MODULATION OF NEURONAL PLASTICITY BY EXTRACELLULAR SERINE PROTEASES AND THEIR INHIBITORS

PROTEOLYTIC CONTROL OF NMDA RECEPTORS

INAUGURALDISSERTATION

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

Mirna Kvajo

aus Buje, Kroatien

Basel, 2004

Friedrich Miescher Institut

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf Antrag der Herren Prof. Dr. Denis Monard, Prof. Dr. Andreas Lüthi und Frau Prof. Dr. Silvia Arber.

Basel, den 08. Juni 2004.

Prof. Dr. Marcel Tanner

Acknowledgements

There are many people whom I would like to thank for their contribution to this work. First of all, I want to express my gratitude to Prof. Dr. Denis Monard, for giving me the opportunity to work in his laboratory, and especially for his constant support and encouragement throughout the years of my Ph.D., as well as for giving me the freedom to explore my own ideas. His support was essential for the progress of my project and development of my scientific interests.

I would like to thank the members of my Ph.D. thesis committee, Prof. Dr. Andreas Lüthi and Prof. Dr. Silvia Arber, for their comments and help during the course of the thesis.

I am especially grateful to all the collaborators in my project, who not only contributed experimentally, but also with their helpful and critical comments and fruitful discussions that shaped some of the ideas presented in this thesis.

Furthermore, special thanks goes to Sabrina Taieb, for her valuable mouse work, and for the great energy and dedication with which she fulfilled my “mouse wishes”. I would also like to thank Elisabeth Fries for technical assistance.

I am grateful to all my lab colleagues for their company and discussions, which contributed to my project. In particular, I would like to thank Xiaobiao Li for her suggestions, Maddalena Lino for her friendship, scientific interest and encouragement, and the great atmosphere she creates, and Marita Meins, for making me feel welcome in the lab, her support and help, and for our discussions on many shared scientific and non-scientific interests.

I am especially grateful to my parents, whom I can thank for setting up the basis of my scientific curiosity and who have always strongly encouraged me in pursuing my

ideas and wishes. Their constant love and support was crucial for my stay in Basel, and the development of my Ph.D.

Finally, all my gratitude goes to Ulrich Hengst for being my lab colleague, friend and partner. I want to thank him for his professional interest and scientific help, but also for his unconditional love, patience and support, that made all the difference in my work.

Table of contents:

Abbreviations

cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
EPSP	Excitatory postsynaptic potential
ECM	Extracellular matrix
LTP	Long term potentiation
LRP	Low density lipoprotein-related protein receptor
NLS	Nucleolar localization signal
NMDA	*N*-methyl-D-aspartate
PAR-1	Protease activated receptor-1
PCR	Polymerase chain reaction
PN-1	Protease nexin-1
$PN\text{-}1^{-/-}$	PN-1 knock-out mouse
$PN\text{-}1^{+/+}$	PN-1 wild-type mice
PBS	Phosphate buffered saline
SDS	Sodium dodecyl sulphate
SEP	Sensory evoked potentials
tPA	Tissue plasminogen activator
uPA	Urokinase plasminogen activator
X-gal	5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside

Summary

Serine proteases are enzymes catalyzing protein cleavage. Because of their capability to cleave a wide variety of substrates, they are involved in many critical physiological processes such as digestion, hemostasis, reproduction and immune response, as well as in developmental processes, signal transduction and apoptosis. In the last years, there is also increasing evidence for their role in the control of plasticity-related events in the adult and developing central nervous system, and involvement in phenomena such as memory and learning.

Protease nexin-1 (PN-1) is a 43 kDa glycoprotein belonging to the serpin superfamily. It strongly inhibits the activity of several serine proteases such as thrombin, tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA), trypsin and plasmin. PN-1 has a complex spatial and temporal expression pattern in the adult and developing nervous system, and its expression is enhanced upon CNS lesions. Mice lacking PN-1 have reduced long-term potentiation (LTP) in the hippocampus, and are more prone to seizures, while mice overexpressing it develop a progressive neurodegenerative disorder. These findings suggest a role for PN-1 in brain function and homeostasis.

In this thesis the role of PN-1 in adult brain plasticity was examined. Activity-dependent expression of PN-1 was demonstrated using an *in vivo* reporter system. PN-1 was found to be crucial for the control of brain proteolytic activity, and PN-1$^{-/-}$ mice displayed decreased levels of the NR1 subunit of the *N*-methyl-D-aspartate (NMDA) receptor in the cortex and hippocampus. Electrophysiological examination of adult PN-1$^{-/-}$ mice revealed decreased NMDA receptor signaling in the barrel cortex, and decreased sensory evoked potentials upon whisker stimulation. Behavioral tests showed that PN-1$^{-/-}$ mice also displayed impaired whisker-dependent sensory motor function. Thus, a tight control of serine protease activity was shown to be critical for the *in vivo* function of NMDA receptors, and the proper function of sensory pathways.

1. Introduction

1.1 Neuronal plasticity in the adult CNS

Not long ago the mature central nervous system was predominantly considered as a static structure in which very little or no structural changes take place. Today, however, it is generally accepted that the brain, especially its cortex, is a dynamic construct, which can be profoundly remodeled by experiences throughout life (Buonomano and Merzenich, 1998; Garraghty and Kaas, 1992; Weinberger, 1995). Brain plasticity can be observed at different levels, from activity-dependent changes in the expression of molecules and intracellular signaling cascades (Barth et al., 2000; Staiger et al., 2000; Brown and Dyck, 2002; Quinlan et al., 1999), to morphological modifications of neurons (Yuste and Bonhoeffer, 2001; Rampon et al., 2000b; Knott et al., 2002; Magarinos et al., 1997) and brain structures (Buonomano and Merzenich, 1998). Currently, considerable research is aiming at decoding the neuronal basis of this plastic potential, and particularly at understanding the mechanisms behind it. As a result of these efforts, we have learned a great deal about the way neuronal activity translates into such functional changes. By now it also became clear that such alterations can at least partially account for phenomena such as memory and learning (Silva et al., 1998; Chen and Tonegawa, 1997), as well as for encoding sensory information (Fox, 2002). However, establishing a causal relationship between forms of plasticity and these events has proven to be a difficult task, and still much work is needed to understand all of its aspects.

One way to learn more about the mechanisms of activity-dependent processes is by studying the molecules regulating them. In the last decade a vast amount of work has extended our understanding of the molecular basis of plasticity. However, while in the past most of the research focused on intracellular mechanisms and their regulators such as ion channels, receptors and structural proteins (Chen and Tonegawa, 1997; Lisman and McIntyre, 2001; Fox, 2002), more recently extracellular and cell-surface

interactions came in the center of attention. A large proportion of brain volume is thought to consist of extracellular space, filled by an extracellular matrix (ECM) composed of collagens, proteoglycanes and glycoproteins, and a vast number of other molecules with different, and still largely undefined functions (Ruoslahti, 1996). The ECM plays a prominent role in tissue architecture and homeostasis. Molecules of the matrix not only interact with each other, they also activate signal transduction pathways through various cell-surface receptors. These pathways orchestrate inputs from diverse ECM molecules controlling the stability of cell environment, and enabling proper cell-cell communications to take place (Dityatev and Schachner, 2003). Accordingly, many studies have shown their importance for cell proliferation, migration, as well as morphological and biochemical differentiation of cells (Jones and Jones, 2000; Nishio et al., 2003; Pesold et al., 1999). It is, thus, not surprising that ECM and cell-surface interactions caught the attention as potential determinants of adult brain plasticity (Dityatev and Schachner, 2003; Wright et al., 2002), bringing molecules controlling them into the research spotlight.

