^{2+} stores. The ubiquitous nature of Ca^{2+} in second messenger systems, signal transduction pathways, and phosphorylation/dephosphorylation cascades, highlights the importance of Ca^{2+} in the induction and maintenance of LTP. The importance of Ca^{2+} is further illustrated by the recent finding that both LTP and LTD could be induced or abolished by the perturbation of Ca^{2+} levels with the Ca^{2+} chelator EGTA (Cho *et al* 2001). Additional studies have revealed that activation of calpain (Lynch and Baudry, 1984; Stauble *et al* 1988; Denny *et al* 1990), protein kinase C (Stauble *et al* 1988; Colley *et al* 1990), Ca^{2+}-calmodulin kinase type 2 (Malenka *et al* 1989; Malinow *et al* 1989), and the release of Ca^{2+} from intracellular storage pools (Bortolotto *et al* 1995), also contribute to hippocampal LTP. Thus, Ca^{2+} appears to play a critical role in synaptic plasticity.

3.2 Synaptic Strengthening in LTP

The precise mechanism(s) underlying long-term memory storage remains unclear, however, it is widely assumed that specific reconfiguration in the strength of synaptic connections are of paramount importance, and LTP in some way facilitates such plasticity changes (Bailey and Kandel, 1993; Bliss and Collingridge, 1993; Doherty *et al* 1995; Geinisman 2000). There is accumulating evidence that LTP leads to deviations in the number and shape of synaptic spines (Weiler *et al* 1995; Sorra and Harris, 2000; Muller *et al*

2002; Nikonenko *et al* 2002). Some years ago two research groups (Lee *et al* 1980; Chang and Greenhough, 1984) described increases in the number of shaft synapses or dendrites of CA_1 neurons following the induction of LTP. Similarly, Desmond and Levy (1983, 1986, 1988) reported increases in the number of concave spine synapses within the dentate gyrus. Kirov and colleagues (1999) compared fresh hippocampal slices with perfusion-fixed hippocampal slices and noticed 40-50% increases in spine numbers in fresh slices taken from adult rats, and a 90% increase in slices taken from postnatal day 21 rats. These results were interpreted to suggest that new spines are produced in response to loss of synaptic activity when the slice is removed from normal brain circuitry. In contrast, when LTP was induced by high frequency stimulation of the Schaffer-collateral pathway while recording from CA_1 pyramidal neurons, no such measureable deviations in dendritic spine stock occurred, although there was an increase in the number of shaft synapses (Lee *et al* 1980). This same experimental configuration was shown to produce increases in the number of stumpy spines by Chang and Greenhough (1984). When LTP was chemically induced using a supernatant containing elevated Ca^{2+}, reduced Mg^{2+}, and TEA to block voltage-gated K^+ channels (Turner *et al* 1982; Bliss *et al* 1984; Anikszteyn and Ben-Ari, 1991), there were elevations in numbers of small spines and an alteration in the angular displacement of spines (Hosokawa *et al* 1995).

LTP induction in cultured hippocampal slices has been shown to produce new spines, while prevention of LTP with the NMDA receptor antagonist APS (D-2-amino-5-phosphonovaleric acid) prevented new spine formation (Engert and Bonhoeffer, 1999). Similar spine changes have been measured *in vivo* following induction of LTP in the dentate (Van Harreveld and Fifkova 1975; Fifkova and Van Harreveld, 1977). These new spines began emerging within 2 minutes following stimulation. Toni *et al* (1999) observed that LTP produced multiple spines making axonal-dendritic connections. These new spines did not appear to split-off from existing spines but were newly formed (Fiala *et al* 2002; Harris *et al* 2003).

The significance of these morphological changes may lie in their influence upon Ca^{2+} influx. Shortening or widening the spine base influences Ca^{2+} influx into the dendrite (Volfovsky *et al* 1999; Majewska *et al* 2000). For example, glutamate sensitivity increases at spines with larger heads (Matsuzaki *et al* 2001; Smith *et al* 2003); and are linked with larger pre-synaptic terminals possessing more vesicles (Harris and Stevens, 1989; Schikorski and Stevens, 1997). Finally, polyribosomes are predominantly translocated to large spines during spine reconfiguration suggesting the presence of local protein synthesis (Ostroff *et al* 2002). Thus, these morphological increases in spine numbers and size appear to enhance neuronal transmission thus providing the increased efficacy of signaling that is presumed to underlie the synaptic bases of memory consolidation.

3.3 LTP and Dendritic Ca^{2+} Levels

The mechanisms underlying these stimulation-induced post-synaptic morphology changes are not well understood, however, intra-dendritic elevations in Ca^{2+} accompanying LTP appear to trigger a series of changes in cytoskeletal proteins. These elevations in Ca^{2+} appear to occur via synaptically activated NMDA-type glutamate receptors (Alford *et al* 1993; Perkel *et al* 1993), potentially augmented by Ca^{2+}-induced release of additional Ca^{2+} from internal stores (Jaffe *et al* 1994) and/or via influx through VDCC (Alford *et al* 1993). The actin- and calmodulin-binding protein, brain spectrin (Fodrin), is a major form of submembrane cytoskeleton and is degraded by synaptisomal Ca^{2+}-activated protease calpain I. Siman *et al* (1984) suggest that post-synaptic activity-driven accumulations of Ca^{2+} could trigger cytoskeletal mechanisms resulting in reasonably long lasting alterations in the size and shape of dentritic spines. Thus, cytoskelton proteolysis may result in decreases in spine rigidity and promote alterations in spine orientation, thus permitting synaptic plasticity (Hosokawa *et al* 1995). Several cytoskeletal proteins, including actin (Crick 1989), myosin (Morales and Fifkova, 1989), microtubule-associated protein (Aoke and Siekevitz, 1988), and calpain (Lynch and Baudry, 1987) have been implicated as contributors to changes in total spine area and shape associated with the induction of LTP. Accumulating evidence supports a correlation between release and/or activation of proteases capable of cleaving ECM proteins, and the stabilization of synaptic connections during LTP. For example, tPA increases soon after the induction of LTP (Huang *et al* 1996) and appears to facilitate synaptic growth within the mossy fiber pathway (Baranes *et al* 1998). Also, both NCAM and amyloid precursor protein have been shown to increase in the dentate gyrus within 90 minutes following the induction of LTP (Fazeli *et al* 1994).

Elevations in spine Ca^{2+} levels have been associated with activation of two protein kinases that serve as compliments of post-synaptic density. These include protein kinase C, and Ca^{2+}/calmodulin dependent protein kinase II (Lovinger *et al* 1986; Silva *et al* 1992), that act on several cytoskeletal proteins present in spines including actin-binding protein, MAP-2, and myosin light chain. Several investigators have proposed that alterations in spine actin networks, produced by phosphorylation and activation of actin depolymerizing proteins, may contribute to activity-dependent alterations in spine morphology (Coss and Perkel, 1985; Fifkova and Morales, 1991; Lauri *et al* 1999). Dendritic protein synthesis can occur locally in the dendrite (Rao and Steward, 1991; Torre and Steward, 1992; Steward 1997), consistent with activity-dependent plasticity (Steward and Banker, 1992). Feig and Lipton (1993) have shown that ^{3}H-leucine incorporation into protein within CA_1 dendrites rapidly occurs after high frequency afferent stimulation (Schaffer-

collaterals), but not with low frequency stimulation, in the presence of carbachol. This label incorporation could be blocked by atropine, or the NMDA glutamate receptor antagonist, D-aminophosphonovalerate.

Two reports (Shi *et al* 1999; Zamanillo *et al* 1999) support the notion that accompanying tetanic synaptic stimulation there is the rapid redistribution of an intracellular pool of alpha-amino-3-hyroxy-5-methyl-4-isoxasole proprionic acid (AMPA) receptors to dendritic shafts and spines. Such post-synaptic delivery appears to be dependent upon NMDA receptor activation and occurs within 15 minutes of tetanic stimulation-induced LTP in the CA_1 field. Of the four AMPA receptors thus far described Glu^{R1} to Glu^{R4} (Hollman and Heinemann, 1994), CA_1 neuron AMPA receptors evidence predominantly the Glu^{R2} subunit in heteromeric complex with either the Glu^{R1} or Glu^{R3} subunits (Morales and Goda, 1999). Thus, Shi and colleagues (1999) found tetanus to result in the accumulation of Glu^{R1} AMPA receptors in the dendritic shaft with some also present in the spine. These results have been interpreted to suggest that the mechanism underlying LTP may be "an awakening of silent synapses" during LTP facilitation by the movement of Glu^{R1} receptors to NMDA-receptor activated post-synaptic membrane (Morales and Goda, 1999). This accumulation of Glu^{R1} receptors may in turn contribute to an increase in synaptic current thus potentiating LTP. Additional Glu^{R1} receptors were observed to accumulate at the base of the spine where they could serve as a reserve pool. Zamanillo *et al* (1999) extended this inquiry by preparing Glu^{R1} knock-out mice. Despite the loss of this receptor subunit these mice displayed normal development of dendrites and synapses, gross anatomy, and behavior. However, LTP in the CA_1 field was significantly impaired in mutant mice as compared with wild-type mice. Surprisingly, the ability of mutant mice to solve the circular water maze task was unimpaired. These results may question the importance of hippocampal NMDA receptor-dependent LTP to successful performance of spatial learning tasks. Along these lines, Geinisman *et al* (2001) have recently shown that an associative learning task promoted synaptogenesis in the form of significantly increased synaptic boutons in the CA_1 hippocampal field of rabbits.

Taken together these findings suggest that, under some conditions, LTP can induce the formation of new synapses between stimulated axon terminals and newly available dendritic spines, however other investigators have failed to measure such changes in synapse numbers. LTP has also been suggested to promote transformation of affected synapses into a more efficient state, perhaps via conversion of "silent synapses" into active ones. Thus, it appears that at least two processes potentially underlie the phenomena of learning and memory, synaptogenesis and synaptic remodeling.

3.4 Cell Adhesion Molecules in LTP

It is clear that synaptic activity can differentially regulate the expression of CAMs (Bailey *et al* 1992; Mayford *et al* 1992; Itoh *et al* 1997), and activation of NMDA receptors facilitate serine protease-induced cleavage of CAMs (Hoffman *et al* 1998). Two classes of CAMs are thought to contribute to separate phases of LTP. NCAMs are suggested to be involved in the initial formation of LTP, i.e. induction (Luthl *et al* 1994; Ronn *et al* 1995), while integrins are proposed to contribute to the long-term maintenance of LTP (Xiao *et al* 1991; Bahr *et al* 1997). The NCAM related changes appear to occur rather quickly, perhaps within 30 seconds (Gustafsson *et al* 1989), while the changes induced by integrins take longer (Lynch 1998). This delay may be due to the time required to activate integrins from a quiescent state to an active adhesion state (Newton *et al* 1997). Staubli and colleagues (Bahr *et al* 1997; Stauble *et al* 1998) have used an integrin peptide antagonist (GRGDSP) injected onto hippocampal slices, prior to, or after the induction of LTP. Application of the antagonist 10 minutes prior to, or 10 minutes after induction, accelerated the decay rate of LTP. However, treatment of the slice 25 minutes after induction had no effect. These results suggest that integrin activation is important during a specific time period for maintenance of synaptic potentiation following the induction of LTP.

Cadherins are also important to the maintenance of LTP. Both neural and epithelial cadherins are present at the synapse. Hippocampal slices pretreated with antibodies against the extracellular domain of either neural or epithelial cadherins, blocked LTP (Tang *et al* 1998). High extracellular Ca^{2+} levels prevented blockade of LTP by this antibody, or by the peptide antagonist HAV. These results suggest that electrically stimulated depletion of extracellular Ca^{2+} triggers the destabilization of cadherin-cadherin interactions, and thus initiate ECM reconfiguration.

3.5 Proteases and LTP

There is growing evidence that proteases are directly involved in LTP. It was initially shown that calpains, Ca^{2+}-dependent cysteine proteases are activated by titanic stimulaltion. Lynch and Baudry (1984) demonstrated that μ–calpain activates fodrin to allow cryptic glutamate receptors positioned within the post-synaptic membrane to surface. However, recent evidence supports the importance of calpain's contribution to LTP, but not as originally envisioned by way of fodrin cleavage-induced glutamate receptor uncovering (Tomimatsu *et al* 2002). Specifically, calpain has been suggested to proteolytically digest large N-terminal fragments of the three NR2 subunits (NR2A,B and C) as well as smaller C-terminal fragments of the NR2 subunit

of NMDA receptors (Gutmann *et al* 2001). Calpain has been proposed to influence second messenger modulation and/or turnover of NMDA receptors. It has also been proposed that μ-calpain degrades glutamate receptor-interacting protein (GRIP) that appears to function as an AMPA receptor-targeting/synaptic-stabilizing protein (Lu *et al* 2001). In this way the breakdown of GRIP could dissolve the GRIP-GluR2 interaction permitting structural and functional reconfiguration (Tomimatsu *et al* 2002).

As mentioned earlier plasminogen activators such as tPA and uPA are serine proteases that catalyze the process of plasminogen conversion to plasmin, also a serine protease. TPA has been implicated as one of several immediate-early genes that correlate with kindling, seizuring, and LTP (Qian *et al* 1993). The presence of tPA appears to be necessary for LTP maintenance in CA_1 and CA_3 hippocampal pathways (Huang *et al* 1996; Baranes *et al* 1998). Further, tPA over expression has been correlated with enhanced LTP (Madani *et al* 1999). Nicole *et al* (1994) have reported that tPA facilitates NMDA receptor-mediated signaling by cleaving the NR1 subunit of the NMDA receptor. These results point to an important role for tPA in the maintenance of LTP.

Thrombin is also a serine protease that influences G-protein-coupled protease-activated receptors via signal transduction mechanisms. Thrombin facilitates LTP (Komai *et al* 2000) and enhances NMDA receptor-mediated responses in CA_1 neurons (Gingrich *et al* 2000). This thrombin effect could be prevented by hirudin, a selective thrombin antagonist, and mimicked by the peptide agonist SFLRN via activation of the protease-activated receptor-1 (PAR-1, Gingrich *et al* 2000). PAR-1 has been shown to couple with the G_q family of the G_α –proteins that in turn activate phosphoinositide hydrolysis and PKC. Activation of PKC is thought to reduce voltage-dependent Mg^{2+} blockage of the NMDA receptor, thus facilitating LTP (Chen and Huang, 1992).

A final serine protease, neuropsin, is expressed in the hippocampus and appears to facilitate the expression of LTP. The application of neuropsin enhanced expression in the CA_1 field while antibody-induced blockade of neuropsin reduced expression of LTP (Komai *et al* 2000). These investigators argue that neuroposin may have its influence upon LTP via its proteolytic degradation of fibronectin, an ECM protein and ligand for integrin (Shimiza *et al* 1998). In contrast with this proposal, Davies *et al* (2001) have shown that neuropsin-deficient mice displayed normal hippocampal LTP. Resolution of these differences in findings awaits further investigation.

4. POSSIBLE MECHANISMS OF MEMORY CONSOLIDATION

As discussed in the Introduction, the processes of learning and memory are believed to be mediated by structural changes in the CNS as represented by neuronal plasticity. However, such molecular changes are transient and may account for short-term memory lasting seconds or perhaps minutes, but cannot by themselves account for long-term memory where consolidation is thought to be necessary (Lamprecht and LeDoux, 2004). Thus, long-term memory requires structural changes in synaptic morphology and protein synthesis. There are several candidate matrix molecules and preoteases that could contribute to the formation of long-term memory engrams. The following are likely, but certainly not the only, possible mediators of new memory formation. CAMs, and especially integrins, cadherins, and NCAMs, have been the focus of considerable research attention for several years. There is no doubt that NCAMs are powerful regulators of neurite outgrowth during development, however their role in the adult CNS is less clear. tPA and the MMPs are equally impressive candidates given their ability to breakdown the ECM, thus presenting the potential to initiate neuronal reconfiguration.

4.1 Cell Adhesion Molecules

Although the precise mechanism(s) underlying long-term memory has not been identified, it is clear that CAMs are of major importance. Figure 1 presents the basic configuration of a glutamatergic synapse including the location of ECM (wiggly lines), integrins (α and β subunits), NCAMs, α-amino-3-hydroxy-5-methyl-4-isoxaxolepropionic acid (AMPAR) and N-methyl-D-aspartate (NMDAR) receptors, VDCC, and receptor signaling pathways. With increased release of glutamate and activation of AMPA and NMDA receptors on the post-synaptic membrane, as seen during LTP, there is a presumed increase in the expression and activation of MMPs. This elevation in MMPs could serve at least two critical roles that relate directly to the efficiency of synaptic remodeling and accompanying neural plasticity. First, the transient MMP-dependent destruction of the inter-synaptic ECM molecules could lead to a relaxation of crosslinks that orient pre- and post-synaptic elements thus allowing for their favorable reorientation. Second, disruption of ECM-cell adhesion molecule interactions could lead to altered intracellular signaling processes mediated by CAMs such as integrins and syndecans that have been functionally linked to synaptic processes requisite for LTP and learning. The consequence of such a disruption would be expected to manifest post-synaptically as alterations in the pattern of gene

transcription and cytoskeletal processes that direct dendritic structure and the localization of critical receptors, kinases, and associated scaffolding proteins. This process could then be halted by TIMPs and returned from a dynamic back to a static configuration. Several excellent recent reviews provide greater depth of discussion on these possibilities, and the roles of LTP and LTD in these processes (Kaczmarck *et al* 2002; Dityatev and Schachner, 2003; Stamenkovic 2003).

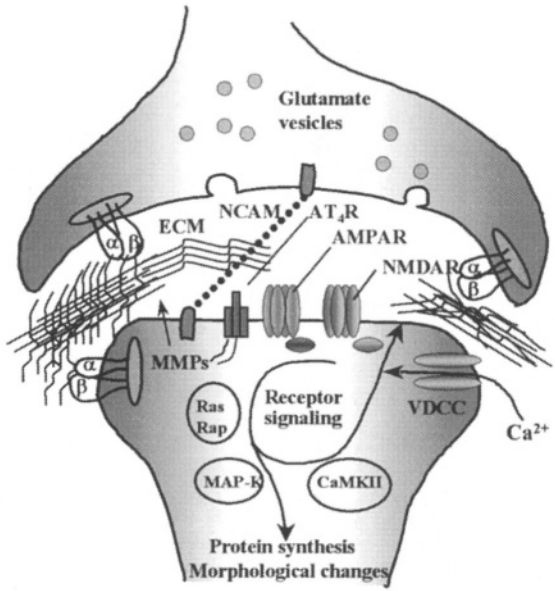

Figure 1: Hypothetical model depicting the possible relationship among ECM molecules, and several receptor systems in the control of neuronal plasticity. Cell surface receptors such as integrins (α and β subunits) and NCAMs interact with receptor tyrosine kinases (not shown) at pre- and post-synaptic appositions thus influencing receptor and cytoskeletal functioning. Glutamate release activates AMPA and NMDA receptors ultimately facilitating Ca^{2+} entry into the cell. Tenascin-C is also thought to interact with VDCCs to further facilitate Ca^{2+} entry. Reconfiguration of ECM molecules appears to be triggered at least partially by Ca^{2+} signaling. Cellular signaling may also occur through Ras and Rap. Adapted from Wright and Harding (2004).

Morphological changes have been described following the induction of LTP, including alterations in numbers of shaft synapses located on dendrites of CA_1 neurons, and increases in the number of concave spine synapses in the dentate gyrus. Elevations in the numbers of small spines have been observed with chemically-induced LTP (reviewed in Doherty *et al* 1995; Abel and

Kandel, 1998; Benington and Frank, 2003). Kirov *et al* (1999a) have noted dramatic increases in spine numbers in hippocampal slices taken from both adult and young rats that have experienced a loss of input stimulation to these circuits. Dendrites of the CA_1 pyramidal neurons treated with glutamate receptor antagonists to block synaptic transmission became much spinier than dendrites activated by low-frequency or tetanic stimulation (Kirov *et al* 1999b). This is an important observation in that the salient parameter for morphological alterations may be a change in activity within these circuits either increases or decreases, rather than absolute levels of input.

Lynch and colleagues have investigated how induction of LTP is transformed to maintenance and then consolidation thus producing a long lasting potentiation. As described above, the activation of integrins appears to be required to stabilize the maintenance phase. It is this maintenance phase that presumably permits synaptic reconfiguration. However, prior to synaptic reorganization the system must be destabilized in order to breakdown the established configurations. Lynch and colleagues (Vanderklish *et al* 1995; Vanderklish *et al* 1996; Lynch 1998) believe that the primary molecule involved in this process is the Ca^{2+}-activated cysteine protease calpain. Calpain has been implicated in the breakdown of several structural proteins permitting integrin-mediated reorganization of cytoskeletal proteins (Inomata *et al* 1996; Schoenwaelder *et al* 1997). In addition, calpain appears to play a role in the reorganization of the spectrin complex that accompanies the induction of LTP (Bahr *et al* 1995; Vanderklish *et al* 1995). In this way the intracellular anchors holding the pre- and post-synaptic membranes together are dissolved permitting synaptic reconfiguration. In support of this notion, calpain cleavage of intact talin significantly increased talin binding to the B3 integrin cytplasmic domain, thus promoting clustering and activation of integrin receptors (Yan *et al* 2001). In turn, this would be expected to facilitate cell-to-cell adhesion.

As previously described the NCAMs include several isoforms (NCAM -120, -140, -180 and -200 kDa; Tomasiewicz *et al* 1993), and are well positioned to facilitate synaptic restructuring following the induction of LTP and memory formation (Hoffman 1998a,c; Ronn *et al* 1998). It has been suggested that down-regulation of stable forms of NCAMs (variable alternative spliced exon [VASE+], and polysialic acid [PSA]- isoforms) may be replaced with growth-promoting forms (VASE- and PSA+), thus facilitating synaptic plasticity (reviewed in Doherty *et al* 1995). The level of polysialic acid (PSA) attached to NCAM molecules is believed to influence their adhesiveness. Given the large negative charge of PSA-NCAM it is hypothesized that during synaptic remodeling this negative charge on interacting NCAM molecules provides a repulsive force that drives them apart, thus destabilizing presynaptic-postsynaptic contact. Interestingly, the sialinase responsible for this NCAM modification is Ca^{2+}-dependent, thus

linking it functionally to the intracellular Ca^{2+} increases that are requisite for LTP. Upon restabilization of synapses PSA is removed resulting in a facilitation of NCAM adhesiveness. Doyle and colleagues (1992a) have measured elevations in NCAM -180, 12 to 24 hours following passive avoidance conditioning in rats. The time course was 6-8 hours post-training based on an increment in glycoprotein synthesis (Doyle *et al* 1992b; Scholey *et al* 1993; Arami *et al* 1996). This agrees with the amnesic effect induced by the protein synthesis inhibitor cycloheximide during a 3-6 hour post-training window (Squire and Barondes, 1972). Cellular and morphological changes have been observed to accompany such training. These changes include: 1) increases in ribosome production in the adult rat dentate gyrus; and 2) a 2-fold elevation in dendritic spine numbers in the mid-molecular layer of the hippocampus at 5-7 hours post-training (Vaughn and Bjorkman, 1996). In agreement with these observations Solomonia and colleagues (1998) have measured significant elevations of NCAM-120, -140, and -180 in the forebrain of chicks trained and prescreened as good learners, compared with poor learners and non-trained controls. The intermediate and medial hyperstriatum of the forebrain appear to be sites of recognition memory during imprinting. Finally, Murphy and Regan (1999) have measured significant activation of NCAM polysialylation on dentate neurons 12 hours following training in the circular water maze, and a similar response following training on the passive avoidance conditioning task. However, when animals were sequentially trained on the circular water maze task, immediately followed by training on the passive avoidance task, they recalled successful search strategies in the water maze, but did not recall the passive avoidance conditioned response. Consistent with these behavioral outcomes the levels of NCAM PSA activation were equivalent with those seen in animals trained only on the water maze task. When animals were permitted 2 hours rest between tasks the levels of NCAM PSA equaled the sum of those seen for animals separately trained on each task. These results suggest that limits exist concerning the activation of NCAM PSA with sequential task training.

Antibodies against NCAMs are capable of interfering with hippocampal slice LTP (Ronn *et al* 1995); while intracerebro- ventricular (icv) injection of antibodies against NCAM in chicks has been shown to interfere with passive avoidance conditioning (Mileusnic *et al* 1995; Scholey *et al* 1995). Similar results have been reported using rats (Doyle *et al* 1992a; Alexinsky *et al* 1995). Arami *et al* (1996) have noted poor acquisition of the circular water maze task by rats continuously icv infused with polyclonal antibodies directed against NCAM. Cremer and colleagues (1994) have observed severe deficiencies in the acquisition of a spatial learning task by mice prepared with a functional NCAM gene inactivated via homologous recombination. However, results from an investigation by Qualls and colleagues (1999) utilizing NCAM-180 knockout mice (NCAM TMICWRU mice) revealed no

impairment in acquisition of the 8-arm radial maze task as compared with wild-type mice.

The cell adhesion molecule L1 neural antigen is involved in neurite outgrowth and axon guidance during brain development (Fischer *et al* 1986). L1 and NCAM bind to one another thus increasing L1-mediated adhesion (Kadmon and Kowitz, 1990). Within L1's structure is an RGD amino acid sequence that serves to also act as a ligand for integrin receptors (Ruppert *et al* 1995; Montgomery *et al* 1996). There are indications that L1 may be involved in memory storage. Lüthl *et al* (1994) have shown that antibodies against both L1 and NCAM, when applied to hippocampal slices, interfered with the induction of LTP. Rose and colleagues (Rose 1996; Johnston *et al* 1998; Tiunova *et al* 1998; Rose and Stewart, 1999) have proposed that both L1 and NCAM are involved in the consolidation of memories by way of changes in synaptic configuration. They have shown that intracranial injections of antibodies against L1 in 2-day old chicks disrupted memory retention of a visual discrimination task when tested 24 hours post-training. However, the timing of the antibody injection was important. Memory disruption occurred only if the injection immediately preceded training, at 5-6 hours following training, or 15-18 hours after training. Thus, there appear to be specific time periods during which the L1 glycoprotein participates in events required for memory consolidation. This same research group has shown similar disruption in recall of passive avoidance training in one-day old chicks, retested 24 hours following conditioning when L1 antibodies were administered 30 minutes prior to training, and 5-8 hours following training (Scholey *et al* 1995).

The preparation of an L1 knockout mouse model with a null mutation in the L1 gene resulted in an animal with multiple neural pathologies rendering it unusable for behavioral testing (Fransen *et al* 1998). This animal's locomotor behaviors were characterized by persistent circling similar to that seen in cerebellar lesioned rodents, which appears to be due to hypoplasia of the cerebellar vermis. Although this model may have limited usefulness in studying synaptic plasticity it does seem useful in approximating the developmental dysfunctions seen in the human nervous system due to mutations in the L1 gene (Kamiguchi *et al* 1998; Kenwrick and Doherty, 1998). This research group has also implicated the role of brain-derived neurotrophic factor (BDNF) in memory consolidation and recall in day old chicks (Johnston and Rose, 2001; Anokhin *et al* 2002). However, a role for BDNF in sleep-induced memory formation has not been supported. Specifically, increases in neocortical BDNF and tPA mRNA have been reported following 8 hours of sleep deprivation in rats. In contrast, sleep resulted in decreased levels of BDNF and tPA, while MMP-9 mRNA increased (Taishi *et al* 2001).

Recently, Chan *et al.* (2003) have tested knockout mice with reduced expression of α3 integrin, α3 plus α5 integrins, and α3 plus α5 plus α8 integrins. Their results indicate that interference with expression of α3 is sufficient to disrupt maintenance of LTP in hippocampal CA_1 slices. Mice prepared with reduced α3 plus α5 integrins expression also showed a deficit in paired-pulse facilitation, usually an indication of a lack of post-synaptic signaling. And those mice with reduced α3 plus α5 plus α8 integrin expression revealed problems with hippocampal LTP maintenance and difficulty solving the Morris water maze task of spatial memory. However, these mice were able to demonstrate normal fear conditioning, a response primarily dependent upon the amygdala rather than the hippocampus (eg. Zinebi *et al* 2003; Tsvetkov *et al* 2004).

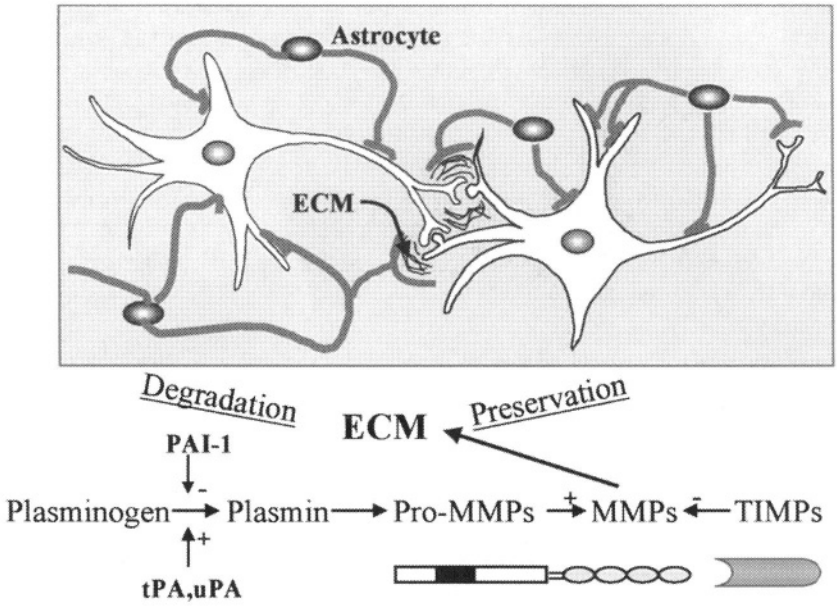

Figure 2: Model describing the influences of PAI-1, tPA, uPA, plasminogen and plasmin upon the conversion of pro-MMPs to active MMPs. Many active MMPs function to degrade the ECM while TIMPs are designed to deactivate the MMPs thus preserving ECM molecules and connections. Adapted from Wright and Harding (2004).

4.2 Potential Roles of tPA and MMPs

Changes in ECM structure alter the synapse in ways that facilitate or inhibit synaptic efficiency, and modulate functional neuronal plasticity. As previously discussed the ECM possesses a mutable nature largely due to the regulated expression and activity of MMPs that function to degrade existing matrix. A cascade of enzymes is involved in the activation of MMPs (Figure 2). The serine protease plasmin, derived from plasminogen by way of the plasminogen activators tPA and uPA, acts to convert pro-MMPs to their active forms capable of degrading the surrounding matrix. This cascade of proteases initiated by plasminogen activators is capable of degrading the vast majority of matrix and basement membrane molecules, interfering with cell-to-cell and cell-to-substrate connections and resultant signaling events, altering neuronal and synaptic morphology, and modifying chemical sequestration within the synapse (Yoshida and Shiosake, 1999). Thus, CNS plasminogen activators have the potential to act as primary enzymes in the control of events such as LTP, LTD, learning and memory.

Increases in plasminogen activator activity within the CNS have been associated with cell migration (Kalderon 1979; Seeds *et al* 1997), growth cone expansion (Krystosek and Seeds, 1981; Pittman *et al* 1989), neural regeneration (Kalderon 1979; Salles *et al* 1990), post-ischemic neurotoxicity (Centonze *et al* 2002b), and LTP (Centonze *et al* 2002a; Tomimatsu *et al* 2002). Expression of the genes encoding tPA and fibronectin are induced in rat dentate gyrus granular cells coincident with LTP (Salles *et al* 1990; Carmeliet *et al* 1994; Gualandris *et al* 1996; Huang *et al* 1996a,b) and seizure (Gilbert *et al* 1993; Tsirka *et al* 1995; Hoffman *et al* 1998d). There appears to be a cause and effect relationship between tPA and LTP given that mice made genetically deficient in tPA reveal a decrease in the maintenance phase of LTP in the CA_1 field (Frey *et al* 1996; Huang *et al* 1996a). In support of this notion Madani and colleagues (1999) have shown that transgenic mice, over-expressing tPA in post-natal neurons, evidenced exaggerated LTP in the CA_1 region of hippocampal slices. These animals also exhibited shorter acquisition times to learn the circular water maze task than wild-type mice, and were quicker to locate the submerged pedestal when it was moved to a new quadrant. Similarly, Seeds *et al* (1995) reported elevations in cerebellar Purkinje cell tPA in rats trained to rapidly traverse an irregular pegged straight runway. Further, hippocampal NMDA receptor stimulation induced proteolysis of cell adhesion molecules by tPA (Hoffman *et al* 1998a). Blocking trypsin-like serine proteases (that include tPA), caused a rapid decline in the duration and maintenance of LTP (Hoffman *et al* 1998b). The serine protease inhibitor, neuroserpin, induced inhibition of hippocampal tPA and significantly delayed the progression of kainic acid stimulated seizure activity in both mice and rats (Yepes *et al* 2002)

Consistent with the above findings, Nagai *et al* (1999) have shown that injections of kainic acid into the lateral ventricles of rats resulted in elevations in tPA activity primarily in the CA_3 field beginning 4 hours after injection. The CA_3 field also revealed the greatest level of neural degeneration within the hippocampus. In contrast, mice deficient in tPA appear to be protected from the neural degeneration typically induced by excitotoxins (Tsirka *et al* 1995, 1996). Plasminogen deficient mice also appear to be protected from excitotoxin-mediated neuronal cell death (Tsirka *et al* 1997).

As described above, a downstream consequence of elevations in plasminogen activators and plasmin is induction of MMP activity (Yong *et al* 1998). This process has been identified in cerebral focal ischemia (Romanic *et al* 1998). As mentioned earlier, within the catalytic domain of MMPs is a cysteine residue that normally interacts with an ion of zinc, the "cysteine switch", revealing a catalytic site that promotes the autocatalysis of a 10 kDa N-terminal pro-region (Springman *et al* 1990). This process results in the conversion of pro-MMPs to active forms (Figure 2). Along these lines, the gelatinase MMP-9 has recently received considerable attention. The active form of MMP-9 (approximately 90-92 kDa) is capable of cleaving Types IV and V collagen (Wilhelm *et al* 1989), fibronectin and gelatin (Davis and Martin, 1990; Netzel-Arnett *et al* 1993), substance P (Backstrom and Tokes, 1995), and β-amyloid (Backstrom *et al* 1996). Thus, many of the substrates for MMP-9 are found within the normal adult CNS. In addition, several of these substrates have a direct relationship with diseases that affect cognitive function. Thus, MMP-9 may play a significant role in normal and dysfunctional cognitive processes. For example, Asahi and colleagues (2000) have reported increases in MMP-9 following focal cerebral ischemia in mice, while MMP-9 knockout mice displayed significantly reduced lesion volumes following ischemia. Pretreatment with the broad spectrum MMP inhibitor BB-94 also reduced ischemia lesion size in wild-type mice, but for some reason not in the knockout mice.

Relevant to the above observations is the finding that hippocampi of Alzheimer's disease patients evidence altered levels of pro-MMPs, and active MMPs (Backstrom *et al* 1992). The implications of these findings are discussed in the next section.

5. CONCLUSIONS

This chapter has presented available information concerning the ECM, LTP, learning and memory in an attempt to both summarize how these research areas have contributed to our understanding of neuronal plasticity in the CNS, and where we must invest future research effort. Several pieces of

information are particularly important: 1) LTP induces elevations in intradentritic Ca^{2+} that initiates changes in protein kinases, and in turn cytoskeletal proteins. The observation that tPA increases following LTP induction supports the notion that LTP triggers synaptic reconfiguration. 2) There is accumulating evidence supporting a relationship between LTP and the synthesis, release, and/or activation of proteases, especially MMPs, designed to cleave ECM proteins. This degradation of existing ECM molecules appears to be prerequisite to synaptic reconfiguration. 3) Increases in hippocampal NCAMs are correlated with successful behavioral conditioning, and icv injections of antibodies against NCAMs interfere with conditioning. Future efforts must focus on the precise molecular changes underlying the synaptic remodeling that serves as the mechanism(s) responsible for learning and memory. Attention must be given to the temporal patterning, location(s), duration, and reconfiguration of the consolidated memory. Proteases will play prominent roles in these scenarios.

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Chapter 11

Serine Proteases and Neuronal Plasticity *in vivo*

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1. INTRODUCTION

Plasminogen activators (PAs) are serine proteases that convert plasminogen to the active enzyme plasmin. Two PAs termed tissue-type (tPA) and urokinase (uPA) possessing consensus sequences but transcribed from different genes, have been identified in mammals (Belin *et al* 1985; Ny *et al* 1988). The tPA is the major serine protease in the mammalian central nervous system (CNS) after birth (Davies *et al* 1998). The presence of tPA mRNA, protein, and its proteolytic activity is observed in neurons and glia cells across brain regions, including neocortex, hippocampus, hypothalamus and cerebellum (Soreq and Miskin, 1981, 1983; Sappino *et al* 1993; Ware *et al* 1995; Tsirka *et al* 1997; Sallés and Strickland, 2002). In contrast, the expression of uPA is extremely low in the postnatal brain (Sappino *et al* 1993; Ware *et al* 1995; Davies *et al* 1998). It is now accepted that tPA participates in a variety of postnatal development events and neuronal plasticity, such as cell migration, experience-dependent circuit refinement and learning. Here, we review these roles for tPA concerning development and plasticity in normal mammalian brain, with special emphasis on the experience-dependent plasticity in the visual cortex.

2. POSTNATAL DEVELOPMENT AND PLASMINOGEN ACTIVATOR

2.1 Cell Migration and tPA

Migration of neurons is a critical phase of brain development. Defects in this process lead to severe ataxia, mental retardation, and seizures. In particular, migration of granule neurons in the cerebellum has been proposed to be tPA-dependent. In rodent, fibrinolytic activity of plasmin, which is converted from plasminogen by PAs, is relatively high at birth and declines during the third postnatal week in the granular layer of cerebellar tissue slices (Soreq and Miskin, 1981). PAs and plasmin are released by granule cells of 7-9 day-old rat cerebellum, but not by 1-month old or adult rat cerebellum (Krystosek and Seeds, 1981; Moonen *et al* 1982), suggesting that the PA-plasmin system participates in the inward migration of granule neurons, accounting for most (95%) of the cells in the adult cerebellum. In neural development at embryonic stages, tPA and uPA may be involved in different modes of neuronal growth (Sumi *et al* 1992).

Interestingly, in the case of granule cell migration, tPA has dominant effects compared to uPA. For instance, specific and potent inhibitors for tPA and plasmin inhibit the inward migration of external granular layer cells in rat organotypic suspension cultures of the 7-day paraflocculus (Moonen *et al* 1982). The molecular weight of the major band following electrophoretic separation (zymography) corresponds to tPA (Soreq and Miskin, 1981). Furthermore, an absence of tPA leads to retardation of granule cell migration. During the most active phase in the second postnatal week, migration rate is slower in mice lacking the tPA gene (tPA KO) compared to wild-type (WT) or urokinase KO mice (Seeds *et al* 1999).

The action of tPA and plasminogen on migration may further be aided by the perinatal expression of a secreted binding partner, amphoterin, which strongly enhances the rate of plasminogen activation and promotes the generation of surface-bound plasmin by tPA at the leading edge (Parkinnen and Rauvala, 1991; Merenmies *et al* 1991). In addition, process extension and migration in embryonic neurons is enhanced through amphoterin binding to receptor for advanced glycation end products (RAGE), a multiligand transmembrane receptor (Huttunen *et al* 2000).

2.2 Neurite Outgrowth and tPA

Nerve growth cones are motile structures located at the tips of growing neurites that facilitate neurite outgrowth and migration of neurons through the surrounding tissue. Interestingly, the activity of PAs is seen in a plasmin-dependent manner at the growth cone bearing neurites of neuroblastoma (N18, NG108) cells (Krystosek and Seeds, 1981b). The PA activity is also found in isolated growth cones from postnatal day-5 (P5) rat brain, and fibrin-agarose zymography clearly shows that tPA is the main PA associated with growth cone structure (Garcia-Rocha *et al* 1994). One might predict tPA to alter growth cone dynamics for neurite extension and/or retraction.

Indeed, the overall length and arborization of neurites are changed in PC 12 cells and cultured sympathetic neurons by inhibition of PAs or over-expression of tPA (Pittman *et al* 1989, Pittman and DiBenedetto, 1995). Neurite arbors are not only longer but also much straighter in sympathetic neurons cultured with serine protease inhibitors. It is, however, difficult to interpret these results in the context of the CNS *in vivo,* because various different conditions, such as basal content of endogenous proteases and inhibitors, extracellular environment or affinity for drugs, are probably all reflected in the results (reviewed in Pittman *et al* 1992). To know the absolute requirement of endogenous proteases for neurite outgrowth in particular brain regions, there are several advantages to utilizing genetically manipulated mice (eg. knockout mice; see Seeds *et al* 1999).

Recently, using this strategy, a critical *in vivo* role for tPA has become clear in hippocampal mossy fiber reorganization (Wu *et al* 2000). It is well established that mossy fiber sprouting is induced in adult animals by kainic acid (KA) injection into the amygdala (Ben-Ari and Represa, 1990). Although tPA KO mice exhibit no obvious abnormalities and undergo normal development, mossy fiber outgrowth after KA injection is decreased in the supragranular cell layer (GCL) and found as an aberrant band at the GCL border with the molecular layer.

Taken together, these results indicate that tPA accelerates morphological maturation in the developing cerebellum and the activity-dependent axonal rearrangement in adult hippocampus.

3. LEARNING & MEMORY AND tPA

Structural changes in synaptic connections appear with the formation of long-term memory and learning. Interestingly, tPA mRNA and protein expression is increased in rat Purkinje neurons after cerebellar motor learning (runway task) (Seeds *et al* 1995). Consistent with this result, disruption of the

tPA gene (tPA KO) or blocking tPA activity (by tPA-stop or PAI-I) during training dramatically impairs this learning, suggesting that proteolytic tPA activity may facilitate synaptic plasticity during motor learning (Seeds *et al* 2003).

Likewise, tPA mRNA expression is increased within 3-hours in mouse striatum by long-term depression (LTD), which occurs more frequently in WT (16 of 20 cells) compared to tPA KO mice (8 of 20cells) (Calabresi *et al* 2000). Furthermore, the performance on an active avoidance test, which is dependent upon the integrity of the striatum, is worse in tPA KO mice (Huang *et al* 1996; Calabresi *et al* 2000). Thus, tPA may play an important general role in motor control and learning in both the cerebellum and striatum.

Several investigators have also evaluated hippocampus-dependent learning in tPA KO mice using a variety of learning tasks as follows: (1) spatial learning is not defective (Barnes circular and Morris water mazes, Huang *et al* 1996; eight-arm radial maze, Horwood *et al* 2004), (2) habituation of object exploration is impaired (open radial maze; Calabresi *et al* 2000; Ammassari-Teule *et al* 2001), (3) step-down inhibitory avoidance learning is impaired at the first training session, in which tPA action requires a proteolytic step (Pawlak *et al* 2002), and (4) differential reinforcement of low rate of responding (DRL) performance is altered (Horwood *et al* 2001). Conversely, transgenic mice overexpressing tPA learn more rapidly the hidden platform water maze protocol (Morris) and the homing holeboard task (Madani *et al* 1999).

Stress causes neuronal remodeling in the amygdala. Some studies indicate that the basal level of anxiety (elevated-plus maze), locomotor activity and pain threshold (footshock) are similar between WT and tPA KO mice (Huang *et al* 1996, Pawlak *et al* 2002). In contrast, tPA KO mice spend less time freezing in the context test but more time freezing in the cue test (contextual and cue fear conditioning; Calabresi *et al* 2000; Ammassari-Teule *et al* 2001) while they have less stress-induced anxiety (elevated-plus maze) in a plasmin-independent manner (Pawlak *et al* 2003). Controversial results may reflect differences of genetic background, age, sex, breeding environments or testing apparatus.

4. REGULATION OF tPA RELEASE UNDER PHYSIOLOGICAL CONDITIONS

In 1993, tPA was identified, along with c-fos and zif 268 to be an immediate early gene in rat hippocampus (Qian *et al* 1993). The expression of tPA mRNA increases within 1 hour of intense neuronal activity through the NMDA receptor, such as convulsive seizures, kindling and long-term

potentiation (LTP). Increased tPA mRNA is also seen in rat visual cortex by injection of an exogenous noradrenaline precursor (Mataga *et al* 1996) and in mouse cortical cell cultures by BDNF exposure in a time- and dose-dependent manner (Fiumelli *et al* 1999). These results suggest that transcription of tPA is enhanced through the CRE promoter region (Holmberg *et al* 1995). Interestingly, the transcriptional binding factor for this promoter, CREB, is implicated in experience-dependent plasticity in the visual cortex (Mower *et al* 2002). The following evidence, however, indicates that tPA transcription is not always essential for the regulation of tPA release into the extracellular space.

In chromaffin cell culture including PC 12 cells, tPA is packaged into exocytic vesicles with catecholamines or neuropeptide Y and is released by chemical depolarization of the plasma membrane (60mM potassium chloride (high K^+) or 50mM tetraethylammonium (TEA) solution) (Gualandris *et al* 1996; Parmer *et al* 1997; Lochner *et al* 1998; Taraska *et al* 2003). The membrane depolarization induces tPA release through a Ca^{2+}-dependent pathway without affecting tPA gene expression (Gualandris *et al* 1996; Lochner *et al* 1998). Similar observations have been reported for the release of tPA from primary cultures of mouse cortical neuron (6 days in vitro from embryonic day 16-17), where extracellular tPA activity is stimulated by high K^+ or TEA independent of tPA transcription (Fiumelli *et al* 1999; Fernández-Monreal *et al* 2004). In contrast, BDNF selectively enhances the extracellular proteolytic activity of tPA and not uPA in cortical neurons by elevating tPA transcription. Additional tPA release is found upon combining a maximal dose of BDNF with high K^+ solution. Thus, the source of tPA release evoked by BDNF and high K^+ may differ, possibly reflecting de novo synthesis versus pre-packaging into synaptic vesicles, respectively.

5. EXPERIENCE-DEPENDENT PLASTICITY IN VISUAL CORTEX AND tPA

5.1 Classical Approach: Ocular Dominance (OD) Plasticity in Cat Visual Cortex

The change of ocular dominance (OD) distribution following monocular deprivation (MD) is one of the most well characterized models for experience-dependent plasticity accompanied by morphological rearrangement. The majority of cells in cat visual cortex respond to both eyes viewing a moving light slit as it passes through their receptive field

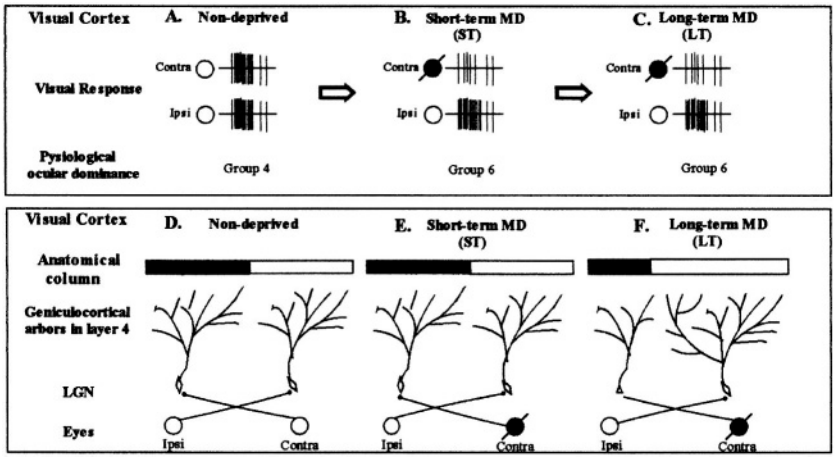

Figure 1: Ocular dominance plasticity is accompanied by morphological changes.
A~C, Typical neuronal response in the binocular zone of visual cortex by single unit recording in vivo. **A**, The majority of neurons normally respond to input from both eyes (open circles, contra- or ipsi-lateral eye to recorded hemisphere). **B and C**, After monocular deprivation during the critical period, physiological responses to the closed eye (closed circle) become weaker. Ocular dominance instead shifts toward the open eye as a result of this competitive plasticity (e.g. on a 7-point scale, neurons classified as group 4 in non-deprived animals shift to an ipsilateral-eye biased group 6 after brief contralateral eye occlusion).
D~F, Anatomical ocular dominance column structure in layer 4 of visual cortex. **D**, Visual inputs from individual eyes reach layer 4 via the lateral geniculate nucleus (LGN) and geniculocortical afferent terminations form alternating columns in the visual cortex (Shatz and Stryker, 1978; Hensch and Stryker, 2004). Column structure is periodic in layer 4 and territories serving each eye are typically equal. **E**, The arborization and bouton density of single LGN axons or overall anatomical column width are not changed by short-term monocular deprivation (Silver and Stryker, 1999). **F**, After long-term occlusion, loss of arbors and shrinkage of column size serving the closed eye are manifest (Antonini and Stryker, 1993; Antonini *et al* 1999).

(Hubel and Wiesel, 1962) (Figure 1A). In contrast, if an eye is deprived of vision in kittens, the closed eye becomes functionally blind as cells in the cortex lose their responsiveness to that input within days during a developmental stage called the 'critical' or 'sensitive period' (Wiesel and Hubel 1963; Hubel and Wiesel 1970) (Figure 1B-C). Neuronal connections are extremely plastic only at this time in life.

It is known that anatomical rearrangement of geniculo-cortical axon terminals eventually follow the more rapid, initial physiological shift of OD in favor of the open eye in kitten visual cortex (Antonini and Stryker, 1993; Trachtenberg *et al* 2000) (Figure 1D-F). Despite four decades of research

since this competitive process was first discovered (Wiesel and Hubel, 1963), the molecular and cellular mechanisms underlying OD plasticity still remain unclear. It is also largely unknown how the rapid physiological loss of responsiveness is coupled to axonal changes or why this plasticity declines with age.

The first neurochemical basis for OD plasticity was proposed to be noradrenaline (Kasamatsu and Pettigrew 1976; Kasamatsu 1991). The powerful technique of direct and continuous drug infusion into visual cortex using an Alzet osmotic minipump (Alza) concomitant with monocular deprivation was introduced (Pettigrew and Kasamatsu 1978, Kasamatsu and Schmidt 1997). Using a similar strategy, various neurotransmitters (glutamate, GABA, acetylcholine, serotonin), neuromodulators (BDNF, neurotrophin-4/5) and signal transduction molecules (protein kinase A, ERK, CREB) have been identified among the essential molecules for regulation of OD plasticity in cat visual cortex (reviewed in Gu 2002; Daw 2003; Hensch 2003, 2004). All of these molecules may be orchestrated for the full induction or maintenance of OD plasticity, but there are also undeniable technical limitations to interpreting their endogenous hierarchy based on a purely pharmacological approach (Kasamatsu *et al* 1998; reviewed in Gu 2002,).

5.2 Reduced OD Plasticity in Kitten Visual Vortex by tPA Inhibitors

As mentioned above, the loss of responsiveness to an eye deprived of vision is mediated by a rapid functional disconnection followed by anatomical rearrangement within kitten visual cortex (Antonini and Stryker 1993). Extracellular proteolysis is one mechanism with which to remodel neuronal circuits by degradation of extracellular matrix proteins or cell adhesion molecules. We, therefore, hypothesized that the protease tPA system acts to degrade cell surface receptors, cell adhesion molecules or other surface and extracellular factors, thus permitting morphological changes and synaptic remodeling dependent on visual experience.

Indeed, the MD effect is significantly suppressed in kitten visual cortex by blocking intracortical tPA function using an osmotic minipump (Alza model 2001, 1 μl/h for 1week from 4 weeks of age), containing an endogenous inhibitor of plasminogen activator (PAI-I) (hexapeptide for the reactive center of PAs, 100μM; Mataga *et al* 1996). Instead the expected clear shift of responsiveness toward the open eye is found in control hemispheres and control recordings from remote regions (about 5mm from the PAI-I infusion center). We confirmed that the expression of tPA but not uPA mRNA was detectable in control cat and rat visual cortex by Northern blot analysis (Mataga *et al* 1996).It is well known that PAI-I is an endogenous inhibitor for

not only tPA but also uPA. Unfortunately, it is difficult to determine specifically how much proteolytic activity of tPA is locally reduced by PAI-I infusion with limited sample sizes of cat tissue.

In contrast to our findings, Müller and Griesinger (1998) showed a proteolytic cascade consisting of plasmin generated by tPA may selectively mediate cortical plasticity induced by reverse-occlusion without affecting the initial MD response. In the reverse occlusion paradigm, after an initial period of MD, the eye that was originally deprived is re-opened and allowed normal vision, whereas the originally non-deprived eye is sutured (Hubel and Wiesel 1970; Hensch *et al* 1998a). After a few days of reversal, loss of function of the originally open eye and the complementary recovery of responses to the originally deprived eye can be attributed to anatomical changes, namely pruning and re-expansion of geniculocortical connections serving the two eyes, respectively (Antonini *et al* 1998). Müller and Griesinger applied several broad or specific protease inhibitors via osmotic minipumps and found that the initial OD shift by MD was intact following infusion of any inhibitor. In contrast, recovery of visual responsiveness (cortex) and morphology (LGN cell size) for the re-opened input was prevented by leupeptin (100mM, a variety of thiol- and serine-protease inhibitor), plasmin inhibitor (N-alpha-naphtyl sulfonyl-3-amidino-D,L-phenylalanyl-nipecotic acid thiophene-2-methyl-amide, 10 mM), or tPA inhibitor (2,7-bis-(4-amidinobenzyliden)-cycloheptan-1-one, 1mM or 10mM).

From these studies, it remained unclear whether tPA contributes selectively to recovery of lost input following reverse suture (Müller and Griesinger, 1998) or more generally to critical period plasticity (Mataga *et al* 1996). An obvious source of discrepancy lies in the different potencies of tPA inhibitors used, underscoring the drawbacks of the pharmacological approach.

5.3 Novel Approach to OD Plasticity using Transgenic Mice

Competition-based OD plasticity is well-established in cat visual cortex but also observed following sensory deprivation in ferrets (Issa *et al* 1999), rodents (Dräger 1978; Maffei *et al* 1992; Fagiolini *et al* 1994; Gordon and Stryker, 1996) and other mammals including humans (Berardi *et al* 2000; Daw 2003). In rodents, a binocular zone is localized to the lateral one-third of primary visual cortex (Figure 2A). Although the normal OD distribution is strongly biased toward the contralateral eye (Figure 2D), OD shifts can be induced reliably in mice with brief deprivations (>4-days), and this plasticity peaks during a well-defined critical period (around postnatal day, P24-28) (Figure 2B, 2C, and 2E). There are numerous similarities between mice and cats in competitive mechanism (Gordon and Stryker, 1996; Hensch *et al*

1998a), molecular basis (reviewed in Berardi *et al* 2003; Hübener 2003; Hensch 2003, 2004) and ultimately morphological plasticity of geniculocortical afferents during the critical period (Antonini *et al* 1999; Fig.2).