1.2 Extracellular serine proteases

As described above, plastic changes are characterized by structural and morphological remodeling, requiring rapid changes in the ECM and receptor signaling (Dityatev and Schachner, 2003; Pizzorusso et al., 2002). Thus, extracellular enzymes catalyzing protein modifications emerged as likely candidates for the control of such events. In particular, serine proteases, capable of cleaving a great variety of substrates, may play important roles in the plastic remodeling of the central nervous system.

1.2.1 Structure and properties of serine proteases

Serine proteases (Hedstrom, 2002) constitute one third of all proteases found in nature. This mechanistic class of enzymes was originally defined by the presence of three residues, aspartate, histidine and serine in the catalytic site, forming a hydrogen bonding system often referred to as the “charge relay system” or “catalytic triad”. The

triad can be found in at least four different structural contexts, indicating that this catalytic machinery has evolved on at least four separate occasions. These four clans of serine proteases are typified by chymotrypsin, subtilisin, carboxypeptidase Y, and the Clp protease, according to the MEROPS protease database nomenclature (Rawlings et al., 2004).

Serine proteases associated with the central nervous system such as thrombin, tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA) and plasmin, belong to the chymotrypsin-like proteases. In general, proteases of this class are the most abundant serine proteases in nature, and can be found in eukaryotes, prokaryotes, archae and viruses (Patthy, 1985). Because of their capability to cleave a wide variety of substrates (Perona and Craik, 1995; Hedstrom, 2002), they are involved in many critical physiological processes including digestion, hemostasis (Neurath, 1984), reproduction (Barros et al., 1996), immune response (Sim and Laich, 2000) as well as signal transduction (Coughlin, 2000) and apoptosis (Johnson, 2000) (Table 1). Furthermore, cascades of their sequential activation drive developmental processes (LeMosy et al., 1999), matrix remodeling (Van den Steen et al., 2001), differentiation (Selvarajan et al., 2001) and wound healing (Li et al., 2003).

Table 1. Representative Mammalian Chymotrypsin-Like Proteases (adapted from: (Hedstrom, 2002))

digestive proteases	**blood coagulation**
chymotrypsin	coagulation factor VIIa
trypsin	coagulation factor IXa
pancreatic elastase	coagulation factor Xa
immune response	coagulation factor XIIa
tryptase	thrombin
complement factor D	protein C
complement factor B	**fibrinolysis**
complement factor C	urokinase
complement component 2	tissue plasminogen activator
mast cell protease	plasmin
cathepsin G	kallikrein
neutrophil elastase	**reproduction**
	prostate specific antigen
	acrosein

Chymotrypsin-like proteases are synthesized as inactive precursors (zymogens), which are activated by a proteolytic removal of an N-terminal extension peptide. Structurally, these are mosaic proteins, composed of several distinct modules. Their molecule is generally divided into the substrate-recognition domain, the catalytic, and the zymogen activation domain (Bode and Renatus, 1997). In addition to these proteolysis-related parts, auxiliary regulatory domains (e.g. kringle, epidermal growth factor-like domains) are often present (Patthy, 1993).

The tertiary structure of this family of proteases is best presented on the example of chymotrypsin (Figure 1) (Blow, 1968). Chymotrypsin has 245 residues arranged in two six-stranded ß-barrels. The active site with the catalytic triad is located between the two barrels. In the vicinity is the "oxyanion hole", which is important for the stabilization of the substrate-protease interaction during cleavage (Stubbs et al., 1998).

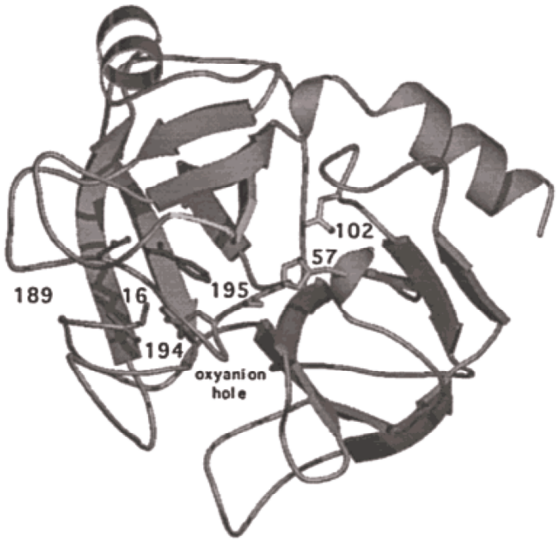

Figure 1. The structure of chymotrypsin. The ß-barrels are depicted as arrows. The numbers denote residues involved in the formation of the catalytic triad and the oxyanion hole (Hedstrom, 2002).

1.2.2 The catalytic mechanism of serine proteases

The catalytic mechanism of cleavage mediated by serine proteases (Figure 2) (Hedstrom, 2002), starts with the binding of the substrate to the active site. In the acylation half of the reaction, the active site serine residue attacks the carbonyl of the substrate, assisted by the active site histidine acting as a general base. This leads to the formation of a tetrahedral intermediate and an imidazolium ion. The oxyanion of the tetrahedral intermediate is stabilized by interaction with the main chain amines of the oxyanion hole. This intermediate breaks down, assisted by histidine-H+ acting as a general acid, to yield the acylenzyme intermediate, an imidazole base and release of an alcohol or amine. The acylenzyme is hydrolyzed through the reverse reaction pathway with the hydroxyl group of water attacking it, assisted by the active site histidine, yielding a second tetrahedral intermediate. This intermediate collapses, expelling the active site serine and carboxylic acid product.

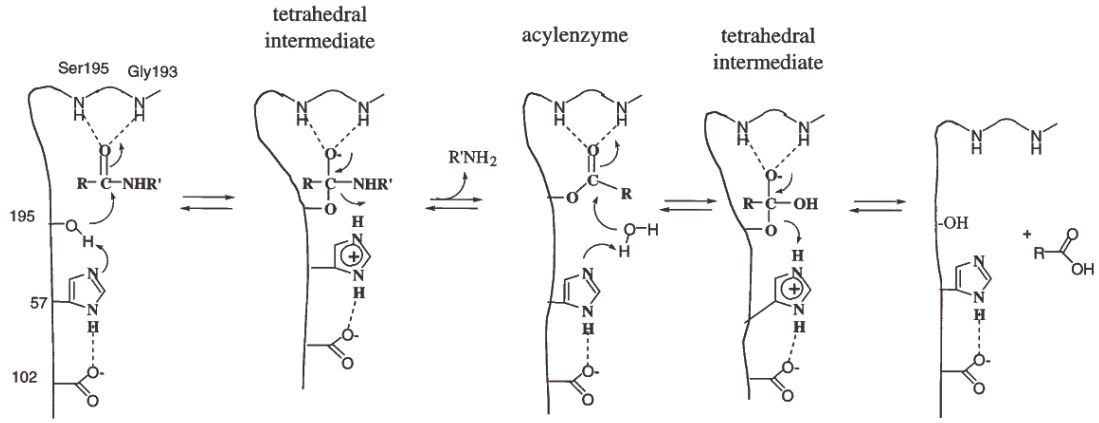

Figure 2. The catalytic mechanism of serine proteases, showing the sequence of events upon binding of the substrate to the active site (Hedstrom, 2002).

1.2.3 Specificity of serine proteases

As all serine proteases use a very similar catalytic mechanism, the specificity for different substrates is, in first place, controlled by the substrate recognition sites on the protease. These sites include a polypeptide binding site and the binding pockets for the side chains of the substrate. Studies of specificity were based on a model defining the amino-acid side chains to both sides of the scissile peptide bond as $P_1 \ldots P_n$ toward the amino-terminus, and $P_1' \ldots P_n'$ towards the carboxy-terminus (Schechter and Berger, 1967). The substrate specificity is thus defined by the amino acid in the P_1 position that can fit in the binding site. Structural studies have shown that chymotrypsin prefers bulky, aromatic residues, like tryptophane, tyrosine, phenylalanine or leucine, trypsin positively charged, such as argining and lysine, while elastase favours small, non-charged amino acid residues, like valine and alanine (Hedstrom, 2002).