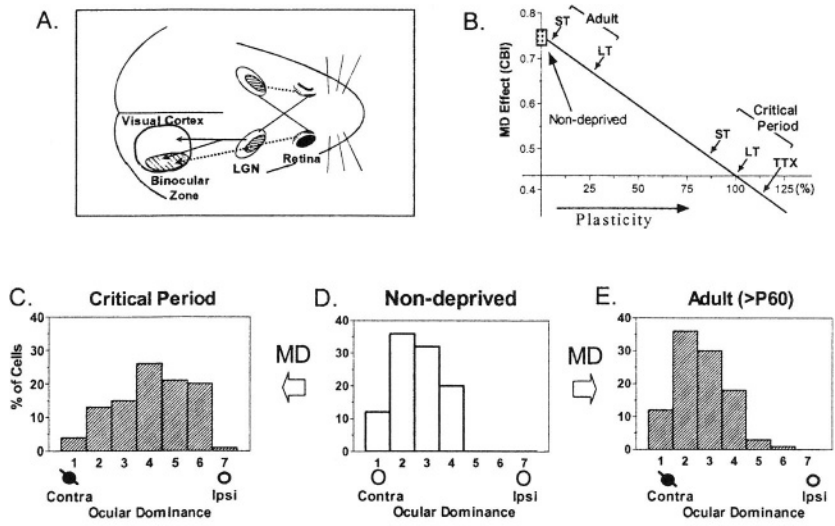

Figure 2: Experience-dependent plasticity in mouse visual cortex.
A, The visual pathway of the mouse. **B**, The level of plasticity can be estimated from ocular dominance distribution after monocular deprivation (MD) using the contralateral bias index (CBI; see Gordon and Stryker, 1996). MD effects in young and adult mice are indicated by arrows. Abbreviations: ST, short-term MD; LT, long-term MD. TTX, monocular tetrodotoxin injection. In this review, we refer to the effect of MD by long-term MD during the critical period as 100 percent plasticity. **C-E,** Typical ocular dominance histogram for 20-30 neurons recorded from the binocular zone in non-deprived (**D**), or monocularly-deprived mice (**C,** during the critical period; **E**, in adulthood). Group 1 and 7 cells are driven exclusively by one eye, group 4 cells are driven equally by both eyes, and groups 2, 3, 5, 6 are binocular intermediates. In the normal ocular dominance distribution, the majority of cells in rodents respond predominantly to the contralateral eye (groups 1-3) (**D**). In contrast, the OD histogram shifts toward the ipsilateral, open eye when the contralateral eye is previously closed during the critical period (**C**) when circuits are plastic, but not later in adulthood (**D**).

Since genetic, biochemical, immunohistochemical and electro-physiological approaches can now readily be performed in the mouse model, we have dissected the trigger cascade for functional OD plasticity and how these initial physiological changes couple to morphological refinements within the binocular zone of mouse visual cortex. We first identified a disruption of OD plasticity in mice lacking glutamic acid decarboxylase (GAD) 65 (Hensch *et al* 1998b). Growing experimental

evidence now supports that the critical period is initiated by appropriate maturation of intracortical ***inhibition*** through $GABA_A$ receptors, especially those containing the $\alpha 1$–subunit (Hensch *et al* 1998b; Huang *et al* 1999; Hanover *et al* 1999; Fagiolini and Hensch, 2000; Iwai *et al* 2003; Fagiolini *et al* 2004).

Interestingly, when synaptic inhibition is enhanced or reduced throughout the critical period in kitten visual cortex, the resultant anatomical OD 'column' spacing of alternating left and right eye axons is bi-directionally altered (Hensch and Stryker, 2004). This finding in the original cat model nicely confirms that a progression of events from GABA function to structural consolidation underlies experience-dependent plasticity (Hensch 2004). Until now, the molecular switch that converts physiological events to structural refinements has remained a mystery. Proteases such as tPA/plasmin may play a vital role in this link.

5.4 Developmental Regulation of tPA in Mouse Visual Cortex

In normal development, there are marked changes in the expression of many molecules in the visual cortex. For instance, mRNA and protein products of immediate early genes, such as egr-1/zif 268 and c-fos, dramatically increase after eye opening (Malaga *et al* 2001; reviewed in Kaczmarek and Chaudhuri, 1997). These gene expressions are regulated by visual activity at any age (Kaczmarek and Chaudhuri, 1997; Yamada *et al* 1999). In contrast, the expression pattern of other molecules, such as NMDA receptor subunits (Roberts and Ramoa, 1999; Quinlan *et al* 1999; Chen *et al* 2000; Erisir *et al* 2003), adrenergic receptors (Liu *et al* 1993) or protein kinase C (Jia *et al* 1995), are tightly correlated with the critical period for OD plasticity. To predict whether a molecule may participate in OD plasticity, dark-rearing experiments are used as a probe, because the critical period is retarded when animals are reared in complete darkness from birth (Cynader and Mitchell, 1980; Mower 1991; Fagiolini *et al* 1994; Fagiolini *et al* 2003; Iwai *et al* 2003).

We, therefore, examined the expression profile of tPA mRNA, protein and proteolytic activity at P8 (before eye opening), P18 (pre CP), P28 (during CP) and P60 (adult) in wild type (WT) mouse visual cortex (Mataga *et al* in revision). Under normal light-dark conditions, constitutive levels of tPA mRNA and protein in visual cortex are highest at P8 and gradually decrease into adulthood. There is no expression peak during the developmental CP. Similarly, proteolytic activity is highest before the CP and gradually decreases until adulthood, and there is no difference between normal and dark-reared adults, which state for plasticity is similar to P18. These results

suggest that tPA proteolytic activity may participate not only in OD plasticity but also in general cortical development requiring structural changes, such as cell migration and neurite outgrowth.

Since the basal expression profile of tPA mRNA, protein and its activity does not reflect expected OD plasticity levels, we assayed tPA regulation by brief MD in the binocular zone of mouse visual cortex (Gordon and Stryker 1996). A significant increase of tPA activity in both hemispheres is induced within two days after MD only in WT mice during the critical period and is maintained at these levels for at least four days (Mataga *et al* 2002), consistent with the saturating functional plasticity of brief MD. Interestingly, elevated tPA activity is initiated prior to morphological changes in thalamo-cortical axonal arbors or boutons (Antonini *et al* 1999; Silver and Stryker, 1999), indicating that tPA may participate in more rapid morphological refinement within cortex during the critical period (see section 8 below).

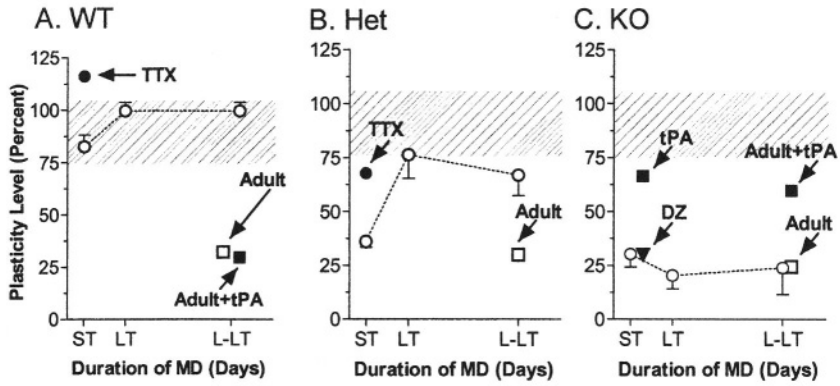

Figure 3: Ocular dominance plasticity in tPA mutant mice.
Using figure 2B as a reference, the level of plasticity is calculated by the contralateral bias index of MD effects in WT (**A**), tPA heterozygous (**B**) and tPA knockout (**C**) mice. Shaded regions indicates normal range of MD effects by monocular suture of WT mice during the critical period. Abbreviations: ST, short-term MD (4-days from P25 or P26); LT, long-term MD (2 weeks from P18); L-LT, extra long-term MD (over 7weeks from P18); TTX, monocular tetrodotoxin injections; DZ, short-term MD with diazepam injections (i.c.v.); tPA, short-term MD concomitant with exogenous tPA injections (i.c.v.) in young animal; Adult, long-term MD in adulthood (2 weeks from P60); Adult+tPA, long-term MD with exogenous tPA injections (i.c.v.) in adulthood.

5.5 OD Plasticity in tPA Knockout Mice

Mice bearing a tPA gene deficiency have been generated by Carmeliet *et al* (1994). These animals develop normally, are fertile and have a normal life

span. In addition, the visual cortex is similarly localized and exhibits equivalent OD histograms across genotypes in the binocular zone (Mataga *et al* 2002), suggesting the structural development of this region shows no gross abnormality in tPA knockout (KO) mice. Figure 3 shows a summary of MD effects in the tPA mutant by single-unit recording *in vivo,* measured against the level of plasticity by long-term MD in WT during the critical period. Plasticity was assayed in several ways: 1) short-term MD during the critical period (Figure 3, ST; 4days from P26), 2) long-term MD spanning the entire critical period (Figure 3, LT; 2weeks from P18), 3) extra-long term MD (Figure 3, L-LT; 6weeks from P18), 4) late-MD in adulthood (Adult, 2 weeks from P60), 5) MD concomitant with diazepam or exogenous tPA injections (i.c.v.) (Figure 3, DZ, tPA), and 6) monocular tetrodotoxin (TTX) injection to block both spontaneous and evoked activity during the critical period (4 days).

As expected, plasticity is high in WT during the critical period (Figure 3A, ST and LT, 75-100% of plasticity). The MD effect is enhanced by TTX injection for 4 days during the critical period compared to eyelid suture (Figure 3A, 130% of plasticity), similar to previous findings (Hensch *et al* 1998). In contrast, plasticity is strongly suppressed in tPA KO mice for all MD paradigms (Figure 3C, ST and LT, less than 25%). Critical period onset is not simply delayed in tPA KO mice, since extra-long MD produced no additional effects compared to MD during the critical period (Figure 3C, L-LT, 23%).To estimate the amount of tPA that might enable OD plasticity, two different 'rescue' experiments were further explored. Gene dosage was titrated in tPA heterozygous (Het) mice, or exogenous tPA injections were made directly in tPA KO mice prior to single-unit recording (Mataga *et al* 2002).

In tPA Het mice, the amount of tPA mRNA (2951±620 arbitrary units, 4 mice each), protein (8.6±1.3 ng/mg, 4 mice each) and proteolytic activity (0.24±0.01 IU/mg protein, 7 mice) are essentially half that of WT. Accordingly, plasticity in tPA Het mice is reduced to an intermediate level. The loss of responsiveness is not found by short-term MD during the critical period (Figure 3B, ST; 35% of plasticity) with no obvious rescue from KO levels (Figure 3C, ST). In contrast, after long-term MD, the OD shift is restored in tPA Het mice at a level similar to WT mice (Figure 3B, LT vs. 2A, LT). This long-term MD effect is significantly stronger than that of adult animals ($p<0.001$) (Figure 3B, LT vs. Adult) indicating normal closure of the critical period.

In contrast, OD plasticity is restored **at any age** to tPA KO mice by exogenous tPA injections (E6010, i.c.v.). In this paradigm, tPA protein level in the binocular zone reaches half that of WT levels but its activity is only one-tenth that of WT within 30 minutes after injection (Figure 3C, tPA, Adult+tPA). Taken together, these results indicate that tPA has a permissive

role for OD plasticity, since regulated release is not required. Moreover, an appropriate amount of tPA is necessary for the full induction and closure of the critical period, similar to our previous observations on the upstream GABA function (Fagiolini and Hensch, 2000; Iwai *et al* 2003).

5.6 Neuronal Activity in tPA KO Mouse Visual Cortex

In rat hippocampus, tPA was identified as an immediate early gene induced by seizures, kindling, and LTP (Qian *et al* 1993). As a further measure of neuronal activation, we previously quantified the expression of an immediate early gene egr-1/zif 268 as a sensory marker (Kaczmarek and Chaudhuri, 1997; Mataga *et al* 2001). In the visual cortex of WT animals, egr-1 mRNA is expressed constitutively in the light and decreased after dark-adaptation (5 days of dark-rearing during the critical period). A two-fold increase is induced by photo-stimulation (30 min., 1Hz, 20J) following dark-adaptation. There is no difference between WT and tPA KO mice in egr-1 gene regulation in the visual cortex across these three conditions (Mataga *et al* 2002), while the response to photo-stimulation is up-regulated when inhibition is suppressed in GAD65 KO mice (Hensch *et al* 1998b) (Table 1). In addition, GABA content is decreased in the visual cortex lacking GAD65 (WT; 1.76±0.15 vs. KO; 1.28±0.09 nmol/mg, $p<0.05$) but is normal in tPA KO mice during the critical period (WT; 1.87±0.26 vs. KO; 1.67±0.12 nmol/mg, n.s.) (Table 1). These results indicate that deletion of tPA does not directly modulate the excitatory-inhibitory balance that triggers plasticity in visual cortex (Hensch 2003).

6. MOLECULAR BASIS OF tPA ACTION IN OD PLASTICITY

6.1 Regulation of Extracellular tPA Activity for OD Plasticity

Visual cortical (OD) plasticity is thus disrupted in both GAD65 KO and tPA KO mice (Hensch *et al* 1998b; Fagiolini and Hensch, 2000; Mataga *et al* 2002; Iwai *et al* 2003) (Table 1). Interestingly, OD plasticity is rescued throughout life in GAD65 KO mice by enhancing GABAergic transmission with benzodiazepine agonists, which bind a subset of $GABA_A$ receptors (Hensch *et al* 1998; Fagiolini and Hensch, 2000; Iwai *et al* 2003; Fagiolini *et al* 2004). Consistent with this view, increased tPA activity by brief MD is not found in GAD65 KO mice and OD plasticity is partially rescued by

exogenous tPA injection (E6010 for 1 week, i.c.v.)(Mataga *et al* 2002) (Table 1). GABA agonists, such as diazepam, are conversely ineffective in tPA KO mice (Mataga *et al* 2002) (Figure 3C, DZ and Table 1). The plasticity remains low in tPA KO mice after MD concomitant with diazepam injection (1 week, i.c.v) (Figure 3C, 30% plasticity).

Taken together, these findings suggest that tPA release lies downstream of functional inhibitory-excitatory balance. In the absence of zymography with high spatial resolution, however, it remains technically difficult to examine where extracellular tPA activity is increased locally in the binocular zone by MD. Further experiments are needed concerning the molecular cascade of tPA release and the regulatory mechanism for tPA activity in the extracellular space under physiological conditions

Table 1. Phenotype comparison of tPA and GAD65 KO with pre-critical period WT mice.

Ages	Critical period (CP)		Pre CP
Phenotype	tPA KO	GAD65 KO	WT
MD Effects on OD (level of plasticity) :short-term MD (4 days)	±	-	-
:long-term MD (2 weeks)	-	++	n.a.
:tPA injection + MD (7days)	++	++	n.a.
:diazepam injection + MD (4days)	±	+++	++
Enhanced Activation (vs. WT during CP) :egr-1 expression	-	++	n.a.
:GABA/Glutamate	-	--	±
(% of the cells) :prolonged discharge	-	+++	++

Plastic response to MD is indicated as follows: -, no effect (0~25%); ±, slight effect (26~50%), ++, partial effect (51~75%); +++, full effect (76~100%). Degree of enhanced neuronal activity with respect to mature WT mice: --, dramatic decrease (~50%); ±, slight decrease; -, normal; ++ significant increase (~50%) +++; dramatic increase (~75%). Data adapted from our previous reports (Hensch *et al* 1998b; Fagiolini and Hensch, 2000; Mataga *et al* 2002; Iwai *et al* 2003; Fagiolini *et al* 2004).

6.2 Inhibitors for tPA in the Brain

The serine proteases with a physiological role in the brain may be regulated by serine protease inhibitors (Shiosaka and Yoshida 2000). The subtle balance between extracellular proteolytic and anti-proteolytic activities may be required for stability and refinement of local circuitry. One major

family of inhibitors is the serpins, which can bind to target serine proteases. Among these serpins, neuroserpin (Osterwalder *et al* 1996, 1998; Hastings *et al* 1997; Kruger *et al* 1997) and protease nexin-1 (PN-1, Lüthi *et al* 1997) are modulated by neuronal activity in the brain as tPA inhibitors.

Interestingly, neuroserpin, which is predominantly synthesized in neurons and secreted from the axon, is sensitive to light deprivation in rat visual cortex during the critical period (Wannier-Morino *et al* 2003). The expression level of neuroserpin mRNA is decreased in the binocular zone contralateral to the closed eye after long-term MD. Although neuroserpin disruption does not result in a disregulation of brain tPA proteolytic activity, efficient inhibition of tPA is confirmed in transgenic mice over-expressing neuroserpin in neurons under control of the Thy-1.2 promoter (Madani *et al* 2003). These results suggest that other inhibitors also regulate tPA activity or that compensation may occur in neuroserpin KO mice.

The major serpins in the fibrinolytic system, plasminogen activator inhibitors (PAI-1 and PAI-2), are undetectable under physiological conditions in the brain (Fiumelli *et al* 1999; Sallés and Strickland, 2002). In contrast, PAI-2 mRNA expression is induced by BDNF exposure at 6 days *in vitro* from El6-17 cortical neuron cultures, while neuroserpin and PN-1 are not affected (Fiumelli *et al* 1999). Several serpins may be differentially orchestrated for regulation of tPA proteolytic activity in the normal brain function, including OD plasticity.

6.3 A Membrane Binding Receptor for tPA

Under pathological conditions, it is clearly demonstrated that there is a plasminogen-independent pathway for tPA action through a membrane binding receptor, such as annexin II (Tsirka 1997; Tsirka *et al* 1997; Siao and Tsirka, 2002). Both tPA and plasminogen (Plg) KO mice are resistant to excitotoxin-mediated hippocampal neuronal degeneration. In contrast, microglial activation is attenuated only in tPA KO mice and can be rescued by inactivated tPA (S478A point mutation)((Tsirka 1997; Tsirka *et al* 1997) via annexin II, which is only expressed in microglia (Siao and Tsirka, 2002).

The tPA also binds a low-density lipoprotein receptor-related protein (LRP), which is a multifunctional endocytic receptor that requires neither the protease active site nor the presence of bioactive inhibitor (Bu *et al* 1992). The tPA-induced late-phase LTP is reduced by receptor-associated protein (RAP), which is an antagonist for ligand interactions with LRP (Zhuo *et al* 2000). Caution is required when using this drug, because RAP also acts as a substrate for calmodulin-dependent enzymes, such as calcineurin and CaM-Kinase II (Petersen *et al* 1996), which is already known to affect OD plasticity (Gordon *et al* 1996; Taha *et al* 2002).

Another candidate receptor for tPA in the brain is the tPA-high affinity binding site on cerebellar granule neurons (Verrall and Seeds, 1989) and PC12 cells (Pittman *et al* 1989). Although not yet well characterized, tPA may bind to these receptors for protection from inactivation by protease inhibitors (Verrall and Seeds, 1989). However, there is still no evidence whether tPA has specific receptors for intracellular signal transduction, which may regulate OD plasticity in the visual cortex

6.4 OD Plasticity in Plg KO Mice

It is therefore an important issue whether tPA acts primarily as a serine protease for plasminogen, since tPA could in principle bind a receptor without any proteolysis. Plasminogen is present in the brain and localized to the cortex and hippocampus of both neonatal and adult mice (Tsirka *et al.,* 1997; Basham and Seeds, 2001; Sallés and Stricklands, 2002). In cat experiments, OD plasticity is reduced by blocking tPA activity using a hexapeptide for the reactive center of tPA, suggesting that tPA may act as a protease. Furthermore in mouse experiments, the MD effect is significantly weakened in Plg KO (Carmeliet and Collen 1996) mouse visual cortex after short-term MD for 4 days ($p<0.001$ vs. WT) or 7 days ($p<0.01$ vs. WT) during the critical period, similar to the tPA KO phenotype (Mataga *et al* in revision). In contrast, plasticity in Plg KO mice is completely expressed after long-term MD for 14 days spanning the critical period (Mataga *et al* in revision) as observed in tPA Het mice (Figure 3B).

There are at least two possible interpretations why OD plasticity is restored in Plg KO mice by long-term MD (Fig 4). First, both tPA and plasmin may independently degrade extracellular matrix proteins or cell adhesion molecules (Sumi *et al* 1992) to refine local circuits by neuronal activity. In tPA KO mice, there are no active enzymes in the tPA-plasmin system (Figure 4, ①,) (similar to a double KO of tPA and plasmin), while tPA activity remains intact in Plg KO mice (Figure 4,) (single KO of plasmin). OD plasticity may be mediated by normal tPA proteolytic activity in plg KO mice but takes longer to degrade the relevant molecules. Second, tPA may yield both proteolytic actions and intracellular signal transduction through specific binding sites (see section 6.3 above; Figure 4,).

It is, however, still controversial whether tPA has its own intracellular signaling pathway independent of plasmin. For instance, phosphorylation of postsynaptic extracellular-regulated kinase (p-ERK 1/2) is increased within 5 min by stress in the amygdala of WT mice. These increases and stress-induced anxiety are absent from tPA KO but not Plg KO mice (Pawlak *et al* 2003). Conversely, tPA-plasmin, but not tPA alone, activates p-ERK 1/2

expression with NCAM degradation in hippocampal culture (Son *et al* 2002). There is, therefore, a discrepancy between these two reports as to how tPA enhances p-ERK 1/2 expression.

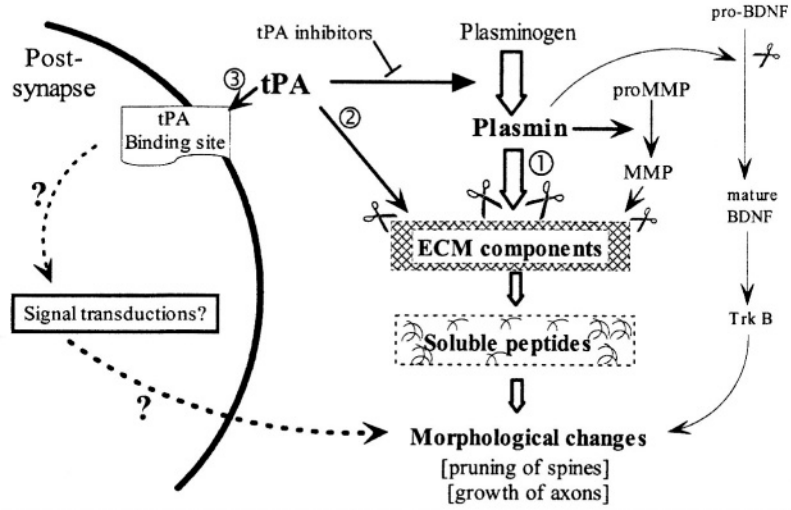

Figure 4: Candidate substrates for tPA action in experience-dependent circuit refinement. ① Main pathway through plasmin with proteolytic activities, extracellular matrix (ECM) components directly degraded by tPA, tPA binding to unknown receptors on the post-synaptic membrane to activate intracellular signal transduction pathways.

An alternative possibility for a plasmin-independent mechanism of tPA is the activation of NMDA receptor-mediated signal transduction. The NR1 subunit of NMDA receptors is cleaved by tPA, causing NMDA-evoked Ca^{2+}influx to increase in cultured cortical neurons, and consequently increasing neuronal cell death (Nicole *et al* 2001; Fernández-Monreal *et al* 2004). Again, this is a controversial finding whether both tPA and plasmin are necessary for degradation of NR1 (Matys and Strickland, 2003). Moreover, it remains unclear whether NR1 is modified under physiological conditions.

There is indirect evidence using an NMDA receptor antagonist that tPA may interact with NMDA receptors for learning and LTP in the hippocampus (operant DRL, Horwoods *et al* 2004; LTP, Neuhoff *et al* 1999) and LTP in the striatum (Centonze *et al* 2002). Prolonged NMDA currents *per se* by targeted deletion of the NR2A subunit reduces OD plasticity with a normal time course of the critical period (Fagiolini *et al* 2003). Rather than inducing neuronal cell death, our findings above show

tPA KO mice by exogenous tPA administration (at clinical doses for stroke patients).

6.5 Candidate substrates for the tPA-plasmin system in brain

In the peripheral non-neuronal system, the primary physiological substrates for tPA-plasmin system are fibrinogen and fibrin (Bugge *et al* 1996). In contrast, fibrin may not be the major substrate for plasmin in the brain. For instance, plasmin functions in neuronal cell death independently of fibrin clearance in the hippocampus (Tsirka *et al* 1997b). Growing experimental evidence suggests various substrates for the tPA-plasmin system in the hippocampus under pathological conditions, such as drug-induced seizure and neuronal cell death [e.g. laminin (Indyk *et al* 2003; Chen and Strickland, 1997; Nagai 1999), NCAM (Endo *et al* 1998, 1999; Hoffman *et al* 1998, 1998b) and proteoglycans (Wu *et al* 2000)]. It is still an open question whether tPA activity modulates p-ERK 1/2 expression and physiological NMDA receptor function via degradation of extracellular components, such as NCAM.

All of the above molecules may contribute to structural stability and synaptic plasticity in the normal brain (Benson *et al* 2000; Dityatev and Schachner, 2003). Among these, proteoglycans may have an interesting role in OD plasticity in the visual cortex. Several classes of chondroitin sulfate proteoglycans (CSPGs) are components of perineuronal nets (PNs), which are observed on interneurons and less frequently on pyramidal cells (Celio and Blümcke 1994; Celio *et al* 1998). Neuronal cell-surface CSPGs, including Cat-301, -315, -316 and neurocan, are first expressed relatively late in development. In the visual cortex, CSPGs emerge around the end of the critical period of synaptic plasticity (Schweizer *et al* 1993; Lander *et al* 1997; Pizzorusso *et al* 2002), suggesting perineuronal nets act as a terminator of OD plasticity. In fact, formation of these nets is retarded in visual cortex by dark-rearing (Guimarães *et al* 1990; Pizzorusso *et al* 2002). Moreover, OD plasticity is reactivated in adult rat visual cortex by chondroitinase-ABC, which can degrade multiple CSPGs (Pizzorusso *et al* 2002).

Our findings indicate that tPA proteolytic activity is decreased in the binocular zone with age. There is an inverse correlation between CSPG expression and dynamics of tPA activity in response to MD. However, PNs bound to wisteria floribunda agglutinin (WFA) appear normally in both adult WT and tPA KO mice, primarily in layer IV/V of the binocular zone (BZ) [47±4, 48±4 cells/field, 4 mice each]. Importantly, PNs were also found in tPA KO mice after 1 week of repeated tPA injection (i.c.v.) that restores sensitivity to MD in these animals [54±3 cells/field, 6KO+tPA]. Over half of

the parvalbumin-positive inhibitory interneurons are similarly enwrapped by PNs in all 3 groups [58±5%, 55±7%, 51±7%, 4WT, 4KO, 6KO+tPA, respectively] despite their differential plasticity levels (Mataga *et al* in preparation). It will be important to determine whether the individual molecular composition of PNs, especially neurocan and phosphcan, is also unchanged between WT and tPA KO mice, as these CSPGs are ligands for NCAM and may be target molecules for tPA (Wu *et al* 2000).

One action of PSA-NCAM could be to sensitize pyramidal neurons to BDNF, thereby modulating activity-dependent synaptic plasticity (Muller *et al* 2000). Late phases of LTP in the hippocampus are mediated by intact BDNF signaling (Patterson *et al* 1996; Korte *et al* 1998; Kovalchuk *et al* 2002). Intriguingly, pro-neurotrophins like BDNF and nerve growth factor (NGF) are directly cleaved by plasmin to become mature ligands at their respective Trk receptors, while the pro-form preferentially binds to the promiscuous p75 receptor (Lee *et al* 2001)(Figure 4). In the absence of tPA or plasminogen, pro-BDNF predominates and L-LTP is impaired unless exogenous mature BDNF is provided (P. Pang and B. Lu personal communication). In the visual cortex as well, excess BDNF enhances LTP at the expense of LTD (Akaneya *et al* 1996, 1997), although it does not block functional OD plasticity (Huang *et al* 1999). The secondary consequences of growth factors on spine proliferation and neurite outgrowth may very well contribute to late structural aspects of OD plasticity (see below; Figure 4).

Similar to pro-BDNF, tPA and plasmin also activate several pro-matrix metallo- proteinases (MMPs) to mature MMPs (Lo *et al* 2002; Lijen and Collen 2003). In the brain, some of these, such as brain specific MT5-MMP, MMP-2 and MMP-9 (Sekine-Aizawa 2001; Lo *et al* 2002), are expressed under physiological and pathological conditions. Since the tPA-plasmin system could link to MMPs (MMP-2 and -9) for neuronal remodeling after injury (Wang *et al* 2003), MMPs may lie down-stream of tPA-plasmin for synaptic plasticity (Figure 4).

7. NEURONAL PLASTICITY AND OTHER SERINE PROTEASES

Although tPA is the major serine protease in the brain, small amounts of other proteases are also detectable, such as neuropsin/brain serine protease 1 (BSP-1) (Shimizu *et al* 1998; Davies *et al* 1998; Shiosaka and Yoshida 2000), RNK-Met-1 (Davies *et al* 1998) and motopsin (Iijima *et al* 1999). Among these, neuropsin/BSP-1 is well characterized to associate with LTP and kindling epileptogenesis (Shiosaka and Yoshida 2000). This protease cleaves the synaptic adhesion molecule L1 (but not NCAM and laminin) in an

NMDA receptor-dependent manner (Matsumoto-Miyai *et al* 2003). Since NCAM and laminin are degraded by the tPA-plasmin axis, several proteases may share the modification of ECM components for local circuit refinement by neuronal activity.