1.2.4 Serine proteases in neuronal plasticity

The early observation that many cell types in the adult central nervous system express serine proteases (Sappino et al., 1993; Ware et al., 1995; Basham and Seeds, 2001), was rather unexpected, as these enzymes were considered to act mainly in blood homeostasis, and in developmental and pathological processes that involve extensive tissue remodeling. Thus, their earliest proposed function was a neuroprotective role during states such as stroke and brain injury (Gingrich and Traynelis, 2000). However, it has been soon recognized that their versatility, broad range of substrates and complex activation cascades makes them good candidates for the regulation of the plasticity-related events.

By now several brain-derived serine proteases have been identified and characterized. Some, such as tissue plasminogen activator, urokinase plasminogen activator

(Sappino et al., 1993), and neurotrypsin (Gschwend et al., 1997), show prominent activity and are expressed throughout the brain, while others, like neuropsin (Hirata et al., 2001), are localized to a specific brain region, such as the hippocampus. Most of the research focused on tissue plasminogen activator, which is at present the best-characterized serine protease in the brain, exemplifying their functions in plasticity-related events. tPA has been first characterized for its role in the conversion of plasminogen into plasmin (Bode and Renatus, 1997), a serine protease with broad substrate specificity, and has since then also been attributed the degradation of some substrates involved in synaptic plasticity, such as laminin (Indyk et al., 2003) and the NMDA receptors (Nicole et al., 2001), and was implicated in the regulation of cell-surface receptor-mediated neuronal signaling (Zhuo et al., 2000).

tPA mRNA and activity can be found throughout the brain (Sappino et al., 1993), with highest levels in the hippocampus (Salles and Strickland, 2002), hypothalamus (Sappino et al., 1993), cerebellum (Seeds et al., 1995) and the amygdala (Pawlak et al., 2003). Cell expression studies revealed that tPA is expressed by various cell types such as neurons and glial cells (Siao et al., 2003; Salles and Strickland, 2002). Within the cell it is produced and stored in secretory vesicles (Gualandris et al., 1996; Lochner et al., 1998; Parmer et al., 1997), which appear to be preferentially transported to lamellipodia and growth cone, suggesting a role in axonal elongation and migration (Krystosek and Seeds, 1981). Interestingly, the release and expression of tPA from secretory vesicles was shown to be dependent on neuronal activity, which was one of the first indications for its involvement in activity-dependent neuronal remodeling. This was found in cell culture studies, where the release could be induced by depolarization (Gualandris et al., 1996; Parmer et al., 1997), and in the hippocampus where tPA upregulation was correlated with electrical stimulation (Qian et al., 1993). tPA expression was also increased after complex motor learning tasks in cerebellar Purkinje cells (Seeds et al., 1995), and upon stress in the amygdala (Pawlak et al., 2003). In addition, it was strongly upregulated in pathological models such as the excitotoxic injury in the hippocampus (Salles and Strickland, 2002) and seizures

(Yepes et al., 2002; Qian et al., 1993). Interestingly, kainic acid-induced seizures could also strongly upregulate the transcription of the serine protease precursor plasminogen (Sharon et al., 2002), suggesting that serine proteases may be commonly regulated by neuronal activity.

Several studies provided evidence for a role for tPA in synaptic plasticity. Especially well characterized was its effect on the hippocampal long-term potentiation (LTP). LTP is a long-lasting, activity-dependent increase in the efficiency of synaptic transmission (Bliss and Collingridge, 1993; Bi and Poo, 2001), and is considered to be a cellular model for certain forms of memory and learning (Martin et al., 2000). The first indications that tPA modulates this type of synaptic facilitation came from studies showing a selective reduction of LTP in hippocampal slices derived from tPA deficient mice (Calabresi et al., 2000; Frey et al., 1996; Huang et al., 1996). In line with these results, LTP was also shown to be enhanced in slices from mice overexpressing tPA (Madani et al., 1999), and upon *in vitro* application of tPA in wild-type hippocampal slices (Baranes et al., 1998). Furthermore, mice overexpressing tPA exhibited improved learning capacity (Madani et al., 1999). Interestingly, when tPA deficient mice were tested for hippocampus-dependent functions such as learning and memory, controversial results were obtained. One study reported normal functions (Huang et al., 1996), while another one decreased hippocampal learning (Calabresi et al., 2000). However, both communications described a disruption of striatum-dependent learning, which is in line with a later published report showing decreased corticostriatal LTP in these tPA deficient mice (Centonze et al., 2002). Further indications that tPA is involved in activity-dependent processes came from studies in the amygdala, where it has been implicated in the control of stress-induced neuronal remodeling and anxiety-like behavior (Pawlak et al., 2003), and in the cerebellum where it is needed for complex motor learning (Seeds et al., 2003). Other findings further supported the functions of serine proteases in plasticity. For instance, mice overexpressing uPA in the brain showed impaired learning (Meiri et al., 1994), while neuropsin deficient mice had abnormalities in

synapses and neurons (Hirata et al., 2001). Interestingly, in humans, a truncating mutation in neurotrypsin was linked to mental retardation (Molinari et al., 2002).

Serine proteases are not only crucially needed for numerous physiological processes, but are also a potential hazard. If uncontrolled, they can destroy the extracellular components of cells and tissues, induce aberrant intracellular signaling (Barnes et al., 2003; Carden et al., 1998; Murer et al., 2001; Naruse et al., 1999), and apoptotic cell death (Siao and Tsirka, 2002; Choi et al., 2003). For these reasons, their activity has to be tightly controlled. Several levels of control have been described. First, as mentioned earlier, the expression and release of serine proteases can be regulated by external stimuli (Gualandris et al., 1996). Second, they can be released as an inactive zymogen, which becomes activated only in the presence of other serine proteases (Bode and Renatus, 1997; Stubbs et al., 1998). Finally, the third level of control is achieved with specific serine protease inhibitors that spatially and temporally regulate their activity, thereby preventing excessive degradation of target molecules (Barrett and Salvesen, 1986).

1.3 Serine protease inhibitors-Serpins

Serine protease inhibitors are grouped into several families. One of the most prominent belongs to the serpin (**ser**ine **p**rotease **in**hibitors) superfamily of structurally similar proteins with diverse functions (Gettins, 2002; Silverman et al., 2001; Ye and Goldsmith, 2001). The serpin superfamily includes protease inhibitors such as protease nexin-1 (PN-1), α1-antitrypsin, antithrombin III, α1-antichymotrypsin and plasminogen activator inhibitor (PAI-1), as well as noninhibitory members such as ovalbumin and angiotensinogen. Serpins are very widely distributed among eukaryotes. They are found in animals and higher plants, but are absent in fungi (Irving et al., 2000). Their existence has been reported in prokaryotes (Irving et al., 2002), and in some viruses (Irving et al., 2000). Serpins are commonly found extracellulary, but some are also intracellular, and others can exist in both intra- and extracellular forms (Mikus et al., 1993; Belin et al., 1989).

Structurally, serpins are small glycoproteins (*M*r 40 000 - 60 000) with a single polypeptide chain and a variable number of oligosaccharide side-chains (Carrell and Boswell, 1986). Their secondary structure is highly conserved, with a single core domain consisting of three ß-sheets, eight to nine α-helices and a reactive center loop (RCL) (Figure 3). The reactive center loop, which contains the protease recognition site, is an exposed, flexible stretch of approximately 17 residues tethered between two ß-sheets.

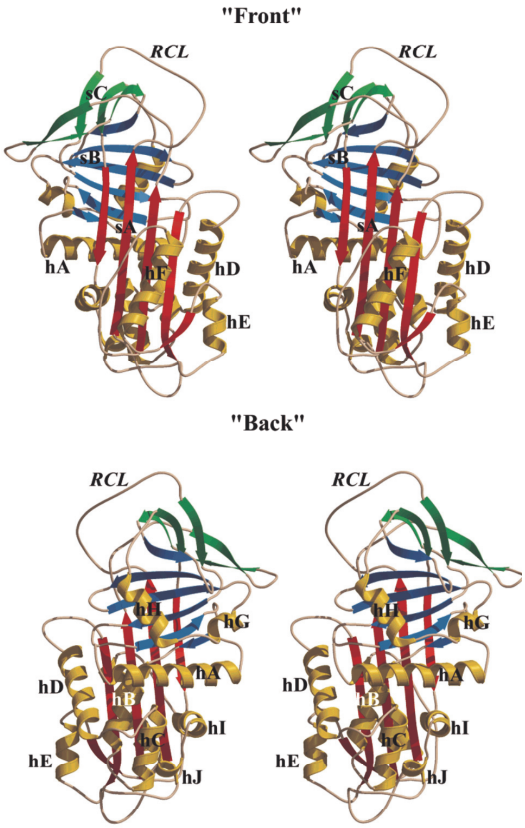

Figure 3. Front and back stereoviews of a typical serpin, illustrating the secondary structural features. ß-sheets (sA, sB, sC), are shown in red, blue and green, respectively. The eight α-helices are labeled hA-hH. The reactive center loop (RCL) is indicated in grey (Gettins, 2002).

Most inhibitory serpins control the function of chymotrypsin-like serine proteases, although cross-class inhibitors, targeting cysteine proteases, caspases and cathepsins,

have also been identified (Gettins, 2002). Their inhibitory mechanism has several remarkable features stemming from their structure. Conformational studies have shown that the active-form structure of serpins is thermodynamically less stable then the conformation adopted in an inhibitory complex (Silverman et al., 2001), and that such metastable conformation is required for their inhibitory activity (Carrell et al., 1991). The inhibition requires several steps during which there is a dramatic structural and thermodynamic change within the molecule, leading to a stable, latent complex. As shown in the A-E panels of Figure 4, the binding of the protease to the reactive loop center (A) leads to the insertion of the loop between the ß-sheets of the inhibitor (B) and to a partial displacement of one α-helix (C), needed to permit the movement of the protease toward the bottom of the serpin (D). When the final complex is formed, the α-helix has returned to its previous position but the protease has been distorted, and consequently fully inactivated (E). As a result, the serpin-protease complex is effectively irreversible in nature (Gettins, 2002).

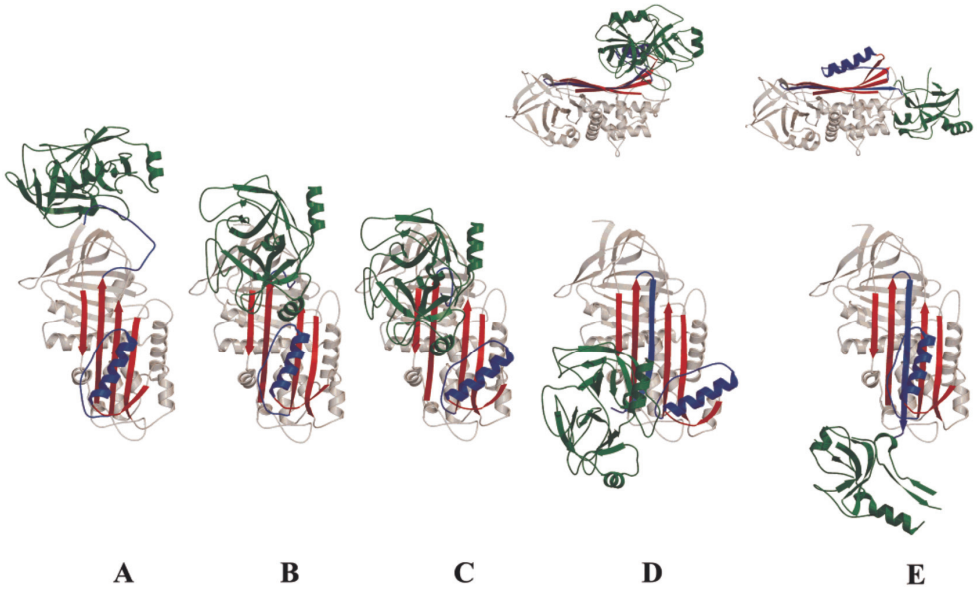

Figure 4. A schematic representation of the conformational states of the serpin-protease complex during inhibition. Individual panels show the changes in conformation described in the text (Gettins, 2002).

A number of studies addressed the fate of serpin-protease complexes. Their half-life was shown to be rather short, compared to the half-life of native serpins (Collen and Wiman, 1979; Ohlsson and Laurell, 1976), suggesting their rapid removal from the extracellular matrix. Further research revealed a specific clearance mechanism relying on receptors present on the cell surface. These are LRP receptors, members of the LDL receptor-related family of proteins (Conese et al., 1995; Kasza et al., 1997; Knauer et al., 1997; Knauer et al., 1999). LRPs are multifunctional receptors, whose main function appears to be the binding and internalization of a broad range of ligands, mainly associated with lipoprotein and protease metabolism, with the purpose of their subsequent degradation (Strickland et al., 1990; Herz and Strickland, 2001). The relevance of this receptor-mediated clearance for protease-inhibitor homeostasis has been first established by *in vitro* studies showing that a rapid internalization from the cell surface is crucial for the regulation of extracellular proteolytic activity (Zhang et al., 1998). However, recent studies suggested that the interaction of LRP receptors and proteolytic complexes may not only have a role in their clearance, but also influence intracellular processes such as neurotransmission (Zhuo et al., 2000; Bacskai et al., 2000), vascular permeability (Yepes et al., 2003) and the expression of other proteases (Wang et al., 2003).

There are several serine protease inhibitors expressed in the brain, such as neuroserpin (Krueger et al., 1997), plasminogen activator inhibitor-1 (PAI-1) (Sharon et al., 2002) and protease nexin-1 (Mansuy et al., 1993). Although the properties and functions of serine proteases in CNS processes have been by now well characterized, the exact roles of serine protease inhibitors in the brain, and the importance of the control of extracellular proteolysis for CNS function is just beginning to be recognized.

1.3.1 Protease nexin-1

Protease nexin-1 (PN-1) is a potent serine protease inhibitor belonging to the serpin superfamily (Gloor et al., 1986; Sommer et al., 1987; Guenther et al., 1985). PN-1, a glycoprotein of 43 kDa, has been first characterized by its ability to induce

morphological differentiation in neuroblastoma cells (Monard et al., 1973), through thrombin inhibition (Guenther et al., 1985; Stone et al., 1987). Further biochemical and kinetic studies established PN-1 as the strongest physiological thrombin inhibitor. In addition, PN-1 strongly inhibits uPA (Crisp et al., 2002), tPA, plasmin and trypsin (Stone et al., 1987), but not chymotrypsin and elastase (Scott et al., 1985).

PN-1 is a secreted protein associated with the extracellular matrix (Halfter et al., 1989; Farrell et al., 1988) where it can bind cell-surface proteoglycans (Herndon et al., 1999), heparin (Halfter et al., 1989), vitronectin (Rovelli et al., 1990), and collagen type IV (Donovan et al., 1994). Interestingly, binding to heparin dramatically increases the affinity of PN-1 for thrombin (Scott et al., 1985). PN-1, alone or in complex with serine proteases, also binds to the LRP receptor which mediates its clearance from the cell surface (Kasza et al., 1997).