8. MORPHOLOGICAL PLASTICITY AND TPA

It is well characterized that tPA is invoved in LTP of hippocampus and striatum *in vitro.* In the hippocampus, tPA mRNA expression is increased by LTP (Qian *et al* 1993), maintenance of late-phase LTP is impaired in tPA KO mice (Frey *et al* 1996; Huang *et al* 1996), application of tPA enhances late-phase LTP (Baranes *et al* 1998) and genetic over-expression of tPA facilitates LTP by a single tetanus train (Madani *et al* 1999). Plasmin is involved in the tPA action for LTP in hippocampus as a serine protease (Mizutani *et al* 1996, Nakagami *et al* 2000), suggesting that tPA and/or plasmin may induce activity-dependent structural changes at the synapse. Indeed, the number of perforated synapses is increased by drug-induced LTP, while this increase is blocked by tPA inhibitors (tPA-stop/PAI-I) (Neuhoff *et al* 1999).

Dendritic spines of pyramidal neurons in the cerebral cortex serve as preferential sites of excitatory synaptic connections and are pleomorphic. Two-photon uncaging of glutamate allows mapping of functional glutamate receptors at the level of the single synapse. Non-NMDA type (AMPA) receptors on the spines of CA1 pyramidal neurons are abundant in mushroom spines but sparse in filopodia, which may serve as the structural substrates of immature, 'silent' synapses (Matsuzaki *et al* 2001). Dendritic spines undergo activity-dependent structural remodelling that has been proposed to be a cellular basis of learning and memory (Yuste and Bonhoeffer 2001). Repetitive quantum-like photorelease (uncaging) of glutamate induces a rapid and selective enlargement of stimulated spines that is transient in large mushroom spines but persistent in small spines, individually following Hebb's postulate for learning (Matsuzaki *et al* 2004).

Thus, small spines appear to be preferential sites for LTP induction, whereas large spines might represent physical traces of long-term memory. Spine shape has further been shown to be highly dynamic by two-photon laser scanning microscopy in living transgenic mice expressing green fluorescent protein (GFP). In the visual cortex, motility of spines decreases with age, but it remains controversial whether rapid spine motility is related to critical period plasticity (Grutzendler *et al* 2002; Majewska and Sur, 2003; Konur and Yuste, 2004). We, therefore, hypothesized that morphological plasticity through the tPA-plasmin system may be initiated at the apical dendrites of pyramidal cells receiving convergent input from both eyes (Figure 5).

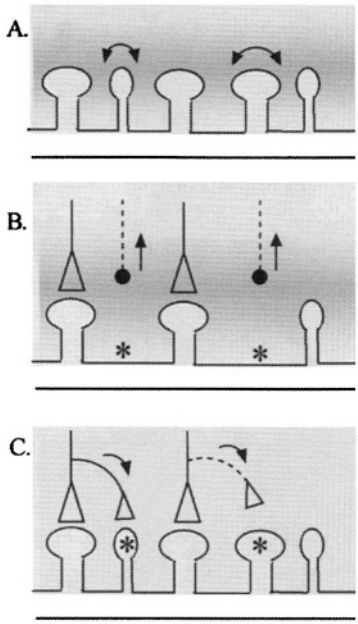

Figure 5: Model of morphological changes by sensory deprivation during the critical period **A,** (step 1) Shortly after monocular deprivation during the critical period, the motility of spines (two-headed arrow) is increased on apical dendrites of excitatory pyramidal neurons within a few days by increased tPA-plasmin proteolytic activities (grey rectangle). **B**, (step 2) With a similar time course to physiologically detectable plasticity (4-day MD; Gordon and Stryker, 1996), the total number of spines is transiently and significantly decreased due to their elimination (asterisks) and retraction of axons (Antonini and Stryker, 1993). Extracellular tPA-plasmin activity is still high. **C**, (step 3) After long-term MD, new spines emerge to receive synaptic input from sprouting open eye axons (triangles), including geniculocortical afferent arbors (Antonini and Stryker, 1993; Antonini *et al* 1999).

We propose three steps to complete morphological rearrangement during experience-dependent plasticity. First, tPA proteolytic activity is increased within 2 days by sensory deprivation (Mataga *et al* 2002). The increased tPA-plasmin degrades cell-adhesion molecules and ECM proteins (Figure 4). Consequently, spines become mobile (Figure 5A, step 1; activity-dependent motility) and are then eliminated along with axons serving the deprived input (Figure 5B, step 2; spine elimination/axon retraction). After synaptic pruning, axonal arbors receiving strong neuronal activity from the preserved input can migrate to free spaces along the dendrite (Figure 2B, asterisks) cleared away by tPA-plasmin. Our current findings support this rapid second step, as the total number of synaptic protrusions are decreased transiently but

significantly in WT mice but not in tPA KO mice by brief MD (4days) (Mataga *et al* submitted).

Finally, the territory representing the stronger sensory input is expanded (Figure 5C, step 3; axon growth/spine emergence). Cortical protein synthesis is required for OD plasticity (Taha and Stryker, 2002). Mature forms of growth factors such as BDNF activated by tPA-plasmin cleavage of their pro-forms could also play an important role here (Lee *et al* 2001). In the visual cortex, it is already known that retraction of deprived thalamic afferents precedes the expansion of open-eye arbors following prolonged MD (Antonini and Stryker, 1993; Antonini *et al* 1999). Thus, physiological plasticity, which is triggered by an appropriate excitatory-inhibitory balance (Hensch *et al* 1998b), may gradually be converted to morphological refinements through the multi-step proteolytic action of the tPA-plasmin system according to visual experience. It will be important to determine how broadly applicable such a model will be across brain regions (Hensch 2004).

9. CONCLUSIONS

Tissue-type plasminogen activator (tPA) is the major serine protease in the postnatal mammalian brain and has been implicated in several physiological functions, such as neuronal development, plasticity and learning & memory. Among these, the plasticity in visual cortex is an excellent model to probe the link from sensory experience to morphological change. Until now, there has been no molecule known to convert physiological events into structural refinements. The tPA/plasmin axis is ideally suited to play this vital role through its diverse proteolytic actions to enable normal cognitive function.

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Chapter 12

Proteases and Synaptic Activity

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1. INTRODUCTION

There is growing evidence that the proteolytic machinery has important roles in regulating synaptic activity in the central nervous system (CNS) (Hegde and DiAntonio 2002, Kaczmarek *et al* 2002; Pawlak and Strickland, 2002; Tomimatsu *et al* 2002). Under the physiological condition, proteases are tightly regulated to prevent the excessive proteolysis. Their proteolytic activities are regulated by at least three mechanisms: the gene transcription, the activation process of precursor forms, and the interaction with endogenous protease inhibitors. Once proteases are activated, they can exert irreversible cleavage of peptide bonds of various proteins. After cleavage, some substrates are inactivated and others are activated to gain new functions. Under the pathological conditions, activated microglia also release proteases to affect neuronal functions (Nakanishi 2003a, 2003b). Therefore, the precise knowledge about the regulatory mechanism of proteases and their target substrates is critical for the better understanding of their physiological and pathological roles in the CNS. Besides proteolysis, some proteases can also exert their functions by non-proteolytic mechanisms. It is becoming evident that proteolytic cascades have crucial roles in synaptic reactions under the physiological and pathological conditions.

2. TISSUE-TYPE PLASMINOGEN ACTIVATOR (tPA)

tPA is a serine protease that catalyzes the conversion of plasminogen into plasmin, which plays an important role in fibrinolysis. The activity of tPA is regulated by members of the serpine family including protease-nexin 1, neuroserpin and plasminogen activator inhibitor 1 (PAI-1). In the CNS, tPA has been reported to distribute discrete regions of the hippocampus, the hypothalamus, the amygdala and the mehingeal blood vessels (Sappino *et al* 1993; Sallés and Strickland, 2002). In the hippocampus, tPA immunoreactivity was found almost exclusively in the mossy fiber pathway. In the amygdala, tPA immunoreactivity was confined to the central and medial amygdala and was almost completely absent in the basal amygdala (Pawlak *et al* 2003). These distributions of tPA immunoreactivity correspond well with those of histological zymographic assay for tPA catalytic activity (Sappino *et al* 1993).

2.1 Plasminogen-Independent Role of tPA in Synaptic Plasticity and Seizure Activity

In 1993, Qian *et al* idendified tPA as a protein encoded by an immediate-early gene, which is induced during seizures, kindling and long-term potentiation (LTP). Since then, much attention has been paid for the function of tPA in synaptic plasticity and seizure activity. Huang *et al* (1996) utilized tPA-deficient mice to show that the late phase of LTP (L-LTP) in both the Schaffer collateral-CA1 and mossy fiber-CA3 synapses in the hippocampus was significantly impaired. On the other hand, tPA-overexpressing mice show an enhanced synaptic plasticity as evidenced by enhanced L-LTP and an improved performance in a classical protocol of spatial learning (Madani *et al* 1999). Furthermore, L-LTP in rat hippocampal slices was suppressed by specific inhibitors for tPA and enhanced by an application of tPA (Baranes *et al* 1998). Although these observations indicate that tPA plays a significant role in neuronal plasticity, the substrates for tPA remain to be determined. One possible substrate is plasminogen that is converted to the broad-spectrum protease plasmin by tPA. However, it has been reported that plasmin-mediated extracellular proteolysis rather impairs the maintenance of LTP by degrading laminin (Nakagami *et al* 2000). The low-density lipoprotein receptor-related protein (LRP), a cell surface receptor for tPA, is another candidate for tPA substrates. Zhuo *et al* (2000) have demonstrated that binding of tPA to LRP activates cyclic-AMP-dependent protein kinase (PKA), which plays a key role in L-LTP.

Besides the role in neuronal plasticity, tPA has been also implicated in the propagation of seizure activity. Yepes *et al* (2002) have reported that unilateral injection of kainate into the amygdala caused upregulation of tPA in the ipsilateral amygdala and hippocampus followed by seizures and hippocampal neuronal death. The propagation of seizure was attenuated in tPA-/- mice but not in plasminogen-/- mice. Furthermore, injection of neuroserpine, a neuron-specific serine protease inhibitor, into the ipsilateral to the amygdala injected with kainate attenuates the seizures. They have demonstrated that tPA is needed for seizure propagation by reorganizing synapses or forming new synaptic contacts through a plasminogen-independent mechanism. One potential substrate for tPA other than plasminogen is the NR1 subunit of the *N*-methyl-D-aspartate (NMDA) receptor. Nicole *et al* (2001) have demonstrated that tPA enhances NMDA receptor-mediated signalings by removing a fragment of approximately 15–20 kDa from the amino terminus of the NR1 subunit of the NMDA receptor. They used co-immunoprecipitation experiments to show an apparent direct association between tPA and the NR1 subunit of the NMDA receptor in membrane preparations from cortical neuronal cultures. The cleaved form of the NR1 was also detected by co-immunoprecipitation assays after exposure of cortical neurons to NMDA. However, as pointed out by Matys and Strickland (2003), possible involvement of plasminogen and its activated form, plasmin, in the degradation of the NR1 subunit cannot be totally ruled out.

2.2 Plasminogen-Dependent Role of tPA in Excitotoxic Neuronal Death

Strickland's group has conducted series of experiments to elucidate mechanism underlying tPA-mediated excitotoxin-induced degeneration in the hippocampus. They have first shown that mice deficient for tPA or plasminogen are resistant to seizure and neuronal death in the hippcampal CA1-CA3 subfields induced by excitotoxins (Tsirka *et al* 1995, 1997). They also examined effects of intra-hippocampal injection of protease inhibitors on kainate-induced neuronal degeneration in the hippocampus of the wild-type mice (Tsirka *et al* 1995, 1997). When tPA inhibitor-1 or α_2-antiplasmin was applied just prior to kainate administration, kainate-induced neuronal death was markedly attenuated. These observations strongly suggest that plasmin formed by tPA-mediated activation of plasminogen promotes neurodegeneration through degradation of extracellular matrix (ECM) proteins. Finally, Chen and Strickland (1997) have demonstrated that laminin is the target ECM protein for plasmin and its degradation is responsible for excitotoxic neuronal death.

Although hippocampal CA1 pyramidal cells are extremely sensitive to tPA-mediated excitotoxin-induced degeneration, the existence of tPA synthetic cells in the CA1 subfield is still controversial. Tsirka *et al* (1995, 1997) combined a variety of approaches to show that tPA is synthesized by both hippocampal neurons and the satellite microglia that overlie the neuronal cell layer. More recently, Siao *et al* (2003) have generated mice that exhibit restricted expression of tPA through introduction of tPA transgenes under the control of neuronal- or microglial-specific promoters into tPA-deficient mice. Using these animals, they observed that tPA, initially secreted from injured neurons, activates microglia at the site of injury. These activated microglia secrete additional tPA, which promotes ECM degradation leading to neurodegeneration by plasminogen-dependent mechanism. However, Sallés and Strickland (2002) have reported that the CA1 subfield is essentially devoid of detectable tPA protein and activity. Furthermore, they could not detect tPA in the CA1 subfield even after excitotoxic injury. They have proposed several possible reasons why CA1 pyramidal cells that are devoid of tPA are most sensitive to tPA-mediated excitotoxin-induced neurodegeneration: (1) CA1 pyramidal cells may express the undetectable-level tPA that is crucial for neuronal degeneration; (2) neuronal death is likely to be attributable to a confluence of many factors; (3) the presence of mossy fiber tPA may be required for neuronal death of CA1 pyramidal cells, possibly through feedback potentiation of electrical activity.

2.3 Non-Proteolytic Role of tPA in Microglial Activation

Tsirka and her colleagues have investigated the mechanism underlying the failure of microglial activation in tPA-deficient mice during excitotoxic neuronal death (Tsirka *et al* 1995, 1997). Rogove *et al* (1999) have found that infusion of catalytically inactive tPA into the hippocampus of tPA-deficient mice restored microglial activation but not neuronal death after injection of kainate, suggesting that tPA mediates microglial activation through some mechanisms other than the conversion of plasminogen into plasmin. More recently, Siao and Tsirka (2002) extended their finding by demonstrating that tPA mediates microglial activation through its finger domain, which most likely interacts with annexin II, a cell-surface receptor, to initiate an intracellular signaling cascade. Thus, tPA appears to associate with excitotoxic neuronal death through two pathways (Tsirka 2002; Pawlak and Strickland, 2002). The first is proteolytic pathway for the activation of plasminogen to plasmin that degrades plasmin leading to neuronal death. The second is non-proteolytic pathway for the activation of microglia that contribute to neurodegeneration through releasing neurotoxic molecules after cellular activation.

3. THROMBIN

Thrombin is a serine protease that is critical to blood coagulation by catalyzing the conversion of fibrinogen into fibrin and including platelet aggregation. Proteolytic activation of the G-protein-coupled protease-activated receptors by thrombin activates many parallel mechanisms of signal transduction. In the CNS, Dihanich *et al* (1991) have shown that prothrombin is expressed in the olfactory bulb, cortex, superior colliculus, inferior colliculus, corpus striatum, and thalamus. Although it is still not clear whether prothrombin is ultimately converted into thrombin within the CNS, blood-derived thrombin may also contribute to synaptic activity during blood-brain barrier breakdown.

Several studies have provided evidence for a role of thrombin in neuronal plasticity and synaptic activity. Komai *et al* (2000) have reported that serine proteases including thrombin with the concentration of nanomole orders enhance LTP in the Schaffer collateral-CA1 synapses of the hippocampus. Furthermore, Gingrich *et al* (2000) have shown multiple experimental paradigms to demonstrate that thrombin potentiates NMDA receptor-mediated responses in the hippocampal CA1 neurons. They have reported that hirudin, a selective thrombin antagonist, or D-Phe-Pro-Arg-chloromethylketone, an irreversible thrombin inhibitor, blocks the effects of thrombin. On the other hand, a peptide agonist (SFLLRN) that activates protease-activated receptor-1 (PAR-1) can mimic the effects of thrombin. PAR-1 is known to couple to the G_Q family of $G\alpha$-protein, which can stimulate phosphoinositide hydrolysis and activate intracellular protein kinases such as PKC. After activation, PKC contributes to the induction of LTP by reducing the voltage-dependent Mg2+ blockade of NMDA receptor-channels (Chen and Huang, 1992). Taken together, it is conceivable that thrombin potentiates NMDA receptor-mediated responses including LTP through proteolytic activation of PAR-1.

4. NEUROPSIN

Neuropsin is a trypsin-like serine protease exclusively expressed in the CNS, especially in the hippocampus and associated limbic structures. Komai *et al* (2000) examined effects of recombinant neuropsin on LTP in the Schaffer collateral-CA1 synapses of the hippocampus. They obtained a bell-shaped dose-dependent curve; low concentrations of recombinant neuropsin enhanced the magnitude of the early phase of LTP (E-LTP) whereas higher concentrations inhibited it. They also used neutralizing antibody and the in vivo continuous intraventricular infusion of an antisence oligonucleotide

against neuropsin to show that neuropsin plays a pivotal role in E-LTP. Neuropsin has been suggested to act as a regulatory molecule in E-LTP through degradation of fibronectin, a major ECM protein and a ligand for integrin that play an important role in the maintenance of LTP (Barh *et al* 1997; Shimizu *et al* 1998). By contrast, recent evidence obtained from neuropsin-deficient mice indicates that deficiency for neuropsin dose not affect LTP in the Schaffer collateral-CA1 synapses or spatial learning (Davies *et al* 2001). They also found that neuropsin-deficient mice exhibit an increased susceptibility for hyperexcitability, suggesting that neuropsin regulates seizure activity rather than neuronal plasticity.

5. PROTEASOME

Proteasome is a large multisubunit complex (26S proteosome) that comprise a 20S core particle that is the catalytic component, and two 19S regulatory complexes that are attached to either end of the catalytic core. Proteasome degrades the polyubiquitinated proteins to small peptides.

5.1 Ubiquitination and Proteasome-Mediated Degradation

The role of ubiuitin-proteasome cascade in synaptic plasticity was first observed during the course of experiments to elucidate the mechanism underlying the long-term facilitation (LTF), a model of learning and memory in *Aplysia.* Serotonin-induced LTF is dependent on persistent PKA activity. Furthermore, the ubiquitin-proteasome-mediated proteolysis of the regulatory subunit of PKA was found to be responsible for the persistent activation of PKA (Hedge *et al* 1997; Chain *et al* 1999). The ubiquitin-proteasome cascade is also required for synaptic plasticity in vertebrates. Lopez-Salon *et al* (2001) have shown that bilateral infusion of a specific proteasome inhibitor, lactacystin, into the CA1 subfield of the rat hippocampus caused complete retrograde amnesia for one-trial inhibitory avoidance learning.

More recently, Ehlers (2003) has found a robust turnover of total proteins in the postsynaptic density (PSD) in neurons that are accelerated by neuronal activity. Using biochemical approaches, he concluded that the ubiqutin-proteasome cascade is a primary mechanism for activity-dependent remodeling of the PSD. PSD proteins undergone selective activity-dependent ubiquitination are the multidomain scaffolding proteins Shank, GKAP, and AKAP79/150. Shank and GKAP act as adaptors that bind to each other and to a variety of proteins in the PSD, while AKAP79/150 anchors PKA and PP2B to complexes containing glutamate receptors and PSD-95 family

members. Selective removal of these multivalent adaptor proteins by activity-induced ubiquitination may cause a profound rearrangement of PSD proteins by enforcing some signaling pathways and destabilizaing others. These modifications, in turn, reciprocally alter synaptic signaling to the downstream of two major signal cascades, CREB (cyclic AMP response element binding protein) and ERK-MAPK (extracellular signal regulated kinase-MAP kinase). These observations indicate that the ubiquitin-proteosomal proteolytic system has an essential role of in activity-dependent reorganization of the postsynaptic apparatus that regulates the synaptic strength.

5.2 Ubiquitination and Endocytotic Degradation

Besides its role as a tag that marks protein for proteasome-mediated degradation, ubiquitin also work as a tag for endocytic degradation of membrane proteins (Craig 1998; Terrell *et al* 1998; Hicke 2001). Ubiquitinated receptors bind to epsins, which in turn interact with adopter proteins that are bound to clathrin-coated pits. Through the clathrin-mediated endocytosis, the ubiquitinated receptor are incorporated and degraded in the endosomal/ lysosomal system.

Several lines of evidence suggest that the levels of neurotransmitter receptors at synapses are regulated through ubiquitination and subsequent endocytic degradation. In *Xenopus* oocytes, α1 glycine receptors are polyubiquitinated in the plasma membrane upon agonistic stimulation (Buttner *et al* 2001). GLR-1, an α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionate (AMPA) receptor of nematodes is ubiquitinated *in vivo* and mutations in this protein that prevent ubiqutin conjugation result in increased accumulation of GLR-1 at postsynaptic sites (Burbea *et al* 2002). It has been also reported that Plic1, an ubiquitin-like protein, can bind to and stabilize the $GABA_A$ receptor (Bedford *et al* 2001). Plic1 may inhibit the ubiqutination and subsequent endocytic degradation of the $GABA_A$ receptor. It is thus reasonable to consider that the ubiquitin-mediated endocytic degradation has an important role in regulating synaptic activity in the CNS because the synaptic strength depends on the abundance of neurotransmitter receptors at the postsynaptic membrane.

6. μ–CALPAIN

Calpains are a family of calcium-dependent cysteine proteases and expressed as the precursor form that undergo autocatalytic processing to yield the mature form via a Ca^{2+}-dependent mechanism (in micromolar and millimolar concentrations for the μ-calpain and m-calpain, respectively). The

level of calpain activity is regulated by interaction with calpastain, a specific endogenous inhibitor. In the CNS, μ-calpain immunoreactivity is localized predominantly in soma and proximal dendrites of neurons (Hamakubo *et al* 1986; Fukuda *et al* 1990; Perlmutter *et al* 1990).

Lynch and Baudry (1984) have first suggested that tetanic stimulation causes an increase in postsynaptic Ca^{2+} concentration, which activates μ-calpain. Then μ-calpain cleaves fodrin, which allows glutamate receptors located deep in the postsynaptic membrane to move to the surface. Their hypothesis is based on the following findings: (1) calpain-mediated degradation of synaptic membrane proteins mimics LTP, (2) activation of NMDA receptors leads to the production of calpain-specific fodrin breakdown products, (3) calpain inhibitors blocks the induction of LTP. However, several experimental results are at variance with this model. It is now generally considered that μ-calpain activation is necessary for LTP formation in the mechanism other than the increase in glutamate receptors by fodrin cleavage. Although the precise pathway by which μ-calpain participates in the generation of LTP remains unclear, several molecules have been suggested as potential substrates for μ-calpain. It has been reported that μ-calpain is involved in limited proteolysis of protein kinase Cζ to form a second messenger-independent, constitutively active form which is necessary for the maintenance of LTP (Hrabetova and Sacktor, 1996). Recently, μ-calpain has been reported to directly induce proteolytic digestion of large N-terminal fragments of all three NR2 subunits (NR2A, NR2B and NR2C) and smaller C-terminal fragments of NR2A subunit of NMDA receptors (Guttmann *et al* 2001). Although the basic functional properties of NMDA receptor channels are not influenced by these cleavages, μ-calpain has been suggested to regulate localization, modulation by second messenger, or turnover of NMDA receptors. Furthermore, it has been demonstrated that μ-calpain degrades the glutamate receptor-interacting protein (GRIP) that has been proposed to function as an AMPA receptor-targeting and synaptic-stabilizing protein (Lu *et al* 2001). Thus it is conceivable that degradation of GRIP by μ-calpain eliminate GRIP-GluR2 interaction resulting in the structural and functional reorganization necessary for generation of LTP.

7. CASPASE-3

Caspases, designated as cysteine-dependent aspartate-specific proteases, are family of proteases with the characteristic that they cleave after aspartate residues in their substrates. Among caspase family, a great deal of data has shown that caspase-3 activation plays a central role in apoptosis to cleave various important cytoplasmic and nuclear proteins. Caspase-3 is synthesized

as precursor forms. Upon apoptotic stimulation, changes in mitochondria membrane permeability leads to release of mitochondrial intermembrane proteins including cytochrome *c*. Cytochrome *c* together with Apaf-1 and caspase-9 form the apoptosome mediating the proteolytic activation of caspase-3 in an ATP-dependent manner. The activation and activity of caspase-3 are regulated by Bcl-2 family and inhibitor of apoptosis proteins (IAPs), respectively.

Besides the function as the executioner of apoptosis, there is growing evidence that caspase-3 can be activated in dendrites and synapses in response to activation of glutamate receptors and may play roles in modulating neuronal activity and plasticity in the absence of cell death (Mattson and Duan, 1999; Chan and Mattson, 1999). Glanzner *et al* (2000) have demonstrated that caspase-3 directly cleaves the GluR1 subunit of the AMPA receptor. When caspase-3 was activated in response to apoptotic stimuli, AMPA channel activity recorded from cultured rat hippocampal neurons was selectively decreased (Lu *et al* 2002). These observations suggest roles for caspases in the modulation of neuronal excitability and plasticity. Gulyaeva *et al* (2003) used a cell-permeable specific inhibitor for caspase-3, *N*-benzyloxy-carbonyl-Asp-Glu-Val-Asp fluoromethylketone (z-DEVD-fmk), to show the involvement of caspase-3 in LTP of the Schaffer collateral-CA1 synapses of the hippocampus. After prolonged pre-incubation (e.g. 3.5–6.9 hr) with z-DEVD-fmk, the tetanus failed to induce LTP without affecting basal indices of synaptic plasticity and short-term plasticity (population spike amplitudes and paired pulse facilitation). One potential substrate for caspase-3 to promote the induction of LTP is calpastatin, an endogenous calpain inhibitor. Wang *et al* (1998) have shown that calpastatin was degraded by caspases during apoptosis, and caspase inhibitor could reduce calpastatin breakdown in culture cells. Thus, it is possible that caspase-3 also indirectly activates μ-calpain through degradation of calpastatin during LTP.

8. CATHEPSIN D

A group of proteases in the endosomal/lysosomal proteolytic system is designated as cathepsin that is derived from the Greek term meaning "to digest" (Willstätter and Bamann, 1929). Cathepsins are synthesized on membrane-bound ribosomes as *N*-glycosylated precursor forms and are transferred into the endoplasmic reticulum and later into the Golgi complex. During transport to the Golgi complex, pro-cathepsins acquire modification of their carbohydrate moieties, which includes the formation of the mannose 6-phosphate (M6P) residues. Following the binding to M6P-specific receptors (MPRs), the enzyme-receptor complexes exit the *trans*-Golgi network in

clathrin-coated vesicles and are transported to the late endosomes (Kornfeld 1992). Upon fusion with the late endosomes, the dissociation of ligands occurs. The delivery of pro-cathepsins to lysosome is accompanied by a series of proteolytic cleavages into their mature forms. In addition to an M6P-dependent targeting system, cathepsins can be also targeted to the lysosomes in an M6P-independent manner (Glickman and Kornfeld, 1993).

Cathepsin D (CD), a major intracellular aspartic protease, is widely but unevenly distributed in almost all neurons and glial cells as a lysosomal enzyme in the CNS (Nakanishi *et al* 1993; Nakanishi *et al* 1994). Recently, we have provided evidence that the loss of CD activity causes a novel type of lysosomal storage disease associated with massive neurodegeneration (Koike *et al* 2000, 2003; Nakanishi *et al* 2001; Nakanishi 2003c). The most striking feature found in CD-deficient (CD-/-) mice of the CNS was a profound storage of autophagosome/autolysosome-like bodies with part of the cytoplasm, granular osmiophilic deposits, and fingerprint profiles in the CNS neurons and retinal photoreceptor cells (Koike *et al* 2000, 2003). These neurons contained large autofluorescent bodies, indicating the accumulation of ceroid/lipofuscin in the lysosomal structures. These ceroid/lipofuscin loaded lysosome contained subunit *c* of mitochondrial F_1F_0-ATP synthase, a common storage material of neuronal ceroid lipofusinosis (NCL) except for the infantile form of NCL. Interestingly, however, the protein and activity levels of tripeptidyl peptidase I, whose deficiency causes late-infantile NCL, were rather increased in the brains of CD-/- mice (Koike *et al* 2000).