In vitro, PN-1 is secreted by a wide variety of cell types, including fibroblasts, astrocytes, glioma (Guenther et al., 1985), neuroblastoma (Vaughan and Cunningham, 1993), astrocytoma (Kasza et al., 2001), and primary Schwann cells (Bleuel et al., 1995). In the developing and adult central nervous system PN-1 has a very complex spatial and temporal expression pattern. During embryonic development it is present in several brain structures. Its expression reaches its peak during early postnatal age, with a sharp decline towards adulthood (Mansuy et al., 1993). In the mature intact nervous system it is mainly expressed by neuronal cells (Mansuy et al., 1993; Reinhard et al., 1988; Reinhard et al., 1994), being especially prominent in the olfactory system (Reinhard et al., 1988) and in cortical cell populations such as layer V pyramidal neurons (Mansuy et al., 1993).

The transient and variable expression of PN-1 during development suggests a structure- and function-dependent expression. This is further emphasized by experimental data showing that PN-1 expression can be modulated in response to pathological states. For example, *in vitro* injury models of dorsal root ganglion explants, sciatic nerve segments and isolated Schwann cells provided evidence for

enhanced PN-1 transcription, regulated through angiotensin II receptors (Bleuel and Monard, 1995). These results have been complemented by *in vivo* studies showing that a lesion of rat sciatic nerve leads to a large increase in PN-1 expression in cells localized distal to the lesion site (Meier et al., 1989). Interestingly, when facial nerve lesion was inflicted, PN-1 levels in the facial nucleus were decreased (Niclou et al., 1998). PN-1 was also upregulated upon lesions in the CNS, for example after the toxic lesion of the substantia nigra (Scotti et al., 1994), and during selective degeneration of CA1 hippocampal cells induced by transient forebrain ischemia (Hoffmann et al., 1992). In addition, PN-1 expression levels were also modified in neuropathologies such as Alzheimer's disease (Vaughan et al., 1994; Choi et al., 1995) and scrapie (Cavallaro et al., 1999).

PN-1 expression during pathological states may be regulated by some of the factors identified by cell culture studies. In particular, molecules secreted during injury and inflammation, such as cytokines interleukin-1 (IL-1) and tumor necrosis factor (TNFα), and growth factors such as transforming growth factor (TGF) and platelet-derived growth factor (PDGF) strongly stimulated the secretion of PN-1 in a mixed neuronal-glial cell line (Vaughan and Cunningham, 1993) and in human fibroblasts (Guttridge et al., 1993). Similarly, interferon gamma and epidermal growth factor (EGF) regulated the expression of PN-1 in human astrocytoma cells (Kasza et al., 2001). Furthermore, fibroblast growth factor 2 (FGF-2) maintained PN-1 expression in primary cultures of met-/mesencephalic cells (Kury et al., 1997). To gather more information about the molecular mechanisms controlling PN-1 expression, studies of the *PN-1* gene promoter structure and activity were performed, revealing a very complex molecular organization. Binding sites for several transcription factors were found, such as those belonging to the Krox family (Krox-24, Krox-20 and NGFI-C), the Upstream regulatory factor (USF), Engrailed transcription factors (En), as well as SP1 binding sites, and an octamer binding site for POU transcription factors (Mihailescu et al., 1999; Erno and Monard, 1993; Erno et al., 1996a; Erno et al., 1996b).

The dynamic expression of PN-1 and its inhibitory properties suggested an involvement in brain homeostasis and function. Thus, to gain further insights about its roles, transgenic mouse models were created, namely mice overexpressing PN-1 in postmitotic neurons, under the control of the Thy1 promoter, and mice lacking the *PN-1* gene (Luthi et al., 1997). Both mouse models showed normal development and brain morphology, and no gross differences in behavior and appearance. However, electrophysiological studies revealed enhanced LTP and compensatory changes in the GABA-mediated inhibition in hippocampal slice preparations from PN-1 overexpressing mice. PN-1$^{-/-}$ mice-derived hippocampal slice displayed reduced LTP, which could be correlated with decreased NMDA receptor signaling (Luthi et al., 1997). Both lines were more susceptible to kainic-acid induced seizures, and had increased *in vitro* epileptiform activity (Luthi et al., 1997). Further studies on adult PN-1 overexpressing mice revealed a progressive neuronal and motor dysfunction, with histopathological changes reminiscent of the early stages of the human motoneuron disease (Meins et al., 2001). The mechanisms leading to these changes were not clear, but they possibly originated from synaptic alterations.

1.4 NMDA receptors

The discovery that adult PN-1$^{-/-}$ mice showed decreased NMDA (*N*-methyl-D-aspartate) receptor signaling (Luthi et al., 1997), suggested that changes in brain proteolytic activity can affect the electrophysiological properties of neurons. NMDA receptors are cation channels gated by glutamate, the main excitatory neurotransmitter in the mammalian CNS (Dingledine et al., 1999). They belong to a family of ionotropic glutamate-gated receptor ion channels, sharing structural and pharmacological similarities with AMPA and kainate receptors (Figure 5). Their signaling properties are controlled by well-defined ligands, thus the way through which extracellular proteolytic activity could alter their function is enigmatic. However, two *in vitro* studies offered an attractive mechanism, showing that the

serine proteases tPA and thrombin can induce a cleavage in one subunit of the receptor (Gingrich et al., 2000; Nicole et al., 2001) and change its signaling (Nicole et al., 2001). Additionally, binding to the thrombin receptor PAR-1 (Gingrich et al., 2000) and the LRP receptor (Bacskai et al., 2000), were also shown to influence NMDA receptor signaling *in vitro*.

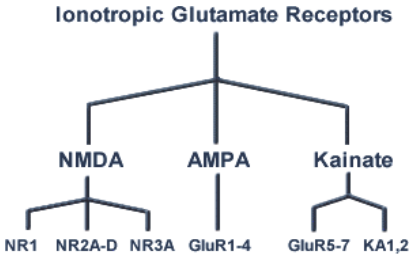

Figure 5. Classification of the ionotropic glutamate receptors.

The interest in NMDA receptors as targets of serine proteases is highlighted by the fact that these receptors are vital for brain function (Forrest et al., 1994), and central to many activity-dependent changes in synaptic strength and connectivity that are thought to underlie the formation of memory and learning (Bliss and Collingridge, 1993). Furthermore, extensive evidence showed that changes in NMDA receptor activity can determine neuronal survival and apoptosis (Hardingham and Bading, 2003), as well as thalamocortical patterning during development (Iwasato et al., 2000). NMDA receptors were also implicated in a whole range of pathological states such as ischemic stroke (Hoyte et al., 2004), neuropathic pain (Parsons, 2001), Parkinson's disease (Loopuijt and Schmidt, 1998), and schizophrenia (Mohn et al., 1999), thus representing particularly interesting targets for the development of pharmacological agents.

The diverse and critical functions of NMDA receptors are stemming from their unique functional and structural features (Dingledine et al., 1999). One of them is their dual dependence on agonist binding and membrane potential. The physiological agonist of NMDA receptors is glutamate, with glycine as an essential co-agonist (Benveniste

and Mayer, 1991). At resting membrane potentials NMDA receptors cannot be activated because they undergo a rapid channel block by extracellular Mg^{2+}, which considerably reduces their contribution to synaptic currents. When neurons become depolarized, the voltage-dependent block by Mg^{2+} is partially relieved, allowing ion influx through activated receptors. The resulting Ca^{2+} entry can trigger a variety of intracellular signaling cascades which can ultimately change neuronal function through activation of various kinases and phosphatases (Dingledine et al., 1999). Such a mechanism of action and other unique features, like the very slow activation and deactivation kinetics (Wyllie et al., 1998) and very high Ca^{2+} permeability (Rogers and Dani, 1995), have attracted considerable interest, since they associate NMDA receptors with molecular basis regulating synaptic plasticity (Ozawa et al., 1998).