At the terminal stage, CD-/- mice manifested generalized seizure, a common clinical features in all childhood-onset forms of NCL. The mice began to manifest generalized seizure from approximately P20; they began to tremble and move on their tiptoes with a stiff tail. To elucidate the mechanism for generalized seizure in CD-/- mice, electrophysiological analyses were performed in the hippocampus, which has been implicated as being important in epileptogenesis. In the hippocampal slices prepared from CD-/- mice after P20, spontaneous burst discharges were recorded from both the CA1 and CA3 subfields (Fig.1 A). These spontaneous burst discharges recorded from the regions were well synchronized, indicating an epileptiform nature of the bursting. The stimulation of mossy fibers also induced burst-like activity in CA3 pyramidal cells (Fig. 1B). On the other hand, no spontaneous activity was recorded from hippocampal slices obtained from control littermates at P23. When a surgical cut was performed between the CA1 and CA3 subfields, spontaneous discharges in the CA3 subfield were unaffected, but those in the CA1 subfield were abolished. This indicates that spontaneous burst discharges in the CA1 subfield are driven by burstings in the CA3 subfield. Burst discharges in the CA3 subfield still remained after an additional cut was made between the CA3 subfield and the dentate gyrus. Furthermore, spontaneous burst discharges were inhibited by 3-dihydroxy-6-

nitro-7-sulfamoyl-benzo[f]quinoxaline (NBQX), a specific inhibitor for AMPA/kainate (KA), but not by D-2-amino-5-phosphonovaleric acid (APV), a specific inhibitor for NMDA. These observations strongly suggest that AMPA/KA receptor-mediated excessive excitatory circuitry in the hippocampal CA3 subfield is responsible for the synchronized spontaneous bursts, which may be directly involved in the generalize seizure in CD-/- mice.

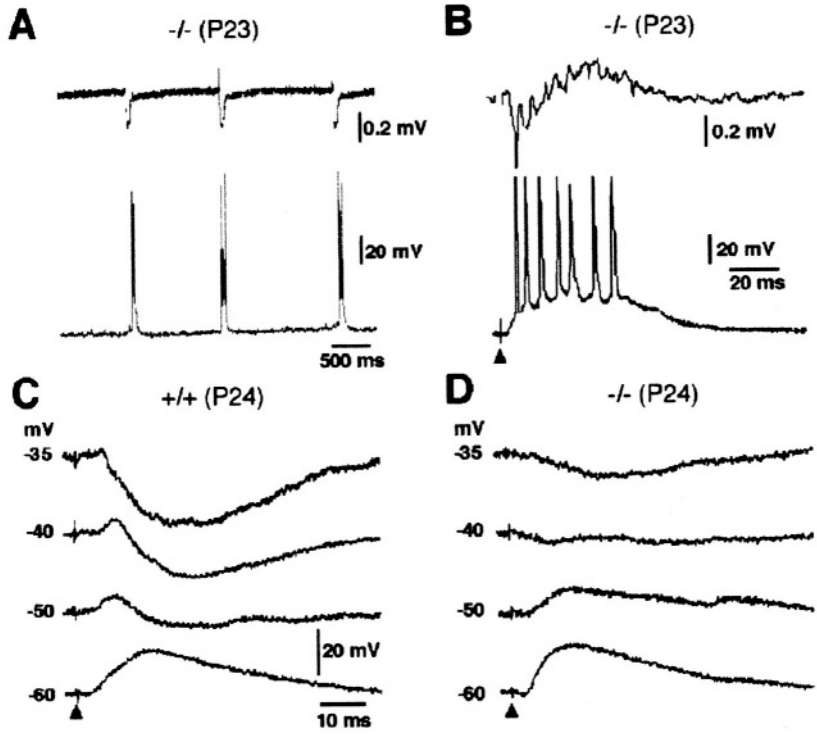

Figure 1: Spontaneous and evoked bursts of CA3 pyramidal cells in the hippcampal slice preparations from CD-/- mice. A: simultaneous extracellular and intracellular recordings of spontaneous bursts from the CA3 subfield of CD-/- mouse at P23. B: simultaneous extracellular and intracellular recordings of the mossy fiber-evoked burst responses from the CA3 subfield of CD-/- mouse at P23. C, D: synaptic responses evoked by the stimulation of the mossy fiber recorded from CA3 pyramidal cells in wild-type (C) and CD-/- mice (D) at P24. Synaptic responses were recorded at different four membrane potentials. It was noted that the amplitude of IPSP recorded from CA3 pyramidal cell of the CD-/- mouse was markedly attenuated as compared with that of the wild-type mouse.

We have attempted to elucidate a possible link between the deficiency for CD and the generation of epilepsy. One possible mechanism underlying the induction of the synchronized burst activity in the CA3 subfield is a disinhibition, because it is well documented that CA3 pyramidal cells generate synchronized burst discharges in the absence of GABA-mediated inhibition (Wong and Traub, 1983; Hablitz 1984). Intracellular recordings of postsynaptic potentials from CA3 pyramidal cells evoked by the stimulation of the mossy fibers revealed that the amplitude of fast inhibitory postsynaptic potentials (IPSPs) mediated by $GABA_A$ receptors recorded in CA3 pyramidal cells from CD-/- mice at P24 was significantly smaller than that of their wild-type lirtermates (Fig. 1C, D). It was also noted that the amplitude of slow IPSPs mediated by $GABA_B$ receptors was also markedly attenuated in *CD-/- mice.* Thus the disinhibitory mechanism contributes to the induction of pacemaker-like synchronized bursts in the CA3 subfield of the hippocampus.

It has been reported that high concentrations of reactive mitochondria were observed within somata and proximal dendrites of CA3 pyramidal cells and various interneurons (Kageyama and Wong-Riley, 1982), suggesting that CA3 pyramidal cells and interneurons are particularly metabolically active. It has been reported that hippcampal CA2/CA3 neurons shows sever ballooning due to accumulation of storage material in patients with Northern epilepsy (CLN8) (Haltia *et al* 2000). Furthermore, severe morphological changes and a loss of GABAergic interneurons are detected in various animal models of NCL including *mnd/mnd* mice (Cooper *et al* 1999), *Cln3*-deficient mice (Mitchison *et al* 1999), and the ovine NCL (Oswald *et al* 2000). The major component of neuronal storage material in most forms of NCL including CD-/- mice is subunit *c* of mitochondrial F_1F_0-ATP synthase, which is a common constituent of ceroid/lipofuscin. Moreover, parvalbumin (PV)-positive GABAergic interneurons were intensely immunostained with the anti-CD antibody in the hippocampus (Fig. 2A, B, arrows). Based on these observations, it is reasonable to speculate that CA3 pyramidal cells and interneurons are most severely affected due to the deficiency for CD. In fact, both ceroid/lipofuscin (Fig. 2C) and subunit *c* (Fig. 2D) are distributed especially in the CA3 subfield at P24. The ceroid/lipofuscin granules are considered to localize mainly in CA3 pyramidal cells because these autofluorescent granules are only occasionally found in PV-positive GABAergic interneurons (Fig. 2E, F; arrows). Furthermore, there is no significant difference in the number of PV-positive cells between CD-/- mice and their wild-type littermates. These results strongly suggest that the disinhibition in the hippocampus of CD-/- mice is not due to a loss of GABAergic interneurons.

Based on findings of morphological alterations and reduced mitochondrial oxidase activity, the mitochondrial dysfunction has been proposed to be the pathogenic mechanism for neuronal death in NCLs (March *et al* 1995; Jolly

et al 2002). March *et al* (1995) have demonstrated that defective recycling of subunit *c* ultimately leads to altered structural and abnormal senescence of existing mitochondria, suggesting that neurons affected by lysosomal storage have defects in energy metabolism. Therefore, the disinhibition in the hippocampus of CD-/- mice may be related with the mitochondrial dysfunction of CA3 pyramidal cell, because the reduction of ATP decreases the $GABA_A$ response in acutely dissociated hippocampal neurons (Stelzer *et al* 1988; Harata *et al* 1997). Further study will be necessary to elucidate the precise mechanism for the disinhibition in the hippocampus of CD-/- mice.

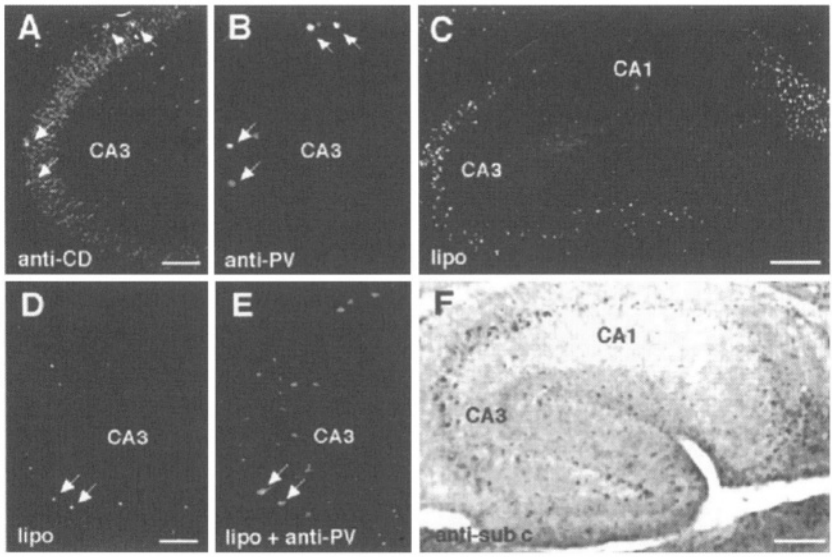

Figure 2: Distributions of cathepsin D (CD), ceroid/lipofuscin (lipo), and subunit *c* of mitochondrial F_1F_0-ATP synthase (sub c) in the hippocampus. A, B: immunohistochemical stainings of CD (A) and parvalbumin (PV) (B) in the same section from the wild-type mouse. Arrows show that intense granular immunoreactive products of CD were found especially in PV-positive cells. Bar in **A = 250μm.** C: autofluorescent lipo granules accumulated in the hippocampus of CD-/- at P24. **Bar = 500μm.** D, E: autofluorescent lipo granules (D) and immunohistochemical staining of PV (E) in the same section. Arrows show that lipo granules are only occasionally accumulated in PV-positive cells. Bar in **D = 250μm.** F: immunohistochemical staining of sub c in the hippocampus of CD-/- mouse at P24. **Bar = 500μm.**

9. MATRIX METALLOPROTEASES (MMPS)

MMPs are initially synthesized as the precursor form with Zn2+ of the catalytic domain bound to the cysteine residue of the propeptide region. Various factors can activate proMMPs through a conformational change that disrupts the cysteine-Zn^{2+} binding (cysteine switch) and leads to express the catalytic site. The intermediate form of MMPs is converted to the mature enzyme through autocatalytic cleavage of the prodomian. The enzymatic activities of MMPs are regulated by tissue inhibitiors of metalloproteases (TIMPs) that can tightly bind to MMPs. In the CNS, MMP-9 (gelatinase B) is synthesized by neurons. MMP-2 (gelatinase A) is secreted from glilal cells as precursor and converted into the active form extracellulary by plasmin or reactive oxygen radicals.

Recent evidence shows that MMPs have play an important role in neuronal plasticity by remodeling synaptic connections through proteolytic modification of ECM proteins. Szklarczyk *et al* (2002) found the unique expression pattern of MMP-9 induced by enhanced synaptic activity and limbic system reorganization. More recently, Reeves *et al* (2003) used the unilateral entorhinal cortical lesion model to show that intraventricular infusion of the MMP inhibitor, FN-439, blocked the deafferentation-induced sprouting in the dentate gyrus. These observations suggest that MMPs play a significant role in neuronal functions through synaptic reorganization. Although it is essential to identify their substrates for better understanding of the role of MMP in the synaptogenesis, typical MMP substrates including laminin and collagens apparently dose not distribute in the extracellular space between neurons. Some ECM proteins including integrins and dystroglycan have been suggested as physiological targets for MMP in the CNS (Kaczmarek *et al* 2002).

10. CONCLUSIONS

The precise knowledge about the regulatory mechanism of proteases and their target substrates is critical for the better understanding of their physiological and pathological roles in the CNS. Figure 3 summarizes in a schematic form of both extracellular and intracellular proteolytic cascades in the CNS. tPA, thrombin, neuropsin and MMP-9 contribute to neuronal plasticity and seizure activity through the extracellular proteolysis of ECM proteins (e.g. laminin, integrin, fibronectin) or cell surface receptors (e.g. NMDA receptors, PAR-1). On the other hand, proteasome, μ-calpain and caspase-3 play important roles in neuronal plasticity through the intracellular proteolysis of regulatory proteins of kinases (e.g. PKA, PKC), PSD proteins

or endogenous protease inhibitors (e.g. calpastatin). Furthermore, malfunction of CD, a typical aspartic lysosomal protease, may induce mitochondrial dysfunction, which leads to the attenuation of GABA-mediated inhibition. It is becoming evident that proteolytic cascades have crucial roles in synaptic reactions under the physiological and pathological conditions. The precise knowledge about proteolytic systems may also aid in developing pharmacological interventions for various disorders of the CNS.

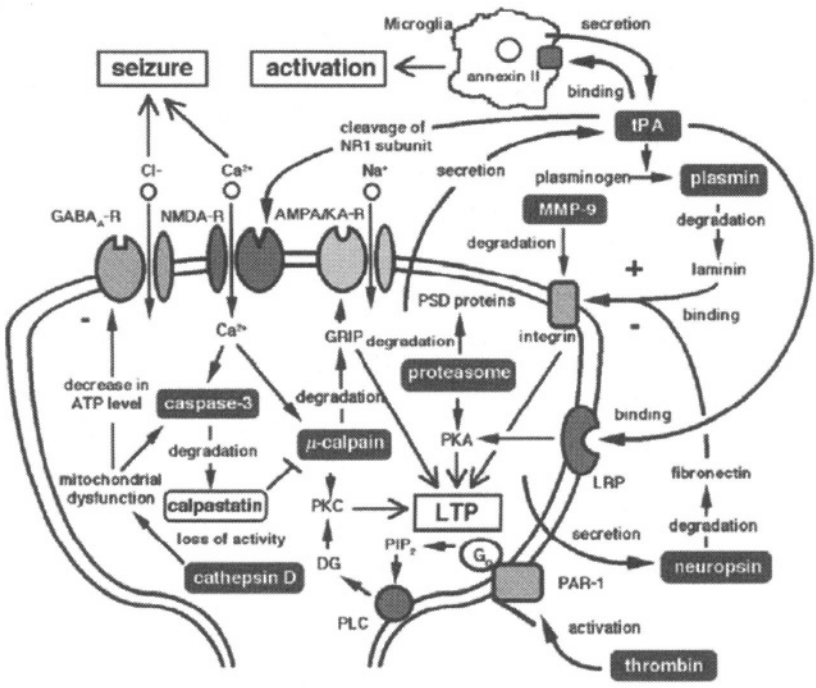

Figure 3: Schematic representation of proteases involved in neuronal plasticity and seizure activity. AMPA/KA: **α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionate/kainate,** DG: deacylglycerol, GRIP: glutamate receptor-interacting protein, LRP: low-density lipoprotein receptor-related protein, LTP: long-term potentiation, MMP-9: matrix metalloprotease-9, NMDA: *N*-methyl-D-aspartate, PAR-1: protease-activated receptor-1, **PIP_2:** phosphatydilinositol 4,5-bisphosphate, PLC: phospholipase C, PKA, camp-dependent kinase A, PKC: protein kinase C, PSD: postsynaptic density, tPA: tissue-type plasminogen activator.

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Chapter 13

Role of Proteinase-Activated Receptors in Brain Function

Thrombin signaling and its interacting players in physiologic and pathophysiologic processes

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1. INTRODUCTION

Thrombin is the ultimate serine protease in the coagulation cascade, and is known as coagulation Factor IIa (FIIa). However, over the last 2-3 decades thrombin's effects on cells, separate from its role in cleaving fibrinogen to fibrin and activation of FXIII (an inactive transglutaminase) to active FXIIIa, has attracted increasing attention. This field of research literally exploded with sequencing and cloning of the first thrombin receptor, now known as proteinase-activated receptor 1 (PAR1). With subsequent identification of other PAR family members and the development of pharmacologic tools to separate thrombin's action, PAR activating peptides (PARAPs), thrombin's effects on neural cells in development and disease of the nervous system are being intensively studied. In particular, the relationship between inflammation and coagulation and the nervous system is being elucidated through studies of thrombin, along with other serine proteases, actions.

One can list the various functions associated with thrombin and determine whether such functions involve one of the three known PARs it activates: PAR1, PAR3, PAR4. Although this review focuses on the PARs, it must be kept in mind that in addition to activation of PARs by a proteolytic mechanism, two peptide sequences that lie outside thrombin's catalytic domain and, thus, are apart from activation of its PARs, were also the attention of studies more than 20 years ago by Bar-Shavit and colleagues.

They found such domains elicited chemotactic responses from clonal and primary activated macrophages. This subsequently was identified as an RGD (Arg-Gly-Asp) sequence. Separately, Carney and colleagues characterized a non-catalytic sequence in thrombin they called thrombin receptor-activating peptide (TRAP-508). This terminology existed prior to the use of TRAP, referring to the new -NH2 sequence after thrombin cleavage of PAR1, subsequently re-named PAR1 active peptide or PAR1AP (see below). These non-catalytic actions of thrombin, in addition to its ability to catalyze the formation of fibrin, may contribute to the potential role of thrombin in the inflammatory response, increasing vascular permeability and promoting transendothelial cell migration of polymorphonuclear neutrophils (PMNs), which is coupled to at least two cellular responses to thrombin: enhancement of adhesion onto endothelial cells (ECs) by increasing cell-surface expression of P-selectin, and a chemotactic action on PMNs. However, it is clear that the initial cloning of the first thrombin receptor, PAR1, has dramatically accelerated knowledge regarding thrombin's cellular effects, and the inter-connectedness of coagulation and inflammatory pathways. This review focuses on the role of thrombin signaling through PARs 1,3 ad 4 in nervous system function and dysfunction and briefly addresses the non-thrombin PAR, PAR2, as well as interacting molecules with thrombin, its serpins and modulators. Excellent general reviews (Cottrell *et al* 2002; Coughlin 2000; Gabazza *et al* 2004; Macfarlane *et al* 2001; Ruf *et al* 2003; Schmidlin and Bunnett, 2001; Trejo 2003) as well as earlier reviews dealing with the CNS and PNS are available (Festoff 2003; Festoff *et al* 1996b, 2001; Hollenberg, 2002; Vergnolle *et al* 2001, 2003; Xi *et al* 2003).

2. THROMBIN: AN INDIGENOUS CNS AND PNS PROTEIN

More than 10 years ago Monard's group showed, using RT-PCR, that prothrombin was expressed in the rat brain in early embryonic development (Dihanich *et al* 1991). They found that expression of this thrombin precursor, previously thought to be specific to the liver, was not detectable after birth. Our group confirmed this and found that prothrombin was also expressed in skeletal muscle, and that both expression of message and active thrombin was detected maximally after birth (Citron *et al* 1997) during the period of elimination of polyneuronal synapses that characterizes both vertebrate and invertebrate CNS and PNS. We also found that prothrombin was expressed in the rat (and mouse) spinal cord, and that both message and protein, as well as active thrombin, were detected in the neonatal spinal cord up to postnatal day 30 (P30) (Citron *et al* 2000). Confirmation of the skeletal muscle expression

of prothrombin and thrombin generation was reported by others (Davenport *et al* 2000; Kim *et al* 1998), who also pursued its functioning in skeletal muscle development (Chevessier *et al* 2001) and disease (Mbebi *et al* 1999, 2001). Thrombin was suggested, based on an *in vitro* model of synapse elimination, to be the responsible protease for activity-dependent synapse elimination since it was inhibited both by protease nexin I (PNI), the tissue-based thrombin inhibitor, and recombinant hirudin, the leech thrombin-specific inhibitor (Liu *et al* 1994a, 1994b). With this information, we utilized an osmotic mini-pump releasing recombinant hirudin in P5 mice that was superfused over the soleus muscles resulting in a delay of elimination by 5 days (Zoubine *et al* 1996). This implicated the muscle-expressed thrombin and a model for the role of thrombin in elimination was proposed (Chang and Balice-Gordon, 1997). Subsequent studies showed this was mediated via activation of specific protein kinase C (PKC) isoforms through PAR1 activation (Faraut *et al* 2003; Lanuza *et al* 2000, 2001; Pindon *et al* 1998).

3. EXTRAVASATION, UPREGULATION AND ACTIVATION OF THROMBIN AFTER CNS INJURY

Prothrombin, as mentioned above, is primarily synthesized in the liver (Degen and Sun, 1998), and circulates in the blood at ~2 micromolar levels (Fenton 1986). Prothrombin is activated to thrombin, through the intrinsic or extrinsic coagulation cascade, by production of Factor Xa. The "prothrombinase complex" consists of Factors Va and Xa, along with calcium and phospholipids. Thus, prothrombin has been considered a potent and abundant early modulator to arrive at the CNS parenchyma after breaching of the blood-brain barrier (BBB) (Cunningham *et al* 1993). It has been estimated that 1 mL of whole blood can produce about 1–2 nmols of alpha thrombin after clotting. This has ramifications, certainly, for traumatic brain (TBI) and spinal cord (SCI) injuries that result in the entry of blood into these organs, including primary intracerebral and intraspinal hemorrhage, with the resultant and immediate production of large amounts of thrombin from cleavage of the prothrombin in the blood.

More than 20 years ago, several groups developed the thesis that serine proteases, such as plasminogen activators (PAs) and thrombin, were important mediators of neural function in the PNS and CNS (Festoff *et al* 1982; Hawkins and Seeds, 1986; Krystosek and Seeds, 1981; Means and Anderson, 1986; Monard *et al* 1983; Romstedt *et al* 1983; Snider 1986). As in the CNS, thrombin is expressed and activated in the PNS (Asfaha *et al* 2002; Bleuel *et al* 1995; Festoff *et al* 1996b; Friedmann *et al* 1999; Lee *et al*

1998; Niclou *et al* 1998; Smirnova *et al* 1996). We found that both prothrombin, as well as activated thrombin, were present within the injured rat spinal cord within 8 h after moderate contusion injury (Citron *et al* 2000). Given the fact that apoptosis occurs following this upregulation of thrombin, as well as PAR1, in the rat SCI model (Yong *et al* 1998), these results have implications for future treatment approaches for human SCI victims (Citron *et al* 2002).

Recently, the issues of prothrombin expression and its activation in the brain (Dihanich *et al* 1991) and in particular, in post-mitotic neurons have been re-visited. The prothrombin gene in mice, coagulation factor II (*cFII*), when inactivated leads to partial embryonic lethality with more than 50% of FII-/- embryos dying between embryonic days 9.5 and 11.5 (Degen and Sun, 1998; Sun *et al* 1998). This 50% embryonic lethality is similar to that found in mice null for PAR1 (c*FIIR*), as reported by the Coughlin (Connolly *et al* 1996; Connolly *et al* 1997) and the RWJPRI groups (Darrow *et al* 1996) and confirmed (Suo *et al* 2002). Transgenic liver-specific overexpression of human FII was sufficient to correct the phenotype of null mice and allow for their development and survival into adulthood (Sun *et al* 2002). Although liver-specific expression rescues them from this lethality it is not clear whether absence of prothrombin expression in brain is required for normal development and functioning.

Monard and colleagues recently generated transgenic mice expressing C-terminal hemagglutinin tagged human prothrombin in post-mitotic neurons (Sinnreich *et al* 2004). Both *in situ* hybridization (ISH) and immunohistochemical analysis (IHC) showed abundant and widespread cerebral expression of the FII (prothrombin) transgene in brain and post-mitotic neurons. However, transgenic prothrombin required added factors, such as snake toxin ecarin or blood components, for its activation to active alpha thrombin in amidolytic assays. This supports the previous finding that although prothrombin is found in the cerebrospinal fluid (CSF) (Lewczuk *et al* 1998, 1999; Smirnova *et al* 1997), derived either from brain and/or blood sources, active thrombin is not present. This suggests that potential function(s) of active thrombin in the adult CNS will depend on either circulating factors arriving from the blood via the BBB, or some endogenous factor that must be co-expressed, for activation. Such endogenous factors might, themselves, be upregulated after stroke, injury or other situations. Such findings lend support to the concept that in non-pathological states an as yet undiscovered thrombin activator, or another ligand, must activate thrombin receptors in the nervous system.

In this regard, the tissue prothrombinase enzyme, also known as fibrinogen-like protein 2 (*fgl-2*), first studied in association with mouse hepatitis virus (MHV-3) infection (Ding *et al* 1997, 1998), is of considerable interest. Fgl-2 is not detected constitutively but within 12-24 h of MHV-3

infection, massive amounts of *fgl-2* gene transcripts were detected in liver and spleen, and focally in kidney and brain. Recently, fgl-2 prothrombinase has been studied in the context of spontaneous abortion and proinflammatory cytokines and does not require MHV-3 infection (Clark *et al* 2001). It is also associated with vascular endothelial cell (EC) damage xenograft thrombosis associated with acute vascular rejection (Ghanekar *et al* 2004). There is no information on whether stroke, injury and/or degeneration upregulates fgl-2 in the brain or spinal cord.

4. THROMBIN AND HYPOXIA

Hypoxia inducible factor (HIF-1α) is a heterodimeric transcription factor (Semenza 2001; Semenza *et al* 2000) that regulates the expression of a number of genes containing hypoxic response elements (HRE) in their promoter regions (O'Rourke *et al* 1997), These include typical hypoxia-associated genes such as erythropoietin (EPO), heme oxygenase-1 (HO-1), thrombomodulin (TM) and serpins such as plasminogen activator inhibitor 1 (PAI-1) and possibly protease nexin I (PNI), as well. PAI-1 is also up-regulated by thrombin, as is PNI, and may be neuroprotective in situations of TBI or SCI, perhaps via its ability to inhibit thrombin and/or other serine proteases (Festoff *et al* 1996a, 1997; Ho *et al* 1994; Rao *et al* 1990). Thrombin has also been shown to increase HIF-1α under normoxic conditions (Festoff *et al* 2001; Gorlach *et al* 2001; Jiang *et al* 2002; Page *et al* 2002; Richard *et al* 2000) suggesting a role for thrombin in regulation of genes that are responsive to hypoxia in plasticity mechanisms. Both HIF-1 α and HO-1 have dual roles in reducing and enhancing injury responses in the CNS, as thrombin.

5. THE SERINE PROTEASE :SERPIN BALANCE AND THE CNS

In any biological situation, either physiological or pathological, the balance between serine proteases and their cognate inhibitors is critical to understanding the roles of each component (Travis and Bangalore, 1993). This applies extremely well to the family of inhibitors known as serpins (Carrell *et al* 1994; Potempa *et al* 1994; Travis and Bangalore, 1993). Figure 1 shows the relationship between a serine protease, such as thrombin, and its cognate serpin, such as PNI, in relationship to neurons and glia (modified from Knauer and Cunningham in fibroblasts). Through specific recognition domains on the pseudo-substrate nexin (Knauer *et al* 1983) serpin (S1) the

protease P1 domains bind and initiate cleavage at the serpin –COOH terminus with release of a 3-5 kDa fragment. Following conformational change, rapid closure of the serpin "hair-pin" loop causes irreversible inactivation of the protease by forming a covalent complex that resists boiling SDS (Carrell and Corral, 2004; Carrell *et al* 1994; Potempa *et al* 1994; Travis and Bangalore, 1993). The figure also depicts the mechanism for internalization of this protease:serpin complex, via a molecule now known to be the low density lipoprotein receptor related protein (LRP) (Conese *et al* 1995; Crisp *et al* 2000). Thus, any consideration of thrombin, or other serine proteases, in brain function and dysfunction, must also take into account not only PNI and other serpins and their regulation at transcriptional as well as translational and even post-translational levels, but LRP as well (Festoff 1990; Festoff *et al* 1996b, 2001).