The functional diversity of NMDA receptors was revealed with a discovery of several distinct receptor subtypes (Cull-Candy et al., 2001) whose different properties were found to depend on subunit composition. All NMDA receptors function as heteromeric assemblies of several distinct subunits: the ubiquitously expressed and obligatory NR1 subunit, a family of four separate NR2 subunits, and two NR3 subunits (Figure 5) (Das et al., 1998; Moriyoshi et al., 1991; Sugihara et al., 1992). Each of these subunits also occurs in different isoforms, owing to the presence of alternative splicing sites (Dingledine et al., 1999). One indicator of the functional consequences of NMDA receptor heterogeneity came from studies examining the expression of the different subunits and isoforms. These experiments revealed that the NR1 subunit is ubiquitously present throughout the brain, while the other subunits and their subtypes display a differential distribution, with patterns of expression changing strikingly during development (Mori and Mishina, 1995; Farrant et al., 1994; Petralia et al., 1994; Monyer et al., 1994). Of special interest were the findings related to the developmental changes of the NR2 subunit subtypes. Throughout development the NR2b subunit becomes gradually replaced by the NR2a (Monyer et al., 1994). As these subunits convey distinct properties to the receptor (Flint et al., 1997), this change has been associated with the developmental shift in signaling that was often

linked with the ability of neuronal circuits to exhibit experience-dependent synaptic plasticity (Constantine-Paton and Cline, 1998). A support for this hypothesis came from studies showing that transgenic mice overexpressing the NR2b subunit in the forebrain exhibited enhanced learning and memory (Tang et al., 1999). This finding indicated that the NR2b-NR2a switch may be responsible for age-dependent gating of the threshold for memory and learning formation. Interestingly, several studies also showed experience-dependent changes in the subunit composition of NMDA receptors (Quinlan et al., 1999; Philpot et al., 2001), further emphasizing the dynamic nature of their signaling and association with plasticity.

The distinct functional properties of NMDA receptor subunits prompted studies addressing their structural characteristics. However, most of them focused on the NR1 and NR2 subunits, while less is known about the differences between the subunit subtypes. Analysis of NR1 revealed a unique transmembrane topology with an large N-terminal extracellular domain, four hydrophobic domains (M1-M4) and a cytoplasmic C-terminal domain (Figure 6) (Wood et al., 1995).

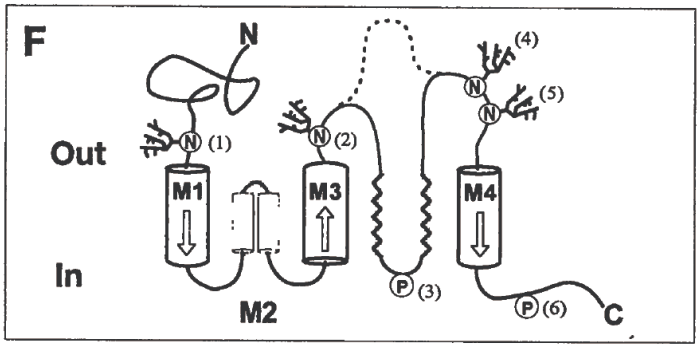

Figure 6. Proposed structure of the NR1 subunit showing the three transmembrane domains (M1, M3 and M4), the glycosylation sites (1-5), and the intracellular phosphorylation site (6) (Wood et al., 1995).

This topology is thought to be representative for other subunits, taking into account some differences that appear to be responsible for their different properties. The extracellular domain of the NR1 subunit features binding sites for the main co-

agonists glycine (Miyazaki et al., 1999), while the binding site for glutamate is located on the NR2 subunits (Figure 7) (Laube et al., 1997), explaining the inability of these subunits to compensate for the loss of each other. The binding of several other extracellular ligands which can modulate NMDA receptor signaling, such as zinc (Smart et al., 1994), protons (Gottfried and Chesler, 1994) and polyamines (Rock and Macdonald, 1995) appears to be jointly controlled by domains on the NR1 and the NR2 subunits (Dingledine et al., 1999). Both subunits have cytoplasmic tails which differ in their length and interaction with numerous intracellular ligands (Sheng and Pak, 2000).

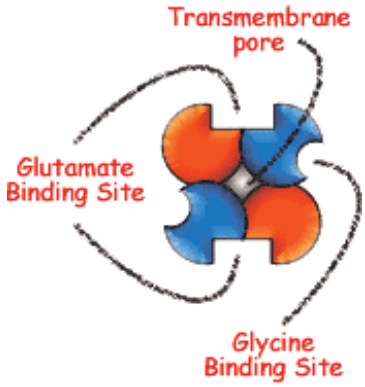

Figure 7. A schematic representation of the binding sites on the NMDA receptor. The NR1 subunits are shown in blue, while the NR2 are in orange. (From: Homepage of the Center for Synaptic Plasticity, University of Bristol (http://www.bris.ac.uk/synaptic/info/glutamate.html))

1.5 Experimental systems for studying plasticity

Studying the involvement of serine proteases and their inhibitors in plasticity-related events, through modulation of NMDA receptor function or other substrates, required a careful selection of experimental systems with certain characteristics. First, in order to investigate activity-dependent expression of molecules, a mean for inducing

neuronal activity was needed, which would provide a non-intrusive, and natural stimulation, and at the same time elicit well-defined plastic changes in brain structures. Second, these systems should provide the opportunity for electrophysiological and histological studies, as well as the possibility to examine functional changes through a behavioral readout. Finally, having information about the contributions of NMDA receptors in the chosen system was important.

For this study, we chose two experimental systems, which fulfill the above requirements, and complement each other. The first one is the enriched environment, and the other is the whisker-to-barrel cortex pathway.

1.5.1 The enriched environment

This experimental concept is based on rearing or exposing laboratory animals to an environment, which is enriched, compared with the standard laboratory housing. In general, the enriched environment contains a complex mixture of motoric, sensory and social stimulations, making the surrounding of the animals more variable and thus more challenging (Figure 8).

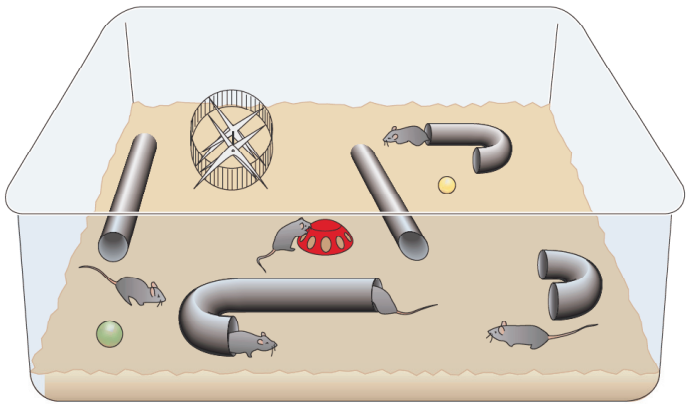

Figure 8. Schematic depiction of enriched environment (van Praag et al., 2000).

This experimental paradigm was introduced in the early 1960s to investigate the effects of experience on the brain (Rosenzweig, 1966; Rosenzweig et al., 1967;

Rosenzweig et al., 1962), and since then has been extensively used because of its advantages, the very wide range of plastic responses elicited, and its non-invasiveness, which is similar to effects triggered by the natural environment (van Praag et al., 2000). Furthermore, over the decades, a wealth of data documenting structural, as well as behavioral effects of enriched environment has accumulated. The evidence that enrichment can induce activity-dependent changes in the adult brain first came from studies showing an increase in the proliferation rate of neurons and glia cells (Altman and Das, 1964), as well as in brain weight and size (Rosenzweig and Bennett, 1969; Diamond et al., 1966). Earlier experiments also showed that enrichment enhances neurite branching (Holloway, Jr., 1966b; Greenough et al., 1985), and synapse formation (Diamond et al., 1975; Greenough et al., 1978; Globus et al., 1973). Changes in biochemical parameters such as DNA/RNA ratios (Bennett et al., 1979), which were later shown to reflect changes in gene expression, were also detected. Specifically, enrichment was shown to have an effect on the expression of the serotonin receptor (Rasmuson et al., 1998) and the synthesis of neurotransmitters (Por et al., 1982; Rosenzweig and Bennett, 1969). More recently gene profiling studies revealed many other genes whose expression changed in response to enrichment. Among those were many linked to structural and synaptical neuronal plasticity, and neurotransmission (Rampon et al., 2000a; Pinaud et al., 2002).