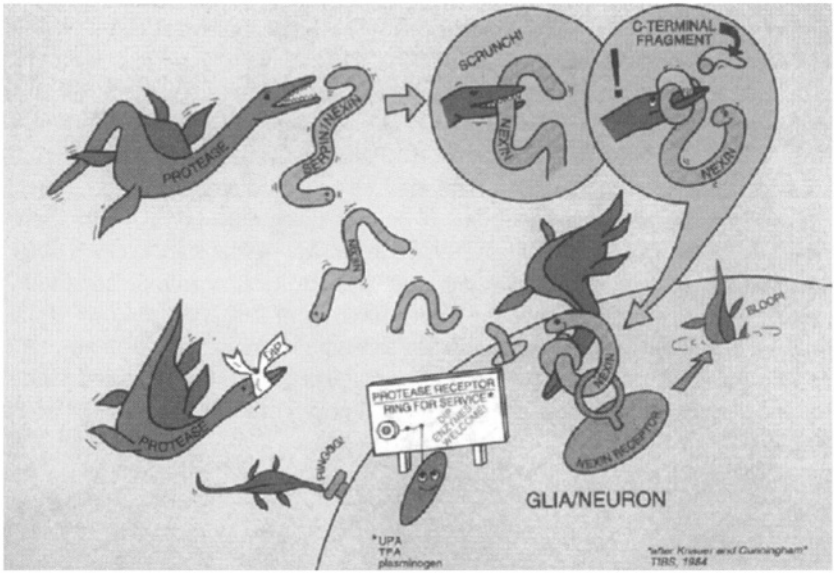

Figure 1: Schematic representation of serine protease interaction with serpin. In this case, the protease can be thrombin and the serpin protease nexin I (PNI). As adapted from various reviews by Knauer and Cunningham.

6. PROTEINASE ACTIVATED RECEPTORS (PARS) AND THE CNS

What must also be taken into account when serine proteases, particularly thrombin, are considered in the CNS is how such enzymes interact with cells. In fact, the last 10-12 years has seen an explosion of studies related to how

"thrombin talks to cells" (Coughlin 1999). Numerous excellent reviews on the mechanisms involved in recognition and activation of a novel family of G-protein coupled receptors (GPCRs) now known as proteinase-activated receptors (PARs) (Cottrell *et al* 2002; Derian *et al* 2002; Dugina *et al* 2002; Gabazza *et al* 2004; Hollenberg 2003; Mackie *et al* 2002; O'Brien *et al* 2001).

Quite early after the initial cloning of the first thrombin receptor (Vu *et al* 1991), now PAR1, studies of thrombin signaling in neural cells and the localization of PARs in the CNS began. Astrocyte phenotypic changes in culture when grown in the presence or absence of serum provided a convenient means for assessing the role of thrombin. In fact, thrombin can replace serum in rapidly causing the loss of processes ("stellation") of astrocytes seen when such brain glia are grown in serum (Cavanaugh *et al* 1990). This reversal of stellation is mediated via PAR1 since the pharmacologic application of PAR1 active peptides (PAR1APs), previously known as TRAPs, mimicked thrombin's effects (Beecher *et al* 1994).

Similarly, mechanistic studies to investigate the effects of thrombin on various neuronal cells: rapid retraction of neurites, cell rounding and neurotoxic effects, began focusing on PAR receptor mediation (Jalink *et al* 1993; Jalink and Moolenaar, 1992; Monard 1993; Suidan *et al* 1992), were undertaken. Furthermore, using ISH and 1HC, efforts to characterize thrombin receptors in the nervous system also appeared (Festoff *et al* 1996b; Niclou *et al* 1994, 1998; Weinstein *et al* 1995). It became clear that thrombin's effects were dose-dependent: at high nanomolar concentrations thrombin induced injury signals in neurons (Brewer 1996; Debeir *et al* 1996; Smirnova *et al* 1998a,b; Turgeon *et al* 1998, 1999) while at low concentration it appeared to induce neuroprotection (Pike *et al* 1996; Vaughan *et al* 1995; Wang and Reiser, 2003; Xi *et al* 2003). Injury at high nM was associated with apoptosis and caspases activation in susceptible cells (Donovan *et al* 1997; Smirnova *et al* 1998a, 1998b; Turgeon *et al* 1998). However, both protection and toxicity appeared to involve activation of the small GTPase Rho (Donovan and Cunningham, 1998; Smirnova *et al* 2001). Thus, in summary, thrombin acting on thrombin receptors and activating multi-signal transduction cascades has dichotomous effects on neural cells.

More recently, other PARs have been explored in peripheral tissues as well as in brain and spinal cord. As indicated in Figure 2, PAR2 is the only known PAR that does not respond to thrombin, having trypsin or a similar protease as the native ligand (Cocks and Moffatt, 2000; Cottrell *et al* 2002; Hollenberg 2003; Macfarlane *et al* 2001; Trejo 2003). The majority of studies on PAR2 and the nervous system have come from the PNS and gastrointestinal nervous system (Gao *et al* 2002; Vergnolle 2003; Vergnolle *et al* 2001, 2003). However, several studies have focused on the hippocampus (Fang *et al* 2003; Olejar *et al* 2002; Pompili *et al* 2004; Smith-Swintosky *et*

al 1997). These have indicated an association of PAR2 in ischemia, toxic or other injury situations.

From the periphery, a wealth of information suggests that PAR2 is involved in neuroinflammation and pain. Using chemical toxicants, Vergnolle and colleagues found that PAR2 was involved in hyperalgesia while PAR1 produced analgesia (Asfaha *et al* 2002; Vergnolle 2001, 2003). PAR2 responds to trypsin and other trypsin-like proteases while the physiologic protease in many situations, especially those that are inflammatory, is likely to be mast cell tryptase (Cocks and Moffatt, 2000; Cottrell *et al* 2002; Hollenberg 2003; Macfarlane *et al* 2001; Trejo 2003). Clearly, more studies of PAR2's roles in CNS function, and especially in neuroinflammtion, are required.

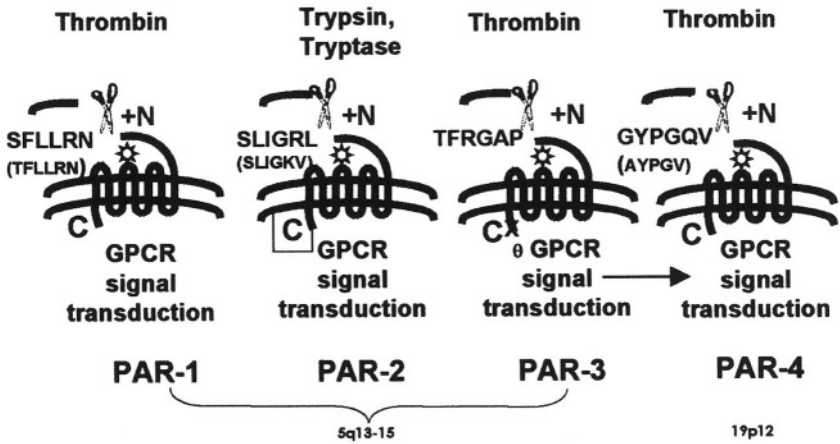

Figure 2: The four known proteinase-activated receptors (PARs). The PARs are typical heptahelical GPCRs except for the novel mechanism of proteolytic cleavage activation of the asparagine-glycosylated extracellular domain. The peptide sequence is specific, after proteolytic cleavage, for each. These PARAPs are used as pharmacologic tools to decipher respective PAR functions. The sequences in parentheses represent more specific PARAPs than the native new –NH2 sequence. The genes for PARs 1-3 constitute a gene cluster on human chromosome 5q13-15, while PAR4 is located on chromosome 19p12. Note that PAR3 does not signal, as its –COOH is inactivated, at least in endothelial cells.

7. THE DICHOTOMY OF THROMBIN'S EFFECTS IN NEURAL CELLS

Until quite recently this represented a conundrum as far as thrombin's actions on neural cells. Since both low (neuroprotective) and high

(neurotoxic) nM thrombin appeared to operate through Rho GTPase activation, this seemingly paradoxical situation and was unsolvable. Most of the studies involved only PAR1, although it was clear that PAR4, at least in platelets and ECs, responded to higher concentrations of thrombin (Asokananthan *et al* 2002; D'Andrea *et al* 2003; Dubois *et al* 2004; Fujiwara *et al* 2004; Henriksen and Hanks, 2002; Kaufmann *et al* 2000; Neaud *et al* 2004; Sabri *et al* 2003). Consequently, once neurons and astrocytes were shown not only to express PAR1 and respond to thrombin, microglia were evaluated for responsive to thrombin in terms of activation (Moller *et al* 2000; Ryu *et al* 2000) and whether nitric oxide (NO), inducible NO synthase (iNOS) or various cytokines such as tumor necrosis factor alpha (TNF-α) or others were measured, thrombin potently activated these brain macrophages. However, when the PAR1AP TRAP was used, activation did not occur despite very high concentrations and these authors concluded microglial activation did not involve PAR1 (Ryu *et al* 2000). In this regard, thrombin induces both proliferation and activation of microglia when injected into rat brain (Xi *et al* 2000, 2001). Therefore, we hypothesized that in microglia thrombin's dichotomy might be explained by differential activation of PAR1 and PAR4. We found, confirming other reports, that PAR1 although participating, did not significantly cause increase in TNF-α release using PAR1 knockout mice (Suo *et al* 2002). However, it was significantly involved in microglial proliferation. On the other hand, once finding that PAR4 was expressed in microglia, we found that it was responsible for thrombin's potent activation (by TNF-α release) of these cells (Suo *et al* 2003a). The mechanism involved was persistent activation of mitogen-activated protein kinase (MAPK) ERK1/2 (Suo *et al* 2003 a). In contrast, although PAR1 also activated ERK1/2, it was transient (Suo *et al* 2002). The persistent MAPK 44/42 activation was responsible for phosphorylation and action of NFκB in these cells, which in turn caused upregulation of TNF-α. Using anti-sense oligonucleotides (ODNs), PAR was down-regulated in microglia and this decreased TNF-α production in a dose-dependent fashion, while the sense ODN had no effect (Suo *et al* 2003a). When thrombin, PAR1AP and PAR4AP were compared for their relative capacity to cause microgliosis and activation in vivo, we injected these separately into rat brain and found the most robust effect was due to PAR4AP, more than thrombin and much more than PAR1AP (Suo *et al* 2003a). Subsequently, all PARs were found to be expressed in mouse microglia (Balcaitis *et al* 2003). Recently, further establishing the link with cytokines, thrombin was shown to upregulate suppressor of cytokine signaling 3 (SOCS3) in microglia through the PKC-δ isoform (Yang *et al* 2004) suggesting this occurred via PAR activation.

This approach has now been applied to neurons, as well, using aggregation of microtubule-associated tau in murine hippocampal neuronal cells exposed to thrombin or these PARAPs (Suo *et al* 2003b). These studies

indicated that PAR4AP, to a much greater extent than PAR1AP, mimicked thrombin's action in causing hyperphosphorylation and aggregation of tau. Again, prolonged MAPK 44/42 activation occurred with the PAR4AP, which resulted in neurofibrillary-like tangles (NFTs) that had β-sheet properties and a specific MAPK inhibitor inhibited both.

Thus, the dichotomy of thrombin's effects, both protective and toxic, on neural cells may relate to the dose-dependent activation of first PAR1 and then PAR4. This, in turn, results in either transient or persistent MAPK activation, with subsequent effects on gene transcription for cytokines and other molecules.

This may impact on disorders of the CNS, both traumatic and degenerative. As was mentioned above, both prothrombin, as well as activated thrombin, were present within the injured rat spinal cord within 8 h after moderate contusion injury (Citron *et al* 2000). Apoptosis occurs in 3 compartments between 8 and 72 h after injury in the rat SCI model (Yong *et al* 1998), after this upregulation of thrombin, as well as PAR1. In a neurodegenerative model, the spontaneous autosomal recessive *wobbler* mouse, increased thrombin is released from spinal cord astrocytes and an 8-fold increase PAR1 is present in large anterior horn alpha motor neurons (Festoff *et al* 2000). Since motor neurons are exquisitely sensitive to the effects of thrombin, and PARAPs (Smirnova *et al* 1998a,b), and can be modulated by downstream effectors (Smirnova *et al* 2001) these results have implications for future treatment approaches both for neurodegenerative diseases (Festoff 1997; Festoff *et al* 2003) and for human SCI victims (Citron *et al* 2002; Festoff,2003) as well.

8. THROMBIN AND NEUROINFLAMMATION: THROMBIN SIGNALING, FIBRIN AND THROMBOMODULIN IN THE CNS

The extravasation of blood into an otherwise healthy CNS leads to the development of inflammatory lesions of the cerebral vasculature, and include: 1) neurogenic inflammation due to massive release of sensory nerve neuropeptides; 2) hemoglobin from lysed erythrocytes, which creates functional lesions of endothelial and smooth muscle cells; 3) activity, expression and metabolites of lipoxygenase, cyclooxygenase (COX) and nitric oxide synthase (NOS); 4) the possible role of endothelin-1 as a pro-inflammatory agent; 5) serotonin, histamine and bradykinin which are especially involved in blood-brain barrier disruption; 6) the multiple actions of activated platelets, including platelet-derived growth factor production; 7) the presence of perivascular and intramural macrophages and granulocytes

and their interaction with adhesion molecules; and 8) the evolution, origins, and effects of pro-inflammatory cytokines, especially IL-1, TNF-alpha and IL-6. However, perhaps central to many or all of these is the prothrombotic and pro-inflammatory action of both thrombin and complement towards the endothelium.

A key feature in this interaction and intersection of coagulation and inflammation connections is the deposition of fibrin, an integral feature of the inflammatory response to a myriad of stimuli that include thermal, chemical and physical trauma. Fibrin deposition requires activation of the coagulation system and the generation of thrombin at sites of inflammation (as shown schematically in Figure 3). Thrombin catalyzes both the conversion of fibrinogen to fibrin, as well as activation of the transglutaminase (TGase) Factor XIII to FXIIIa, which in turn cross-links fibrin into the thrombus. At the same time, FXIIIa, as does a related member of the TGase family, tissue TGase (tTG), activates cytosolic phospholipase A2 (cPLA2) that is required for the synthesis of arachidonic acid, critical to inflammatory signaling.

Traditionally viewed as a molecular bridge between cells, fibrin(ogen) is excluded from the CNS by the BBB and is not synthesized within it. Recent studies have revealed signaling properties of fibrin(ogen), either as monomer or in its polymerized, cross-linked form. As mentioned above, thrombin-activated FXIIIa is responsible for cross-linking the thrombin-cleaved fibrin from fibrinogen. Both interact with cell surface receptors (Fig. 2), which in turn activate protein kinase cascades as well as Rho GTPases. CNS diseases associated with disruption of the BBB are clearly associated with fibrin deposition. The process of neuroinflammation is associated with conditions suitable for fibrin clot formation, and was appreciated 20 years ago in the context of multiple sclerosis (MS) and experimental allergic encephalomyelitis (EAE) (Paterson, 1982; Paterson *et al* 1987). The role of fibrin in neuroinflammatory demyelination has recently been elegantly explored using novel mice depleted for fibrin production in tumor necrosis factor transgenic mouse models of MS (Akassoglou *et al* 2004). Whether they used fibrinogen knockout mice or fibrin depletion pharmacologically with ancrod, the MS mice did better. These results have obvious implications for not only MS but other conditions such as injury or neurodegenerative disease associated with BBB damage. It suggests that fibrin might be a novel therapeutic target in such conditions. Such approaches might be safer than administration of fibrinolytic proteases, such as tissue plasminogen activator (tPA), since these may produce basal lamina degradation, contributing to neuronal death.

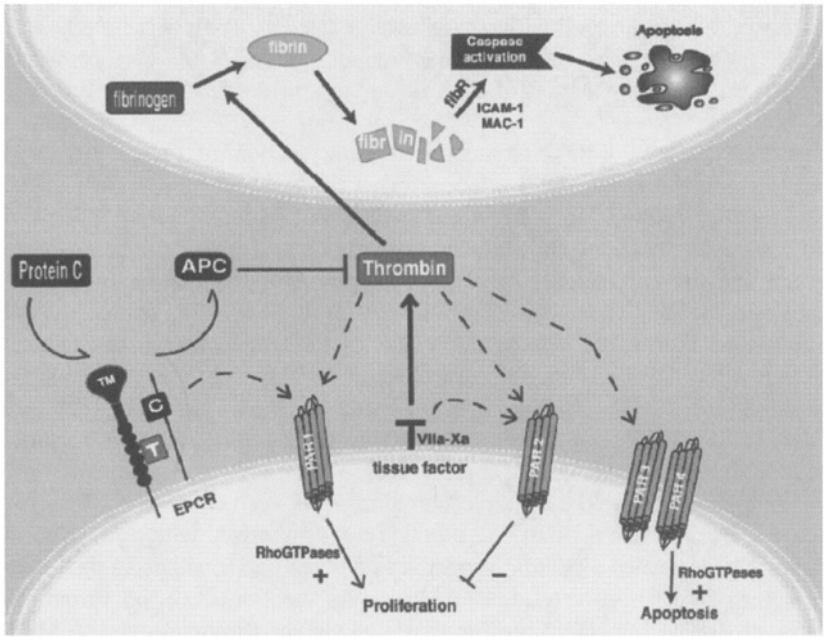

Figure 3: Schematic model of thrombin:TM:APC function along with fibrin in the CNS. Neural cells (neurons, astrocytes or microglia cells) are shown above and below activation of prothrombin to thrombin in brain extracellular space. Tissue factor (TF), coagulation regulators such as thrombomodulin (TM) and endothelial cell protein C receptor (EPCR), as well as thrombin proteinase-activated receptors (PARs), PAR1, 3 and 4, along with PAR2 are shown. PAR activation, as well as fibrin formation and subsequent degradation can mediate neural cell apoptosis and proinflammatory processes (cell above). Anticoagulation therapy, per se, may have adverse effects due to unacceptable bleeding, while specific inhibition of PAR activation, elimination of fibrin(ogen), or inhibition of fibrinolysis, may be therapeutic. Elimination or reduction of TF activity may overcome inflammation and apoptosis. Stippled arrows indicate potential pathways regulating the interaction of neural cells and coagulation components.

Thrombin exhibits both chemotactic and mitogenic activity due, in part, to two peptide sequences that lie outside its catalytic domain and, thus, apart from activation of its PARs. Bar-Shavit and colleagues showed more than twenty years ago that a domain within thrombin, separate from its proteolytic function, elicited chemotactic responses from clonal and primary activated macrophages. This subsequently turned out to be an RGD (Arg-Gly-Asp) sequence (Bar-Shavit *et al* 1991). During this same time frame, Carney and colleagues determined that the non-catalytic sequence in thrombin, called thrombin receptor-activating peptide (TRAP-508), was implicated in wound

healing (Carney *et al* 1986; Glenn *et al* 1988; Norfleet *et al* 2000; Stiernberg *et al* 1993). This terminology pre-dated the use of TRAP that referred to the new –NH2 sequence after thrombin cleavage of proteinase-activated receptor 1 (PAR1), subsequently re-named PAR1 active peptide or PAR1AP. These non- catalytic actions of thrombin, in addition to its ability to catalyze the formation of fibrin, may contribute to the potential role of thrombin in the inflammatory response.

However, it is clear that PARs are involved in the proinflammatory effects of thrombin (Cottrell *et al* 2002; Coughlin and Camerer, 2003; Dugina *et al* 2002; Gabazza *et al* 2004; Mackie *et al* 2002), including the nervous system (Choi *et al* 2003; Suo *et al* 2002, 2003a, 2004). Thrombin enhances PMN rolling in small blood vessels due to effects on adhesion proteins such as intercellular adhesion molecule 1 (ICAM-1) and P-selectin (Clark *et al* 2003; Leirisalo-Repo, 1994; Miyahara *et al* 2003; Reiter *et al* 2003; Sugama and Malik, 1992; Sugama *et al* 1992).

A critical regulator of thrombin's actions, which must be considered along with serpins, such as PNI, and the PARs (1, -3, -4), when discussing regulation of thrombin within tissues such as the CNS, is the cell surface chondroitin sulfate proteoglycan (CSPG) thrombomodulin (TM). TM binds thrombin on EC (Esmon 2000; Weiler and Isermann, 2003), astrocytic (Pindon *et al* 1997, 2000) and other cell surfaces and the complex that TM forms with thrombin is very high affinity (Kd ~0.5 nmol L^{-1}), and serves to inhibit thrombin interaction with both fibrinogen and PAR1 via thrombin's anion binding exosite I. In addition, the TM:thrombin complex is a potent activator of the zymogen protein C (PC) that enhances thrombin-dependent PC activation by more than 100-fold. Activated PC (APC) provides an essential anticoagulant mechanism that prevents amplification of thrombin generation, by proteolytic digestion of activated coagulation factors FVa and FVIIIa (Esmon *et al* 1997).

However, APC not only possesses anticoagulant properties but is significantly anti-inflammatory as well. This is accomplished by roughly half of the extracellular domain that consists near the N-terminal with sequence similarity to C-type animal lectins. This domain is essential for receptor endocytosis (Conway *et al* 1997) and regulates endothelial function in inflammation (Conway *et al* 2002). The remainder of the extracellular portion of TM consists of six epidermal growth factor (EGF) modules, of which modules 1–4, and the CS glycosaminoglycan (GAG) side chains serve to bind thrombin via its anion binding exosites I and II, respectively, as shown schematically in Figure 4.

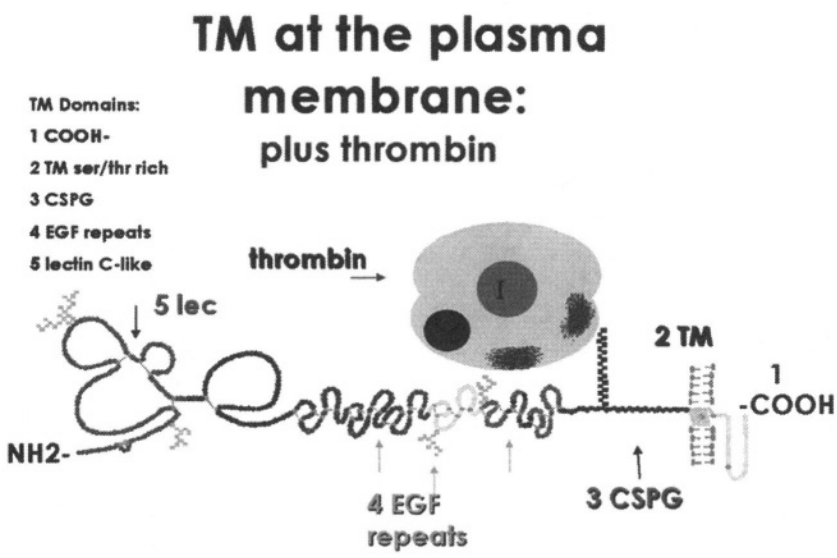

Figure 4: Schema of thrombomodulin (TM) with its five structural domains interacting with thrombin at the cell membrane. Extending from a short cytoplasmic tail (1) and transmembrane domain (2) is a serine/threonine–rich region to which a chondroitin sulfate proteoglycan (CSPG) moiety that optimizes anticoagulant function is attached (3). Next is a domain that consists of six epidermal growth factor (EGF)-like repeats, four of which are responsible for the protein's anticoagulant and antifibrinolytic functions (4). The NH2-terminal domain has two modules. The first, adjacent to the EGF-like domain, is an ~70–amino acid residue hydrophobic region. The second, which is ~155–amino acid residues long, has homology to C-type lectins (5), which in many proteins participate in immune and inflammatory processes.

Both APC and TM have been implicated in anti-inflammatory processes including protection against sepsis (Biernacka *et al* 2003; Esmon 2003; Liaw 2004; Satran and Almog, 2003). Regarding the CNS, TM and APC have been recently implicated in both anti-inflammatory and neuroprotective actions (Griffin *et al* 2004; Taoka *et al* 2000; Zlokovic 1997) and TM may be critical in the astrocytic response to injury (Pindon *et al* 2000). However, the time course for its upregulation is several days after injury and we have found that providing a truncated soluble form of human TM (Solulin) within 1 h and up to 24 h after contusion SCI in the rat affords significant neuroprotection, lesion sparing and positive recovery outcome (Festoff 2000, 2004).

Along with both APC and TM, another molecule not yet studied in the nervous system is the endothelial PC receptor (EPCR) (Crawley *et al* 2002; Esmon 2003; Liaw *et al* 2000). The EPCR is present on brain microvessels,

although it is more expressed in larger vessels and affects both cytosolic $[Ca^{2+}]_i$ as well as regulating apoptosis by inhibiting p53-dependent mechanisms (Cheng *et al* 2003; Domotor *et al* 2003).

9. THROMBIN, CEREBRAL ANGIOGENESIS AND THE BLOOD-BRAIN BARRIER (BBB)

As in other vascular beds, the cerebrovascular endothelium (CVE) participates in the control of hemostasis through mechanisms that involve the synthesis and release of both pro- and anticoagulant factors. Following damage induced by infection, stroke, autoimmunity, traumatic injury or even neurodegeneration, the CVE becomes damaged, and the balance between pro- and anticoagulant factors is upset with a result being a pro-coagulant state on a micro- or local scale, predominating. This intimate cooperation between coagulation and proinfiammatory pathways is becoming clearer within the CNS as it is systemically. Likewise, as in other ECs, brain capillary ECs express active PARs (Bartha *et al* 2000; Domotor *et al* 2002; Kim *et al* 2004). Thrombin as well as plasmin induces contraction of brain ECs, which may increase capillary permeability thereby leading to disruption of the BBB. Thrombin's effects, like bradykinin, are accompanied by cell rounding and inter-EC gap formation (Lum and Malik, 1996), indicating that the predominant transport pathway is a diffusive one (i.e., paracellular transport). PAR1 and $[Ca^{2+}]_i$ are involved, and pretreatment of brain capillary ECs with low (100 pM) thrombin concentration prevented $[Ca^{2+}]_i$ rise in response to high (10 nM) thrombin concentration, but pretreatment with PAR1AP did not prevent subsequent $[Ca^{2+}]_i$ rise to high PAR1AP concentration.

Vascular endothelial cell growth factor (VEGF) stimulates ECs to expose tissue factor (TF), the high-affinity transmembrane receptor and cofactor for cellular initiation of the plasma coagulation protease cascades through the extrinsic pathway, so generating thrombin. Thrombin exerts a number of activities: a) it forms an extracellular fibrin barrier from the VEGF-dependent fibrinogen extravasation; b) it activates progelatinase-A (pro-MMP-2), which destroys the basal membrane, allowing proliferation of ECs in the CNS; finally, c) it induces EC proliferation, potentiating the VEGF effect. Another important factor exposed at the abluminal EC surface is membrane type 1 matrix metalloproteinase (MT1-MMP), a membrane-bound MMP, which also activates progelatinase-A, allowing an alternative pathway to that of thrombin to destroy the basal membrane. In addition, MT1-MMP is also engaged in a direct, cell-associated fibrinolytic activity, essential for tubulogenesis of the novel outsprouting capillary.

P-Glycoprotein (P-gp) is an important BBB transporter macromolecule, a

member of the ATP cassette multi-drug resistance (MDR) protein family. From a pharmaceutical viewpoint, it is important since it prevents accumulation of drugs in the brain by causing brain-to-blood efflux transport of drugs (Begley *et al* 1996; Bendayan *et al* 2002; Sharom *et al* 2001). P-gp is a transmembrane protein encoded by *mdr1a, mdr1b* and *mdr2* genes in mice and its at the BBB has been analyzed using gene knockout mice (Cox *et al* 2001; Jonker *et al* 1999; Schinkel 1997). In humans, P-gp is also expressed at the BBB and is localized at the luminal membrane of human brain capillary ECs, but we are less knowledgeable about its contribution to BBB efflux transport. Other transporters besides P-gp exist, some of which belong to the same ATP binding cassette (ABC) transporter superfamily).

Several characteristic features identify BBB ECs from epithelial cells (EpCs) and peripheral ECs: a) low rate of transcytotic vesicles and a restrictive paracellular diffusion barrier; b) BBB tight junctions (TJs) have distinct morphological and molecular properties, and are more sensitive to microenvironmental than epithelial factors. Finally, ubiquitous molecular TJ components have been identified and characterized: claudins, occludin, ZO-1, ZO-2, ZO-3, cingulin and 7H6 (Dermietzel and Krause, 1991; Wolburg and Lippoldt, 2002). Of interest, another feature distinguishing brain capillary EC compared with EpCs impact GTPase-dependent pathways that regulate intercellular permeability, that appear to operate in an opposite directions. For example, in brain ECs, activation rather than inhibition of the Rho pathway by lysophosphatidic acid (Moolenaar 1995) disrupted the paracellular barrier (Schulze *et al* 1997). Conversely, Rho pathway inhibition LPA-induced increase in permeability (Hirase *et al* 2001). Since thrombin and LPA both activate Rho, and thrombin, as well as cytokines, increases transendothelial permeability *in vitro* by affecting tight and adherens junctions, thrombin:PAR signaling to reorganize the actin cytoskeleton and formation of intercellular gaps in the BBB is likely.

10. CONCLUSION

Thrombin, best known for its role as the ultimate serine protease in the coagulation cascade, is a multi-functional protein acting on neural cells in the brain and spinal cord, as elsewhere, through novel PARs. To date, the vast majority of studies that have emerged deal with thrombin's actions in the mature CNS. In this regard, studies have suggested that thrombin provokes either CNS injury or protection, depending on the concentration of this protease ligand. When nanomolar levels are applied to susceptible neurons, toxic effects are seen, while picomolar levels are protective. This varies somewhat with the sub-population of cells and may be phenotypically easy to

identify just by observation, such as neurite retraction in motor neurons and neuroblastoma cells (Smirnova *et al* 1997, 1998a; Smith-Swintosky *et al* 1995; Turgeon *et al* 1998). On the other hand, it might require more sophisticated analyses, such as confocal microscopy of the NFT-like hyperphosphorylated aggregates of microtubule-associated tau (Suo *et al* 2003b). Previous evidence indicated that thrombin-induced brain "tolerance" or neuroprotection and thrombin-mediated neuronal damage shared the same initial signaling pathway involving activation of PARs and subsequent downstream Rho GTPases (Donovan and Cunningham, 1998). However, this failed to yield satisfactory explanation of the mechanisms involved. Are these effects mediated by the activation of different pathways or a differential stimulation of the same pathway? In this regard, the PAR and non-PAR-mediated mediated pathways may be responsible separately. Or, does thrombin concentrations cause either neuron (brain) injury or protection depending on which PAR is activated? This question has not been fully answered, but more recent studies, both in neurons and in microglia, suggest that this is, indeed the situation. Motor neurons, being exquisitely sensitive to thrombin, appear to primarily respond in a toxic fashion and this is mediated via PAR1 (Smirnova *et al* 1997, 1998a; Turgeon *et al* 1998). In contrast, hippocampal neurons do not respond rapidly to low concentrations of thrombin with obvious phenotypic effects, but PAR1 appears to mediate protection at these low levels (Striggow *et al* 2000). In contrast, at higher concentrations, hippocampal neurons do respond, by evidencing translocation, hyperphosphorylation and aggregation of tau, and this primarily involves PAR4 (Suo *et al* 2003b). Involvement of signaling pathways is clear, since MAPK is transiently activated via PAR1, while in a prolonged fashion through PAR4.

This dichotomy is also seen with brain microglia. When injected into the brain, thrombin elicits both proliferation ("microgliosis") and activation (release of cytokines). Using PAR1 knockout mice as well as PAR-specific APs, we found that PAR1 was largely responsible for the proliferative effects of thrombin while PAR4 mediated the cytokine activation influence (Suo *et al* 2002, 2003a).

It is clear that these studies are gone far, and will continue, to unravel the roles of thrombin signaling in CNS injury, stroke and neurodegeneration. It is hoped that novel drug targets, with agents that affect thrombin signaling (Festoff 1997), will develop. This includes specific PAR antagonists that interfere outside the cell (Ahn *et al* 2003; Anderluh and Dolenc, 2002) as well as novel molecules, known as pepducins (Kuliopulos and Covic, 2003), that block G-protein interaction inside the cell. Finally, there is still the question that remains largely unanswered, and that is what is the normal function of prothrombin, thrombin and thrombin receptors in the brain? (Festoff 1990; Festoff *et al* 1996b; Monard 1993; Zoubine *et al* 1996). Certainly, there

must be a role for thrombin in normal development and in normal physiology of at least the developing brain, and in neuroplasticity, which recapitulates many of development's attributes.

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Chapter 14

Matrix Metalloproteinases and Neuroinflammation in Multiple Sclerosis

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1. INTRODUCTION

Multiple sclerosis (MS) is a leading cause of disability in young adults. Major advances have occurred in elucidating the pathophysiology and in the development of new therapies based on discoveries in neuroimmunology and neuroimaging. Although the etiology underlying the demyelination remains uncertain, it is recognized that inflammation is prominent in the relapsing and remitting form of the illness. This inflammatory response is mediated by invading leukocytes, and results in the destruction of myelin by free radicals, cytokines, complement and antibody fixation, and protease activity. Neuroinflammation is a term used to describe complex molecular cascades, involving diverse gene families that are triggered by ischemia, infection, and trauma. There has been a revival of the older concept that an early, important component of the disease process involves damage to the cerebral blood vessels with opening of the blood-brain barrier (BBB). Interest in this concept, which had been neglected during the intense search for viral and other antigens, was reignited by the findings on magnetic resonance imaging (MRI) with the contrast agent, Gadolinium-diethylenetriaminepenta-acetate (Gd-DTPA). Serial contrast-enhanced MRI scans in patients with relapsing remitting MS showed that the disruption of the BBB could precede the clinical attacks. These insights led to further discoveries in the mechanisms of inflammatory BBB opening, which provided evidence for a role of free radicals and proteases in this process.

One of the main families of proteases involved in the inflammatory damage to the blood vessels is the matrix metalloproteinases (MMPs). MMPs have been shown to be up-regulated in the CSF, brain, and blood of patients with acute MS attacks (Gijbels *et al* 1992; Leppert *et al* 1998; Anthony *et al* 1997). Since the MMPs contribute to cell death and regeneration in other diseases and in cell cultures, it is not surprising that they have been found to be important in these processes in acute MS. Less is known about the role of the MMPs in other forms of MS, such as secondary progressive and primary progressive. In addition, MMPs may be involved in the demyelination seen in other disease processes, such as vascular dementia and viral infection. This review will focus on new findings concerning the role of MMPs in the neuroinflammatory response of MS, particularly on their role in BBB disruption. There are several recent reviews on MMPs in MS and other neurological diseases (Noseworthy *et al* 2000; Yong *et al* 2001; Mun-Bryce and Rosenberg, 1998; Lukes *et al* 1999).

Recent pathological studies have revealed pathogenetic heterogeneity of MS, which may be related to different clinical courses and MRI findings (Lucchinetti *et al* 2000). The hallmark of the illness is the damage to the myelin, which is now recognized to contribute to axonal disruption. Inflammatory substances are released by infiltrating mononuclear cells, neutrophils, and endogenous brain cells. In MS, activated T lymphocytes and reactive blood vessels are present in the early stages of the illness. Venules are the main site of white blood cell adherence prior to passage into the brain. White blood cells are able to cross the normally restrictive BBB because of the release of proteases, including MMPs, which attack the tight junctions and extracellular matrix proteins in the basal lamina. Activated T-cells can cross the intact BBB, and when they do not find a target or antigen they move back into the circulation without causing inflammation (Liblau *et al* 2001). Once within the central nervous system, the white blood cells can be reactivated by CNS antigens and release additional proteases and free radicals, induce expression of endogenous proteases, and amplify the cascade of molecular injury. Genomic factors are important in determining the susceptibility of an individual to the components of the inflammatory response. Genetic make-up is important in experimental models of MS. Different strains of rats and mice, the main animals used in experimental allergic encephalomyelitis (EAE), respond differently to stimuli that elicit an inflammatory response.

Neutral proteases have been shown to be important in disruption of myelin (Hallpike and Adams, 1969). Plasmin causes secondary demyelination in the presence of complement (Brosnan *et al* 1980). The MMPs formed in leukocytes facilitate migration across the blood-brain barrier (BBB), while endogenous MMPs produced by brain cells participate in neuroinflammation with disruption of the BBB and demyelination. In addition to the detrimental effects, the MMPs are involved in the repair and remyelination processes,

making it important to understand the timing of the pathological and repairative responses in order to design therapeutic strategies that block the one without interfering with the other. Another concept that is emerging from studies on the role of the MMPs in the brain is that different cells make different MMPs, and that the coordinated action of several MMPs from different cell types is necessary. Thus, release of MMPs by invading cells and by endogenous brain cells occurs in response to inflammation and continues into the repair processes.

2. EARLY STUDIES

In 1916, Dawson reported that inflammatory cells clustered around veins in the center of the demyelinated plaque (Dawson 1916). Demyelinated areas were seen around the veins that fanned out from the cerebral ventricles, giving the appearance of finger-like lesions. These so-called "Dawson's fingers", which can be seen around the ventricles in the MRI scans, have become a hallmark of the illness. In 1937, Putnam and Adler (1937) showed that fibrosed vessels were centrally located in plaques; they serially reconstructed brains from MS patients, showing the fibrotic vessels surrounded by inflammatory cells. These early studies lead to the theory that vascular disease contributed to the pathophysiology of MS, and this theory was supported by hematological studies. However, over the next fifty years the research focus shifted to viral and immunological causes. This may have been due to the introduction of antibiotics and the increased longevity of MS patients, which resulted in fewer deaths during the acute phase of the illness than had been seen in the earlier days. Rather than observing inflammation around blood vessels, stable MS plaques without perivenous monocyte infiltrations were seen that represented the chronic phases of the illness.

Resurgence in interest in the role of inflammation and the blood vessel response occurred with the introduction of CT and MRI, which showed multiple contrast agent enhancing lesions in the brains of patients with relapsing and remitting disease; Gd-DTPA-enhanced MRI scans showed many more lesions than could be found on enhanced CT scans. In addition, gadolinium enhancing lesions can be found 10 to 15 times as often as the incidence of clinical relapses. Marked inflammatory activity on MRI emphasized the importance of the blood vessels in the pathogenesis of MS. Gadolinium-enhanced MRIs showed waxing and waning of the regions of enhancement, often without corresponding clinical findings, suggesting an active pathological process centered around the blood vessel, confirming the observations of Dawson and Putnam, and leading to renewed interest in the inflammatory response (Miller *et al* 1996).

3. MULTIPLE SCLEROSIS PATHOGENESIS

Multiple sclerosis is an inflammatory disease affecting the central nervous system (CNS). Although it is believed that a key element in the pathological process is the entry of activated T-cells into the CNS, the exact triggers and mechanisms of tissue injury are not well understood. The MS immune reaction is complex and multifaceted, involving a variety of immune cell types and inflammatory mediators, antibodies, cytokines and potentially toxic molecules such as nitric oxide and oxygen free radicals. Inherited polygenic determinants requiring exposure to an unknown environmental trigger are generally accepted as an important component of the disease process. One class of potential environmental triggers is an antigen of viral, bacterial or other origin. Small peptide fragments of viral protein components, with amino acid sequences similar to antigenic segments of myelin proteins, could induce a cross-reactive immune attack on self by a process of molecular mimicry. As an example, immunizing an animal to antigenic myelin components or peptide fragments triggers the animal model of CNS demyelination, experimental allergic encephalomyelitis (EAE). Effective antigens in EAE induction include the major myelin proteins, myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG) and others. Patients with MS, as well as normal individuals, have potentially autoreactive T-cells reactive to these myelin antigens in their peripheral circulation (Chou *et al* 1992; Hohlfeld *et al* 1995; Jingwu *et al* 1992; Ota *et al* 1990; Brocke *et al* 1996).

CNS injury in MS is not restricted to myelin destruction but also involves significant axonal loss, astrogliosis, and oligodendrocyte loss. The axonal loss has been quantitated by confocal microscopy studies, (Trapp *et al* 1998) and can be measured by proton magnetic resonance spectroscopy, which shows loss of the marker metabolite, N-acetyaspartate (NAA). Loss of this metabolite is greatest in lesions, less in perilesional regions and below normal levels even in normal appearing white matter. Changes in diffusion tensor imaging and magnetization transfer parameters reflect loss of structural tissue integrity in brain regions that are normal by typical anatomic imaging sequences. These NMR indications of injury have been shown to occur even before the appearance of gadolinium enhancement and T2 changes typical of new MS plaques on MR imaging. Recent pathological studies have added additional evidence for complexity and pathological heterogeneity in MS and challenge ideas about antigenic and molecular mimickry theories (Lucchinetti *et al* 2000). The autopsy studies of Lucchinetti *et al* showed four distinct subsets of MS plaques and patterns of demyelination. Types I and II were characterized by T-cell mediated immune damage with type II having additional evidence of antibody and complement deposition. Types III and IV suggested a primary oligodendrocyte pathology and type III lesions were

notable in lacking vessel centered inflammation. Multiple MS genotypes producing similar clinical presentations could explain differences in disease severity, progression and responses to immunomodulator therapy. A recent autopsy study by Barnett and Prineas (2004) described acute MS lesions with oligodendrocyte apoptosis in the absence of myelin phagocytosis. One explanation for this loss of oligodendrocytes in the absence of inflammation could be a primary insult to the myelin producing cells. Candidate etiologies could include viral infections, ischemia or vasculitis causing oligodendrocyte injury. In this model the typical inflammatory reaction associated with MS plaques might be a normal or secondary response needed for phagocytosis and removal of myelin and other cellular debris.

Regardless of which pathophysiological model is considered, it must in the end explain the clear genetic susceptibility to MS. Twin studies support the concept of multiple genetic risk factors, triggered by an unknown environmental promoter. The existence of such a trigger is highlighted by the identical twin that has a 75% chance of not getting MS when the sibling is affected. Although MS is often associated with perivascular inflammatory cuffs of lymphocytes and macrophages, the recent pathological studies raise questions about the earliest events in a new MS lesion and complicate the extrapolation of data from the EAE model.

4. PROTEASES AND MS PATHOLOGY

Myelin basic proteins are attacked by lysosomal proteolytic enzymes (Einstein *et al* 1972). Phospholipase A leads to the formation of lysophophatides, such as lysolecithin. Application of lysolecithin to brain sections causes loss of myelin through its cytolytic action. Acid and neutral proteolytic activity increases at the edge of the active plaques of MS (Hallpike and Adams, 1969). Acid and neutral proteases have been detected in the CSF of patients with MS (Cuzner *et al* 1978).

Stimulated macrophages secrete proteases that digest myelin basic protein *in vitro,* and the active substance in the macrophage supernatant was shown to be the fibrinolytic enzyme, plasmin (Cammer *et al* 1978). The original studies were done with frozen myelin basic protein, and subsequent studies in fresh tissue required both complement and plasmin to digest the myelin. Laying a fibrin film on top of brain tissue from MS patients showed the plasmin activity around the blood vessels (Hirsch *et al* 1981). Treatment of animals with EAE with agents that blocked plasmin was shown to have a beneficial effect (Brosnan *et al* 1980, Smith and Amaducci, 1982). There is an increase in the fibrinolytic activity of blood in MS patients with active disease (Menton *et al* 1969).

Gelatinases, a subgroup of the MMPs were shown to be increased in the CSF of patients as part of a neuroinflammatory response (Gijbels *et al* 1992). Mice with EAE have increased CSF levels of MMP-9 during the acute phase of the disease (Gijbels *et al* 1993). Subsequently, the MMPs were shown to digest myelin basic protein, suggesting one mechanism of their action (Chandler *et al* 1995). Treatment of mice with EAE with a broad spectrum inhibitor to MMPs, GM6001, reduced the severity of the symptoms, and limited damage to the BBB (Gijbels *et al* 1994).

Matrix metalloproteinases are ubiquitous enzymes that degrade all the components of the extracellular matrix. They are divided into four major groups: collagenases, stromelysins, gelatinases, and membrane-type metalloproteinases (Nelson *et al* 2000). Collagenases include intersitial collagenase (MMP-1), neutrophil collagenase (MMP-8), and rat collagenase (MMP-13). Collagenases attack type I, II, and III collagen, which are found mainly in connective tissues of the joints, making them a major contributor to arthritic joint destruction (Okada *et al* 1986). The second class contains stromelysin-1 (MMP-3), stromelysin-2 (MMP-10), and matrilysin (MMP-7); they attack most extracellular matrix macromolecules. Gelatinases act on type IV collagen and other components of basement membranes or basal lamina; two gelatinases have been identified, namely, gelatinase A (MMP-2) and gelatinase B (MMP-9). An important, but less well understood group is the membrane-type metalloproteinases (MT-MMPs). MT-MMP is formed in a proform inside the cell; activation occurs by proconvertases, such as furin or plasmin, either in the endoplasmic reticulum or on the cell surface. MT-MMPs participate in a variety of proteolytic activities. The first MT-MMP to be isolated, MT1-MMP, was found to be important in the activation of gelatinase A (Sato *et al* 1996). MT1-MMP forms a trimolecular complex of proMMP-2, MT1-MMP, and TIMP-2 that facilitates the activation of the MMP-2. The importance of this mechanism of activation is that it occurs close to the surface of the cell membrane where it is spatially constrained. This focuses the proteolytic activity, and prevents wide-spread damage. In angiogenesis, for example, the MMPs close to the cell surface, such as activated MMP-2, act to clear the path for the growth of the cells.

Oligodendrocytes grow by secreting MMPs at the ends of the processes, which similarly to the growing blood vessels, allow the processes to extend. Such a mechanism may be important in repair. Mice genetically manipulated to remove the gene for MMP-9 have an impairment in myelin formation after lysolecithin-induced demyelination; this deficiency may be explained by the failure to clear the accumulation of NG2, an inhibitory proteolglycan that retards the maturation and differentiation of oligodendrocytes. Failure to remove the inhibitory proteoglycan impairs remyelination (Larsen *et al* 2003).

Proteases are tightly regulated at various steps in the production and activation process. Without this tight control there would be unwanted tissue

proteolysis. In the case of the MMPs, they are secreted in a latent form with the latency controlled by a propeptide fragment with cysteines that bind to the zinc in the active site of the molecule. This control mechanism is referred to as the "cysteine switch" (Van Wart and Birkedal-Hansen, 1990). Removal of the propeptide fragment exposes the zinc atom that provides for the activity of the protease. In the promoter region of the MMP genes are several transcriptional factors that regulate their production. For example, gelatinase A is a constitutively expressed enzyme that is found in a large number of tissues under normal conditions. In the promoter of the MMP-2 gene are activator protein-2 (AP-2) and SP-1 sites. Gelatinase B is present in very low levels in brain, but it is markedly induced during an inflammatory response. The promoter of the MMP-9 gene contains two sites that are present in many inflammatory-induced genes, namely, the AP-1 and nuclear factor kappa B (NF-κB).

Astrocytes constitutively express proMMP-2, and they can be induced to produce proMMP-9 when stimulated with inflammatory mediators, such as tumor necrosis factor-α (TNF-α) or lipopolysaccharide (LPS) (Gottschall and Yu, 1995). Microglial cells in culture produce proMMP-9, and when stimulated with LPS produce active MMP-9 (Colton *et al* 1993). Immunohistochemistry studies have shown that the microglia make MMP-3, suggesting that it may directly attack extracellular matrix and be involved in the activation of proMMP-9. A broad-spectrum, hydroxymate MMP inhibitor, BB-1101, reduced the activation of MMP-9 in mixed astrocyte and microglia cultures, supporting a role for a metalloproteinase, such as MMP-3, in the activation of MMP-9 by LPS (Rosenberg *et al* 2001). Free radicals may play a role in MMP action either through activation of the latent forms or induction of the mRNA through signaling at the NF-κB site (Yang *et al* 2001). Nitric oxide can form a nitroxylation product with the cysteines in the "cysteine switch," leading to the activation of MMP-9 (Gu *et al* 2002).

Cellular localization of the MMPs has been studied with immunohistochemistry. Astrocytes show immunostaining for MMP-2, which is seen mainly in the foot processes around blood vessels (Rosenberg *et al* 2001). The pattern of MMP-2 in astrocytic processes around blood vessels, beneath the pial surface, and near the ependyma suggests a subpopulation of MMP-2-containing astrocytes that may be involved in fluid regulation at the brain-CSF and brain-blood interfaces. Immunostaining for MMP-9 is normally absent in the brain. In an injury, endothelial cells and neutrophils show MMP-9 immunostaining (Romanic *et al* 1998). Expression of MMP-3 has been seen on immunohistochemistry in microglia, pericytes, and neurons during an ischemic insult (Rosenberg *et al* 2001). The distribution of MMPs in different cell types suggests that activation of the MMP-9 released in a latent form from endothelial cells and astrocytes would be facilitated by the presence of MMP-3 in reactive microglia, and that these extracellular

molecules could diffuse to sites distant from their release and cause greater damage.

Knock out mice have been useful in the elucidation of the mechanism of action of the MMPs. Genetically engineered mice, lacking MMP-9, have smaller infarcts and less BBB damage (Asahi *et al.* 2001). Young, MMP-9 null mice showed less susceptibility to develop EAE than older mice (Dubois *et al* 1999). The role of free radicals in the biology of the MMPs in brain has been studied with mutant mice that over express the free radical scavenger, superoxide dismutase; these mice have reduced production of MMP-9 in cold-injury (Morita-Fujimura *et al* 2000).

5. PROTEASES AND THE BLOOD-BRAIN BARRIER

Early studies showed that the intraventricular injection of neutral proteases opened the BBB (Robert and Godeau, 1974). Bacterial collagenase was the most disruptive of the proteases studied, but other proteases, including plasmin and cathepsin were found to be disruptive of the BBB. Several sites may have been damaged by the proteases with opening of the BBB. Cerebral capillaries have tight junctions, which give them electrical properties similar to epithelial cells. Tight junctions contain specific proteins, such as zona occludens-1 (ZO-1), which is a substrate for MMP-9, and claudins and a number of occludens (Harkness *et al* 2000; Mori *et al* 2002). As with other epithelial cells, cerebral capillaries are surrounded by a basal lamina that contains type IV collagen, laminin, fibronectin, heparan sulfate, and other matrix molecules; these are the substrates hydrolyzed by gelatinases. The function of this layer outside the tight junctions is uncertain. However, injection of proteases that attack components of the basal lamina results in the opening of the BBB, suggesting that the basal lamina may have a barrier function (Armao *et al* 1997). We have shown that mammalian 72-kDa type IV collagenase (MMP-2) opens the BBB when injected into the caudate nucleus of rats and that the reaction can be blocked by tissue inhibitor to metalloproteinase-2 (TIMP-2) (Liotta *et al* 1980). This showed that gelatinases were toxic to the BBB, but did not show that the reaction was important *in vivo*. The role of MMPs *in vivo* was shown by the expression of MMPs in EAE and by their detection in CSF of patients with MS.

A number of studies have documented MMP induction in brain tissues from patients with MS and in animals with EAE. These studies have been done with immunohistochemistry and western blots to identify proteins and with polymerase chain reaction (PCR) to look for mRNA products. In EAE,

there is evidence of MMP-3, -7, and -9 by immunohistochemistry. Human autopsy material shows evidence of MMP-3 and -9 in microglia in the white matter, particularly around blood vessel, and in the macrophages in the MS lesions, there was expression of MMP-1, -2, -3 and –9 (Maeda and Sobel, 1996). In another human study, the authors identified MMP-2 and MMP-7 in microglia/macrophages (Anthony *et al* 1997). MMP-7 showed strong immunoreativity in the macrophages of brains of patients with MS, which was also seen with in situ hybridization for the mRNA product (Cossins *et al* 1997). Quantitative PCR has been done in autopsy material from MS patients. There was an increase in mRNA for MMP-7 and -9, which is consistent with the immunohistochemistry data (Lindberg *et al* 2001).

A novel model of MS in the mouse has been developed that uses the overepression of DM20 (ND4); these mice have a slowly demyelinating process that begins around the fourth month of life and causes an early death (D'Souza *et al* 2002; Mastronardi *et al* 1996). Elevated levels of mRNA for MMP-3 appears prior to the onset of overt disease at a time when MMP-9 is not elevated. The other MMPs, including MMP-11, -2, and -9 failed to show an increase in mRNA levels. MMP-13 showed a small increase. The increase in MMP-3 protein was seen by Western blot. When they cross-bred the ND4 mouse with a TIMP-1 transgenic mouse, the resulting hybrid showed a milder clinical course than the ND4 strain alone, and there was reduced expression of MMP-3 (D'Souza *et al* 2002). These studies suggest that MMP-3 is important at the early stages of the illness.

6. EAE AND THE BBB

In 1935, Rivers and Schwentker showed that injection of homologous brain tissue into monkeys caused demyelination (Rivers and Schwentker, 1935). Kabet and colleagues added Freund's complete adjuvant and enhanced the response (Kabat *et al* 1947). Experimental allergic encephalomyelitis is a monophasic illness induced by the brain products; the pathological hallmark is perivascular inflammation with demyelination. Animals with EAE have a mononuclear infiltrate of lymphocytes and macrophages around small blood vessels. The lesions of EAE are similar to those seen in acute disseminated encephalomyelitis, which is often seen in postviral or post vaccination syndromes.

In EAE, inflammatory T lymphocytes cross the BBB after being sensitized by the peripheral injection of myelin basic protein in Freund's adjuvant. In the early stages of EAE, disruption of the BBB has been shown to occur by studies with radioisotopes (Leibowitz and Kennedy, 1972). Fibrin is deposited in the inflamed vessels, and agents that breakdown the fibrin, such as snake

venom, reduce the injury (Paterson 1976). Damage to the wall of the blood vessel initiates a repair process. Myelin fragments are phagocytized by macrophages. Plasmin/plasminogen is up-regulated to participate in the remodeling of the damaged tissues. Proteases released by the macrophages, however, perpetuate the injury by causing a nonimmunological form of demyelination.

Rats with EAE show the expression of mRNA for MMP-3, MMP-7, and MMP-9, while MMP-2 and MT-MMP are constitutively expressed (Clements *et al* 1997). Immunohistochemistry of rats with EAE shows MMP-9 in blood vessels and neutrophils, and MMP-7 in macrophages (Kieseier *et al* 1998). No information is available on the expression of MMPs in the blood of these animals. Inhibitors to MMPs affect the course of EAE by blocking the disruption of the BBB and altering other actions of the MMPs (Gijbels *et al* 1994; Hewson *et al* 1995; Liedtke *et al* 1998). Because the MMPs are mainly found in the proform merely finding elevated levels is insufficient to determine the role of that MMP in the pathological process. For example, MMP-2 is constitutively expressed and is present at all times in an inactive form. Activation requires the presence of MT-MMP and TIMP-2. However, MT-MMP requires either furin or plasmin for activation. Thus, an injury that leads to the production of uPA and has plasminogen present has the potential to activate the MMP-2. Since there is a large amount of proMMP-2 and a small amount of active MMP-2, detection of the active form may be difficult. Another confounding factor is the multiple sources of the enzymes. MMP-9 may be either released already formed by neutrophils or induced in the brain during inflammation. In the former case, there would be elevated levels of MMP-9 protein that could be detected with zymography, but absence of mRNA for MMP-9. If brain cells produced the MMP-9, which is normally present at low levels, both MMP-9 protein and mRNA would be induced.

Neuroimaging methods have added to the understanding of EAE. A recent study used a novel method for the imaging of macrophage-like activity in the brain. Ultra small particles of iron oxide (USPIO), which accumulate in phagocytic cells, were used as a contrast agent for MRI. The USPIOs are taken up by the macrophages and after entering the brain the presence of the paramagnetic substance produced a local change in magnetic susceptibility that was detected as a loss of signal on MRI. In rats with EAE, the particle laden macrophages were shown to infiltrate into the brain (Floris *et al* 2003). However, enhancement with Gd-DTPA appeared at the onset of illness on day 9, while the USPIOs were best seen at the apex of the illness on day 14. This provides convincing evidence that changes in the BBB precede the infiltration of monocytes into the brain. The iron particles were shown histologically to be inside the microglia/macrophages with iron stains.