Several electrophysiological and behavioral studies attempted to relate these anatomical changes with functional consequences. In hippocampal slices derived from enriched or individually housed rats, excitatory postsynaptic potential (EPSP) slopes detected in the dentate gyrus were greater in the slices from enriched rats (Green and Greenough, 1986; Foster et al., 2000). Similarly, exposing rodents to a new, complex environment enhanced hippocampal field potentials (Sharp et al., 1985). Strikingly, environmental enrichment also has a positive effect on cognitive and sensory functions such as memory and learning (Rosenzweig, 2003; Engineer et al., 2004; van Praag et al., 1999b), as well as delaying the disease progression in

several mouse models of human pathologies such as Huntington disease (Hockly et al., 2002), epilepsy (Young et al., 1999), stroke and brain injury (Pacteau et al., 1989), learning impairments (Kempermann et al., 1998) and ethanol exposure (Rema and Ebner, 1999; Wainwright et al., 1993). These, and many other studies promoted environmental enrichment as a widely used model for studying activity-dependent expression of molecules, and plastic changes, especially in transgenic animals and following brain damage.

1.5.2 The whisker-to-barrel cortex pathway

Since 1970 when it was discovered (Woolsey and Van der Loos, 1970), the whisker-to-barrel pathway has been extensively used for studying developmental and adult plasticity (Fox, 2002). This pathway represents a part of the trigeminal somatosensory system, which relays information acquired by the whiskers via trigeminal and thalamic nuclei to the primary somatosensory cortex (Figure 9) (Paxinos G., 1995; Kossut, 1992).

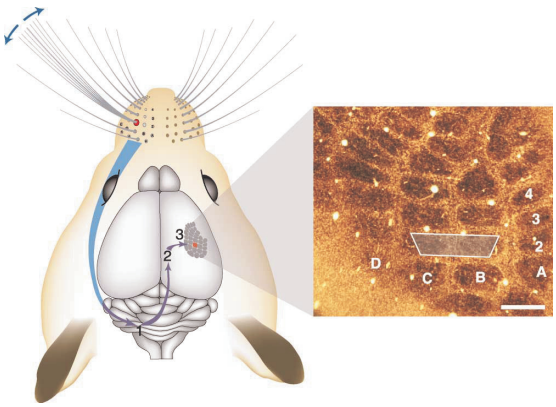

Figure 9. A schematic depiction of the whisker-to-barrel cortex pathway showing the flow of information from the vibrissae to the brain stem (1), the thalamus (2) and the barrel cortex (3). On the right are shown the barrels as detected by cytochrome oxidase staining (Knott et al., 2002).

The whisker-to-barrel cortex pathway was named after the unusual cellular formations in the shape of barrels, found in the first somatosensory cortex (SI) of mice. These multi-neuronal assemblies, located in layer IV, are cortical representations of the whiskers on the whisker pad. As shown in Figure 10, each whisker is anatomically and functionally represented by one single "barrel" (Kossut, 1992). The number of barrels is the same as the number of vibrissae on the contralateral side of the face, and they are arranged in a pattern that corresponds to the topography of the whiskers (Woolsey and Van der Loos, 1970). Their formation is driven by activity: a disruption of whisker input during early postnatal development leads to absence of the barrel corresponding to the damaged vibrissae, while the diameters of the neighboring barrels increase, invading its cortical territory (Welker and Van der Loos H., 1986; Jeanmonod et al., 1981; Andres and Van der Loos H., 1985; Van der, 1977b).

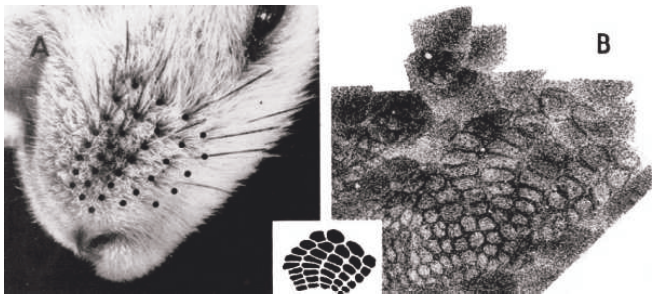

Figure 10. Direct correspondence of the whiskers on the face and the large barrels in cortex. (A) Photograph of the group of mystacial vibrissae from a mouse. Black dots are placed over the whisker follicles for easier identification. (B) Structural organization of layer IV in the somatosensory cortex. Discrete cytoarchitectonic units in shape of barrels are easily identifiable. Insert shows a scheme of the barrels in (B) (Woolsey and Van der Loos, 1970).

Anatomically, each barrel contains about 2000 neurons (Pasternak and Woolsey, 1975), arranged in a cylindrical cell-dense wall around a cell-sparse center. The cells

constituting the walls are smooth and spiny stellate neurons (Woolsey et al., 1975), whose dendrites are restricted to one barrel. The barrel centers are rich in thalamic afferents, and most of the thalamocortical synaptic contacts take place in there (White, 1976). The septa between barrels are densely packed with vertically oriented dendrites and fibers (Figure 11) (Paxinos G., 1995).

Like the rest of the somatosensory cortex, the barrel cortex has a columnar organization. Barrels are considered to be the main responsive elements for whiskers, but actually all cells within one vertical column respond first to the same whisker. Upon sensory activation, cells in layer IV and Vb are the first to respond, followed by layer III, II and Va, and finally layer VI cells, after which the excitation spreads to adjacent columns (Figure 11) (Armstrong-James, 1975).

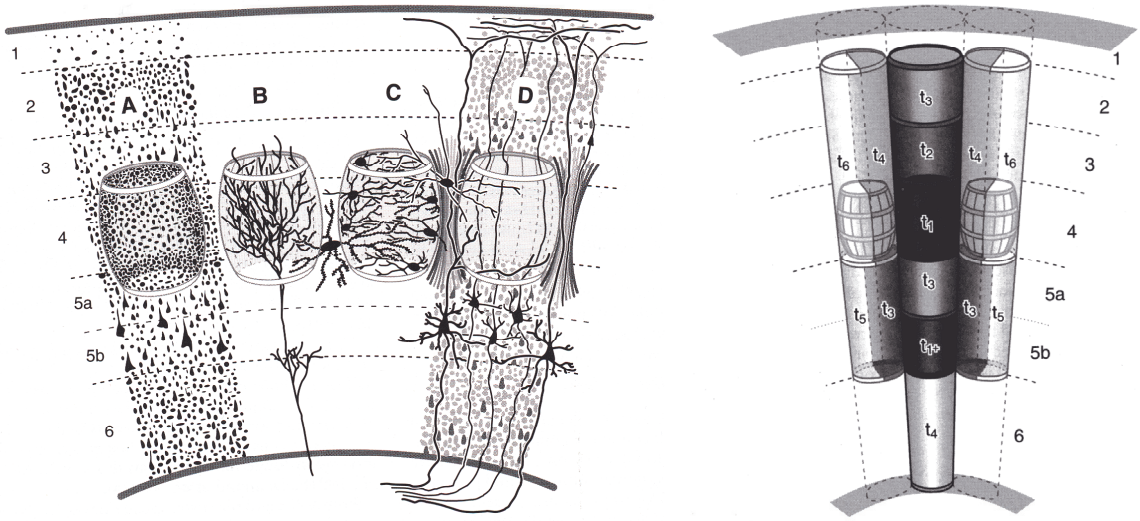

Figure 11. Left: neuronal components of a cortical barrel. (A) the distribution of neuronal somata showing the high density of stellate cells in layer IV, particularly in the barrel sides. (B) An afferent from the thalamus ends predominantly in the barrel center. (C) Morphology of barrel cells and their dendrites. (D) Apical dendrites of layer V cells pass preferentially through the barrel wall and septum. Right: spread of activity within a column in the barrel

cortex. t_1 indicates the earliest whisker-evoked activity occurring in layer IV cells, rapidly followed by activity in layer Vb (t_{1+}). Cells in other layers respond with increasing latencies with the sequence indicated by t_2-t_6 and the level of shading (Paxinos G., 1995).