7. BBB DISRUPTION IN MS

Although there is converging evidence for early damage to the blood vessels in acute MS from pathological studies, the most compelling evidence for a major role of the BBB in the pathophysiology of MS comes from serial MRI scans in patients with relapsing-remitting MS. Patients have BBB changes during symptomatic as well as asymptomatic periods. Even patients with minimal clinical involvement have been observed to have waxing and waning of the contrast-enhancing lesions on MRI (Miller *et al* 1988; Grossman *et al* 1986). The hallmark of the BBB damage in human pathology and in the EAE model are the inflammatory cells that enter the brain at the perivenular regions. In the relapsing and remitting form of the illness, there is active inflammation around the cerebral vessels. Although the trigger that initiates the attack on the venule remains elusive, the cascade of events that leads to the BBB breakdown is more firmly established. The mononuclear cells adhere to the venule lumen. The T cells form several proteases, including MMP-9. In cultured endothelial cells, the proteases degrade tight junction proteins (Harkness *et al* 2000). Endogenous production of the MMPs leads to destruction of the basal lamina macromolecules. The cells that can produce endogenous MMPs include: MMP-9 from endothelial cell, pericyte-derived MMP-3 and MMP-9, and astrocyte-derived MMP-2. Activation could occur from free radicals and plasmin/plasminogen released by the infiltrating cells. Amplification of the proteolytic response would take place through the formation of cytokines, such as tumor necrosis factor-α (TNF-α), and chemokines, which recruit additional white blood cells. The opening is reversible at this early stage without extensive tissue disruption, however, as the process continues the chances of a more permanent injury increase.

Patients with MS have MMPs in the cerebrospinal fluid (CSF) (Gijbels *et al* 1993; Rosenberg *et al* 1996; Leppert *et al* 1998). Latent MMP-2 is a normal CSF constituent, and in neuroinflammatory conditions, MMP-9 is induced. The source of the MMP-9 in the CSF has been debated. One possible source is the neutrophils, which contain MMP-9. While they are most likely a major source of MMP-9 in bacterial meningitis, where large numbers of neutrophils are present in the CSF, this may not be the case in patients with MS since there are increased levels of MMP-9 in the absence of either an increase in white blood cells or protein. One interesting approach to resolve this issue was to index the MMP-9 in the CSF to that in the blood as is done for albumin and IgG. The indexing of MMP-9 in the CSF to that in the blood showed that the MMP-9 was endogenously produced (Liuzzi *et al* 2002).

Leukocytes secrete MMPs to enter the brain; agents that block the action of the MMPs reduce transport across the artificial membranes (Leppert *et al* 1995). For example, interferon-β, which is used in the treatment of MS,

decreases the movement of white blood cells across artificial membranes by blocking the release of the MMPs (Stuve *et al* 1996). MMPs have been found to be elevated in the blood of patients with MS, particularly during the acute phase of the illness, and there is a correlation between the levels in the blood and an acute attack of MS (Waubant *et al* 1999; Lee *et al* 1999). Levels of serum MMPs fall during treatment with interferon-β (Trojano *et al* 1999), and mRNA for the MMPs from blood-derived leukocytes is reduced by such treatment (Galboiz *et al* 2001).

High-dose intravenous steroids reduced BBB breakdown in acute exacerbations of MS. Levels of MMP-9 in the CSF correlated with the presence of enhancement with Gd-DTPA on the MRI (Rosenberg *et al* 1996). We found that treatment with high-dose intravenous methylprednisolone lowered the levels of MMP-9 in the patients that had enhancement on MRI, but not in those without enhancement. Steroids block the action of the FOS/JUN dimer, preventing activation of the AP-1 site in the MMP-9 gene (Jonat *et al* 1990). However, another study showed that a trial of oral high-dose steroid treatment in patients with MS and optic neuritis who had elevated levels of MMP-9 in the CSF failed to show a reduction in MMP-9 (Sellebjerg *et al* 2000). Further studies are needed to identify the optimal way to use high-dose steroids in the acute exacerbations of MS.

A recent study of MMPs in white blood cells in MS was published (Bar-Or *et al* 2003). The authors studied the expression of 23 MMP family members in circulating leukocytes in MS patients. Real time PCR was performed on multiple cell types. MMP-11, MMP-26, and MMP-27 were enriched in B cells. MMP-1, MMP-16, MMP-24 and MMP-28 were prominent in T lymphocytes. Monocytes showed expression of MMP-1, MMP-2, MMP-3, MMP-10, MMP-14, MMP-17, MMP-19 and MMP-25. Stimulated monocytes from MS patients express MMP-2, MMP-14, and TIMP-2, which form a tripartite complex for the activation of MMP-2. The MMP-14 is bound to the membrane and functions as a receptor for the MMP-2 or the MMP-2/TIMP-2 complex. These studies show the differential expression of the MMPs in various monocytes, and that in the acute MS attack, the monocytes cross the blood vessel wall through secretion of a variety of MMPs. They also show that strategies designed to suppress MMP expression may limit the extent of leukocyte infiltration into the brain.

In addition to the relapsing and remitting form of MS, there is a primary progressive forms. Less inflammatory activity is seen around the blood vessels in the primary progressive form than in the relapsing/remitting and secondary progressive forms. In the progressive forms, pathological studies show that the blood vessels become hypertrophic with thickening of the collagenous material around the blood vessel (Revesz *et al* 1994). There are fewer enhancing regions seen on Gd-DTPA MRI in the primary progressive form than in the relapsing and remitting forms. An inflammatory response is

seen around the blood vessels in the secondary progressive form, which is more than is seen in the primary progressive form (Revesz *et al* 1994). The differences in pathological findings in the various forms of MS have lead some investigators to question whether they are due to one disease process. Multiple forms of MS have been described based on the findings on the MRI and at autopsy (Wingerchuk *et al* 2001).

8. MECHANISMS OF DEMYELINATION

Proteolytic demyelination can occur after immunological and nonimmunological stimuli. Several neutral proteases have been shown to attack myelin. These enzymes are released into the extracellular space, where they can be activated. Animals that had been injected peripherally with purified protein derivative, and subsequently had an intraventricular challenge with the same antigen, showed widespread demyelination (Wisniewski and Bloom, 1975). The source of the demyelinating factors was shown to be macrophages that had been attracted to the sensitized regions, where they released neutral proteases that caused the demyelination. This was called "bystander demyelination" to distinquish it from an antigen-driven response. *In vitro* studies showed that macrophages collected from the peritoneum could be stimulated to release the proteolytic enzyme, plasmin. Plasmin was shown to be involved in the breakdown of lyophilized myelin with the generation of myelin basic protein (Cammer *et al* 1978). Complement was necessary along with the plasmin for the break down of myelin in unfrozen tissues. Agents that blocked plasmin were found to reduce the severity of EAE in experimental animals (Brosnan *et al* 1980).

MMPs have been shown to attack myelin *in vitro* with the production of myelin basic protein (Gijbels *et al* 1993; Chandler *et al* 1995). Microglia/ macrophages, which phagocytize fragments of myelin, are found in regions of myelin digestion. Since myelin basic protein is on the inner surface of the myelin sheath, the manner in which the proteases released in the extracellular space lead to the production of myelin basic protein is unclear. It is possible that free radicals and enzymes other than the MMPs participate in the disassembly of the extracellular matrix, exposing the inner sites to enzyme action.

Axonal damage also occurs in experimental demyelination and in MS (Ferguson *et al* 1997). A delayed hypersensitivity reaction due to intracerebral injection of BCG with a peripheral challenge lead to the MMP-mediated opening of the BBB (Matyszak and Perry, 1996). Synthetic inhibitors of MMPs blocked the injury to the BBB. Evidence for an effect of the MMPs on myelin came from the direct injection of MMPs into the white

matter, which resulted in axonal damage that could be blocked by MMP inhibitors (Newman *et al* 2001).

In acute MS lesions in humans, the leakage of plasma proteins into the brain was demonstrated with stains for complement (Gay and Esiri, 1991). Vessel wall damage was found in all acute plaques, and intramural deposition of complement was seen on smooth muscle components. Macrophages infiltrated the region of the plaques, and the blood vessels showed damage to the type IV collagen and laminin, suggesting disruption of the basal lamina. The mechanism of injury suggested that humoral, immune-mediated damage to the myelin was unlikely, but that the myelin was damaged as an innocent bystander rather than as a specific target for the inflammatory reactions. In a study of primary and secondary progressive forms of MS, the majority of the demyelinated plaques were of the chronic, inactive type with few oligodendrocytes and fibrous gliosis, suggesting a late stage of the illness (Revesz *et al* 1994). Thickened, hyalinized blood vessel walls were commonly seen. The secondary progressive MS group showed a higher number of active lesions that contained macrophages with digested myelin degradation products than the primary progressive group. They concluded that the pathological findings showed unequivocally that secondary progressive MS was an inflammatory disease.

Two reports have identified MMPs in acute and chronic MS. In one study, tissue was available from an acute case of MS, which could be frozen for immunostaining. In this tissue, the macrophages in an active MS lesions expressed MMPs, including MMP-1, -2, -3, and -9 (Maeda and Sobel, 1996). Another study of acute and chronic MS lesions observed MMP-7 and MMP-2, but failed to find MMP-3 (Anthony *et al* 1997). Levels of mRNA have been studied by quantitative RT-PCR from regions of normal-appearing white matter and MS lesions. Increased levels of mRNA for MMP-2, -3, -8, and -9 were detected in the regions with lesions, and mRNA for MMP-9 was elevated in normal-appearing tissues (Lindberg *et al* 2001). Immunohistochemistry showed MMP-9 in blood vessels in both normal and abnormal tissues. Levels of TIMPs were similar in control and MS patients, suggesting an imbalance between the proteases and their inhibitors. Since mRNA levels for several of the MMPs were elevated in the tissues but not in the cells in the CSF, an endogenous source was suggested for the MMPs.

A possible mechanism for MMP participation at several levels of the pathological changes can be formulated (Figure 1). Activated T lymphocytes adhere to the sensitized endothelial cells most likely at the venules. Through the secretion of MMPs, these cells cross the endothelial cells. Once within the brain, they release cytokines that activate the astrocytes and microglia, resulting in the amplification of the protease reaction. Astrocytes release MMP-9 and possibly MMP-2, while the microglial cells form MMP-3 and -9. Proteases and free radicals released in the process of the inflammatory

response enter the extracellular space in the white matter where they have access to the myelin sheaths with demyelination. Although several of the steps in this hypothetical mechanism remain to elucidated in vivo, there is sufficient evidence to support a prominent role for the MMPs in the overall process, and to support the use of inhibitors to the MMPs as therapeutic agents at multiple steps in the process leading to demyelination.

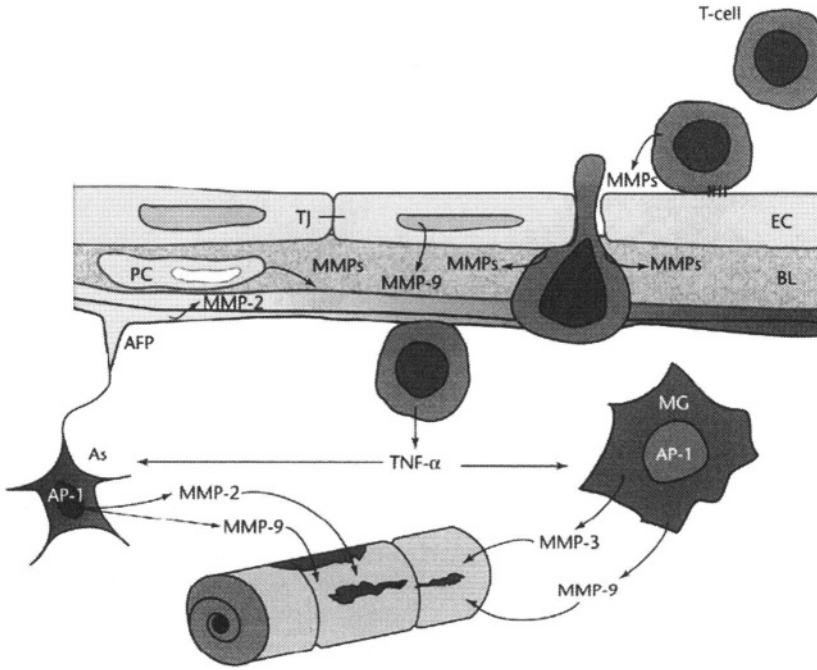

Figure 1: MMPs in MS. Activated T lymphocytes adhere to the sensitized endothelial cells, and secretion of MMPs allows them enter the parenchyma. Cytokines, tumor necrosis factor (TNF) and interleukins (IL) activate the astrocytes and microglia, which release MMP-9 and -2. Microglia cells (MG) release MMP-3 and -9. Proteases and free radicals released in the extracellular space cause demyelination.

9. THERAPEUTIC STRATIGIES TO CONTROL PROTEOLYSIS

Studies in rodents with EAE suggest that MMP inhibitors have a potential role in the treatment of the neuroinflammatory component of the MS attack. However, the current MMP inhibitors, which show excellent effects in animal models, have failed in clinical trials because of joint problems that develop with long-term use (Brown 1998). Tetracycline derivatives possess anti-

inflammatory properties, including suppression of the induction of the MMPs. One of the tetracycline derivatives, minocycline, which is used in the treatment of acne, has been shown to reduce the severity of illness in animals with EAE (Brundula *et al* 2002). These agents are now in clinical trials in several neurological illnesses. They appear to overcome the problems encountered with the use of hydroxymate-based inhibitors, which are poorly soluble compounds with low oral availability. Because the hydroxymates act by blocking the active zinc site of the MMP molecule, they are broad-based inhibitors of the MMPs. Newer agents are under study that are designed from computer models of the pockets into which the inhibitor needs to fit in order to block the active site. Some of these agents are more specific toward certain MMPs. In the treatment of cancer and arthritis, where chronic delivery of the drug is essential, the more specific agents may be beneficial in reducing side-effects. However, in the treatment of acute inflammation where short-term use of the agent may be adequate, a broad-based inhibitor may be sufficient.

Surrogate markers of proteolytic activity in the blood may provide a means for the rational use of the these agents. In the future, it may be possible to select treatments based on the serum levels of protein for the gelatinases or of mRNA levels in the circulating white blood cells, particularly in the acute phase of the illness. In spite of the major advances in our understanding of the MMPs, unraveling the complexity of the interactions of the proteases in the formation of the acute and chronic MS lesions will be a formidable task. Separation of the undesirable components from those that are beneficial will be needed. Studies on the role of inflammation and the blood vessels in MS, which were begun over one hundred years ago, need to continue with the molecular tools that are now available. Once these issues are resolved, the use of MMP inhibitors may be possible in the treatment of MS.

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Index

Aβ, see β-amyloid peptide
AD, *see* Alzheimer disease
ADAM, *see* A disintegrin and metalloproteinase
A disintegrin and metalloproteinase (ADAM)
  ADAM 8 (CD156), 163
  ADAM 10 (kuzbanian), 127ff, 188
  ADAM 17 (TACE), 127ff, 188
Adhesion molecules, 240f, 247, 249f, 324, 335
Advanced glycosylation end products, 184
AGEs, *see* advanced glycosylation end products
Alzheimer, Alois, 1
Alzheimerdisease, 1ff
  Braak staging, 9
  early onset, 7
  risk factor, 4, 220
  sporadic, 9
  therapy, 13f
Amyloid, 2, 179
Amyloid precursor protein, 2
β-Amyloid peptide, 2
Angiogenesis, 155, 159
Angiotensin, 196, 214ff
Angiotensin-converting enzyme (ACE), 3, 198, 216, 219f
Angiotensin-receptor, 214f
Antisense inhibition
  α2-integrin, 157

cathepsin B, 155
MMP-9, 155, 165
proteinase activated receptor 1, 331
uPAR, 157, 160, 165
Apoptosis
mechanisms, 70
APP, *see* amyloid precursor protein
Autoimmunity
Parkinson's disease, 33f
Multiple sclerosis, 110
β-site APP-cleaving enzyme, 5f, 13ff
BACE, *see* β-site APP-cleaving enzyme
Bacterial menningitis, 110, 114
Blood brain barrier, 109, 122, 124f, 326, 333ff, 351ff
Bradykinin
blood brain barrier, 333
metabolism by ectopeptidases, 216f
receptors, 216
Brain ischemia
activation of proteases, 64ff
intracellular Ca^{2+}, 64ff
neuronal death, 65
Brain tumor, 151ff, 158
Calcitonin-gene related peptide, 207ff
Calpain
activation, 64, 66, 69, 84
in Alzheimer disease, 7, 9
in brain ischemia, 64ff
in CNS infection, 117f
in CNS inflammation, 116f
in LTP,251
in Parkinson's disease, 37ff
inhibitors of, 39f, 46, 48f, 66f, 83
neuroprotection, 67f
prion protein processing, 189
structure of, 39
substrates of, 43ff, 66, 82, 116
synaptic activity, 309f
Calpastatin, 39, 41,89, 311
Caspases
activation by PrP^{Sc}, 192f
activation cascade, 69, 86f
in Alzheimer disease, 9

in brain ischemia, 68ff
inhibitors, 71
in multiple sclerosis, 111 ff
knock-out mice, 111, 114
substrates, 86
synaptic activity 310f
Cathepsin
Cathepsin A, 204
cathepsinB, 10, 12f, 84, 91ff, 119, 152ff
cathepsin C, 91
cathepsin D, 7f, 10, 12, 91ff, 153, 311ff
cathepsin E, 12,91
cathepsin G, 119
cathepsin H, 14, 153
cathepsin L, 12ff, 84, 91, 153
cathepsin G, 95
cathepsin S, 12ff, 153
cathepsin X, 84
in cancer progression, 154
CGRP, *see* calcitonin-gene related peptide
CJD, *see* Creutzfeldt-Jakob disease
Creutzfeldt-Jakob disease
sporadic (sCJD), 179ff
hereditary, 179ff
iatrogenic, 179ff
new variant (vCJD), 179ff
Cystatin, 13,91, 120, 154f
Death-inducing signaling complex, 70
Diabetes mellitus
risk for dementia, 4
dipeptidyl peptidase IV
function, 140
in CNS inflammation, 139ff
inhibitors, 141, 145
multiple sclerosis, 139ff
structure, 139
substrates, 140,218
T cell function, 141f
DISC, *see* death-inducing signaling complex
Down syndrom, 6, 9, 14
DP IV, *see* dipeptidyl peptidase IV (CD26)
EAE, *see* experimental autoimmune encephalomyelitis
ECM, *see* extracellular matrix

Elastase, 95
EMMPRIN, *see* Extracellular matrix metalloproteinase inducer
Enkephalin
  degrading enzymes, 211f
  precursor peptide, 205
  sequence, 208
Experimental autoimmune encephalomyelitis
  role of fibrin, 333
  mouse model, 109, 112, 333
  patients, 116
  rat model, 112
Extracellular matrix (ECM)
  degradation, 93, 139f, 254
  tumor cell function, 151
Fatal familial insomnia, 179, 183
FFI, *see* fatal familial insomnia
Gertsmann-Sträussler-Scheinker disease (GSS), 179, 183
Glioma, 151 ff
GSS, *see* Gertsmann-Straussler-Scheinker disease
6-hydroxydopamine (6-OHDA)
  model of Parkinson's disease, 29
IDE, *see* Insulin-degrading enzyme
Inflammation
  Parkinson's disease, 32ff
  Multiple sclerosis, 109ff
Inhibitors
  2,7-bis-(4-amidinobenzyliden)-cycloheptan-1 -one, 278
  α1-antichymotrypsin, 12,96
  α1-antitrypsin, 123
  α2-antiplasmin, 305
  α2-macroglobulin, 12,158
  AK275, 68
  AK295, 68, 83
  batimastat (BB-94), 125, 166
  BB-94, 256
  BB1101, 129
  BE16627B, 166
  CA-074, 91, 154f
  calpain inhibitor II, 83
  calpain inhibitor I, 67
  calpastatin, 39, 41, 89, 311
  captopril, 216
  CP-1,91

cystatins, 13, 91
D-Phe-Pro-Arg-chloromethylketone, 307
E-64-c, 67, 154f
FN-439, 316
GM-6001, 125
K11017, 155
leupeptin, 67, 278
Lys[Z(NO_2)]-thiazolidid, 141f, 145f
Lys[Z(NO_2)]-pyrrolidid, 141f, 146
marimastat, 166
M826, 71
MDL 28170, 46, 49, 67f, 83f
neuroserpin, 255, 285, 304
N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK), 96
serpin, 95, 285, 327f, 335
SJA6017, 83f
suramin, 161
TMC-2A, 143
TSL-225, 143
z-DEVD-fmk, 71,84, 88f, 311
z-FA-fmk, 84
Z-Phe-Gly-NHO-Bz, 153
z-VAD-fmk, 71,84, 88f, 114
z-YVAD-fmk, 84, 88
Integrin, 240, 250, 254
Insulin, 4
Insulin-degrading enzyme, 3f
Knock-out mice
caspase-1, 114
caspase-11, 111
cathepsin B, 12f
cathepsin D, 12f, 312ff
cathepsin L, 12f
Cln3, 314
CREB, 242
fibrinogen, 333
GAD65, 279, 283ff
Glu^{R1} (AMPA receptor), 246
insulin-degrading enzyme, 4
integrins, 254
interferon-γ, 111
L1 (adhesion molecule), 253
MMP-9, 94, 126, 256, 356, 358

NCAM, 252
neuropsin, 248
parkin, 35
plasminogen, 286ff
presenilin-1 and -2, 7
proteinase-activated receptor (PAR), 326, 331
prothrombin (cFII), 326
α-synuclein, 35
tau protein, 9
tissue-type plasminogen activator (tPA), 272ff, 281ff, 304
tumor necrosis factor-α, 111, 128
Kuru, 179f
Lewy bodies, 25, 44
Long Evans rat, 66
Long term potentiation
adhesion molecules, 247
dendritic calcium levels, 245f
phases, 241 ff
synaptic function, 243ff, 304
LTP, *see* long term potentiation
Mitogen-activated protein kinase (MAPK)
induction of atrial hypertrophy
Matrigel, 153, 155, 159f
Matrix metalloproteinase (MMP)
activation, 239
classification, 94, 121, 161, 190f, 238
gene expression, 94, 121, 161
inCSF, 191f
inhibitors, 166
knock-out mice, 94, 122, 126, 256, 356, 358
membrane-type (MT-MMP), 162, 166, 337f
natural inhibitors, 94, 121, 165, 190f, 239
neuronal plasticity, 316
regulation of activity, 94, 190f, 237
role in multiple sclerosis, 121ff
tumor growth, 164
Memory
adhesion molecules, 249
MMPs, 249f, 254ff
TPA, 255f
MMP, *see* matrix metalloproteinase
MPTP, *see* N-methyl-4-phenyl-l,2,3,6-tetrahydropyridine
MT-MMP, *see* MMP, membrane-type

Multiple sclerosis
animal model, 109, 359
cell death, 117, 119
disruption of blood brain barrier, 361ff
hallmarks, 111
fibrin deposition, 333
lesions, 112f, 353, 355, 364
pathogenesis, 109, 125, 354ff
patients, 111ff, 122
protease function, 120, 125, 355ff
Mutation
amyloid precursor protein, 7
presenilin-1 and -2, 7
prion protein, 168, 183ff
ubiquitin B, 9
Neovascularization, 160
Neprilysin, *see* neutral endopeptidase
N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)
model of Parkinson's disease, 31, 41
Neuritic plaque, 2
Neurofibrillary tangles, 8ff
Neurokinin, 208, 209ff
Neuronal plasticity, 235ff, 250
Neuropeptide
biosynthesis, 204f
processing, 204f
propeptide cleaving enzyme, 204f
Neuropeptide Y, 217f
Neuropsin, 307f
Neutral Endopeptidase
in Alzheimer disease, 3, 4f
neuropeptide processing, 210f, 219ff
Nociceptin, 213f
OD plasticity, *see* occular dominance plasticity
6-OHDA, *see* 6-hydroxydopamine
Opioid peptides, 205f, 211f
Opiate receptor, 206, 211
Ocular dominance plasticity, 276ff
Orphanin FQ, 213f
p35, 43f
p53, 46f
PAR, *see* proteinase-activated receptor
Paraquat, 31

Parkin, 35
Parkinson's disease
  clinical signs, 27
  disease rating index, 27
  history, 25f
  incidence of, 26
  Lewy bodies, 25f
  models of, 28ff
  morphology, 26
  therapy, 28
PC, *see* precursor convertase
Plasmin, 3
Plasminogen
  knock-out mouse, 286ff
  OD plasticity, 286ff
Plasminogen activator, 95, 156f
  cell migration, 272
  classification, 271
  developmental regulation, 280f
  gene expression, 276
  knock-out mouse, 272ff, 279, 281f, 304
  inhibitors, 277, 284ff, 304f
  memory, 273f
  neurite outgrowth, 273
  over-expression, 273
  receptor, 285f, 304
  seizure activity, 304ff
  substrates, 288f, 304
  synaptic plasticity, 304ff
  tPA inhibitors, 277, 278
  transgenic mice, 278ff
  visual cortex, 276ff
Plasminogen activator inhibitor (PAI), 156, 158f, 277, 304, 327
Polymorphism
  α1-antichymotrypsin, 12
  α2-macroglobulin, 12
  angiotensin-converting enzyme, 220
  cathepsin D, 7
  neutral endopeptidase, 5
  prion protein, 168
  ubiquitin B, 9
Precursor convertase (PC), 204f, 219ff
Prion disease

clinical classification, 179
molecular classification, 193ff
Prion protein
biosynthesis, 184
conformation, 183
copper binding, 181, 183
metabolism, 185f
polymorphism, 180
protease resistance, 183
proteolytic processing, 187f
PrP^{c}, 179ff
PrP^{Sc}, 179ff
shedding, 187ff
structure, 182
trafficking, 186f
Prion protein gene (PRNP)
mutation, 180, 182ff
physiological role, 181
polymorphism, 180
structure, 180f
PRNP, *see* prion protein gene
Prodynorphin, 205f
Proenkephalin, 205f
Proopiomelanocortin, 205f, 219f
Proteasome, 308ff
Proteinase-activated receptor
activation, 324, 325f, 329
classification, 323, 330
expression, 324ff
knock-out mouse, 326, 331
role in long term potentiation, 248
Reactive nitrogen species, 68f
Reactive oxygen species, 68f, 181, 358
RNS, *see* reactive nitrogen species
ROS, *see* reactive oxygen species
Rotenone, 31f
Secretase
α-secretase, 3f
β-secretase, 5f
γ-secretase, 6f
Shedding
of **TNF-α**, 127, 188
of IL-2 and IL-6 receptor, 95

of prion protein, 187
Single nucleotide polymorphism (SNP)
ACE promoter, 220
siRNA
cathepsin B, 165
MMP-9, 165
SLPI, *see* secretory leukocyte protease inhibitor
Somatostatin, 4
Substance P, 4, 206, 208, 209ff, 256
SUMO-1 and -2, 14
Stefin, *see* cystatin
Synaptic activity, 303ff
Tachykinin, 209ff
Tau protein, 9f, 189
TBI, *see* traumatic brain injury
TGF-β1, 95
Therapy
Alzheimer disease, 13ff
Parkinson's disease, 28
bacterial meeningitis, 126
multiple sclerosis, 365f
Thimet oligopeptidase, 3
Thrombin
activation, 327
angiogenesis, 337
blood brain barrier, 333f, 337f
coagulation cascade, 325
enhancement of adhesion, 324, 335
expression, 324f
receptor activation, 324
hypoxia, 327
TIMP, *see* tissue inhibitors of matrix metalloproteinase
Tissue inhibitors of matrix metalloproteinase
anti-oncogenic activity, 165
classification, 161
overexpression, 165
TNFα, *see* tumor necrosis factor-α
Transmissible spongiform encephalopathies, 179ff
Transgene expression
calpain, 41
calpastatin, 41
DM20 (ND4), 359
plasminogen-activator-1, 159

PTEN (phosphatase and tensin homologue), 165
α-synuclein, 45
tumor necrosis factor-α, 333
tPA, 248, 255, 306
Traumatic brain injury
calpain, 81ff
caspases, 85ff
cathepsins, 84
experimental models, 81
Thrombin, 307
TSE, *see* transmissible spongiform encephalopathies
Tumor necrosis factor-α, 70, 92, 95, 111,115, 128, 331, 333
Vascular endothelial growth factor (VEGF), 157f, 325