The discovery of barrels and their relation to the vibrissae offered a new and remarkable system for studying mechanisms of development, plasticity and flow of sensory information in the central nervous system. Studies dealing with different forms of plasticity and investigating structural and functional events occurring as a consequence of changes in sensory input could take particular advantage from the one-to-one whisker representation and clear columnar structure of the barrel cortex. (Trachtenberg et al., 2002; Welker and Van der Loos H., 1986; Andres and Van der Loos H., 1985; Van der, 1977a; Fox, 2002). Furthermore, the possibility of a natural and selective activation of an easily identifiable peripheral sensory input, was especially suited for precise monitoring of the effects of the stimulus on specific neurons, and studying activity-dependent expression of molecules (Staiger et al., 2000; Rocamora et al., 1996; Brown and Dyck, 2002; Barth et al., 2000).

2. Aim and course of the work

During the course of this research we investigated the role of extracellular serine proteases and their inhibitors in adult brain plasticity. In particular, I focused in the importance of the control of extracellular proteolysis for normal brain function and homeostasis, and the mechanisms through which serine proteases contribute to activity-dependent plastic changes in the adult brain.

To address these questions we first examined whether the expression of PN-1 can be modified by neuronal activity, as it is the case for some serine proteases. For this purpose I used a previously created reporter mouse (PN-1 KI mouse), with a construct containing the ß-galactosidase marker gene inserted in the locus of PN-1. Environmental enrichment was used as a mean of inducing widespread neuronal activity. As a strong upregulation of PN-1, both on the transcriptional and protein level, was detected upon enriched environment, we further investigated whether the overall brain proteolytic activity was affected by this increase. In wild-type mice, enrichment strongly decreased the proteolytic activity, in line with PN-1 upregulation. In contrast, PN-$1^{-/-}$ mice showed a strong increase in brain proteolytic activity, indicating that they have a perturbed control of activity-dependent brain proteolytic activity. As PN-$1^{-/-}$ mice previously showed decreased NMDA receptor signaling in hippocampal slices, and the NR1 subunit of the NMDA receptor can be cleaved *in vitro* by serine proteases, we investigated whether the increased brain proteolytic activity could affect this receptor. Immunoprecipitations of synaptosomal preparations and immunohistochemical stainings of brain sections revealed decreased levels of the NR1 subunit in PN-$1^{-/-}$ mice. To find out if this decrease could be correlated with enhanced proteolytic activity, PN-$1^{-/-}$ mice and their wild-type littermates were exposed to enriched environment. This induced a further decrease in NR1 immunoreactivity in PN-$1^{-/-}$ mice, but not in wild-type mice, suggesting that uncontrolled proteolytic activity may lead to its degradation. These findings raised questions about the consequences of modified NMDA receptor levels on CNS

function. To investigate this issue, we performed electrophysiological and behavioral tests in the whisker-to barrel cortex pathway. In collaborations with the groups of Dr. Petersen at the EPFL in Lausanne and Dr. Kiss at the University of Geneva, measurements of NMDA receptor-dependent synaptic currents in barrel cortex slices and epicranial sensory evoked potential recordings were performed, revealing decreased NMDA receptor signaling in PN-1$^{-/-}$ mice and decreased sensory evoked potentials upon whisker stimulation. PN-1$^{-/-}$ mice were also tested for performance in whisker-dependent behavioral tasks and displayed impaired sensory motor function.

3. Materials and Methods

Experimental animals

To create a PN-1$^{HAPN\text{-}1\text{-}lacZ/HAPN\text{-}1\text{-}lacZ}$ mouse (PN-1 KI mouse), exon II of the *PN-1* gene was replaced by a construct containing the PN-1 secretion signal sequence (56 bp) in front of a HA-tagged mature PN-1 cDNA (1137 bp). This was coupled by an IRES (internal ribosome entry site) sequence to the LacZ-pA sequence containing the nucleolar localization signal (NLS), and to the TK-Neo-pA cassette. The targeting construct was inserted by homologous recombination into 129SV embryonic stem cells. The targeted clones were produced by morula aggregation, producing germline chimeras. Animals heterozygous for the targeted *PN-1* allele were established with a mixed genetic background. Four PCR primers were used to screen for construct insertion. Primer HA23 annealed within the genomic DNA, while HA26 annealed within the HA-tag sequence, leading to a PCR product only in mice carrying the insertion. HA15 annealed within genomic DNA, while HA25 annealed within the sequence of PN-1 in the wild-type and that present in the insert. A difference in band size due to the HA-tag present in the inserted PN-1 sequence allowed distinction of homozygote and heterozygote mice. These reporter mice had been generated by Dr. Hugo Albrecht, a postdoctoral fellow previously working in the laboratory.

PN-1$^{-/-}$ mice (Luthi et al., 1997) were backcrossed for more then 12 generations in the C57BL/6 line. Heterozygous mating allowed the use of PN-1$^{-/-}$ and PN-1$^{+/+}$ littermates. Genotyping was performed on DNA from tail biopsies by PCR.

C57BL/6 mice were purchased from Charles River (France). All experimental animals were 4-8 months old. All animal experiments followed the regulation of the Swiss Law on Animal Experimentation, and had been approved by the Swiss Veterinary Office.

Sensory deprivation

The “single spared whisker” deprivation pattern was imposed as described (Barth et al., 2000) with the difference that the whiskers were trimmed and not pulled out. The “unilateral” deprivation pattern was done in the same way, except that all whiskers on one side were removed.

Enriched environment

Prior to the experiment, all mice were single-housed in standard small cages for at least 3 weeks. During exposure to enriched environment, mice were individually housed in a large cage equipped with objects of different shape and texture, such as tunnels, grids, plastic objects, aluminum foil and paper. When mice were housed in the enriched environment for more than 24 h, the objects were replaced or displaced on a daily basis. Control mice were single-housed in standard small cages.

ß-galactosidase histochemistry on sections and whole mount staining of brains

Mice were anesthetized and transcardially perfused with ice-cold 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), and with ice-cold PBS. Brains were rapidly dissected out, equilibrated in sucrose and quickly frozen in Tissue-Tek O.C.T (Sakura Finetek, USA). Cryostat (50 or 60 µm thick) and microtome (400 µm thick) sections were briefly air-dried, fixed for 20 minutes in 4% PFA, washed 3 times for 15 minutes in a solution containing 2 mM $MgCl_2$, 0.02% Nonidet P40, 0.01% sodium deoxycholate in PBS, and incubated for 6 h or overnight at 37°C in the same solution, supplemented with 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 0.5 mg/ml 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal) (Roche, Switzerland). Whole mount staining of brains was done essentially as described (Barth et al., 2000).

Proteolytic assays

Mice were perfused with ice-cold PBS and brains were rapidly dissected out and homogenized in a brain-homogenizing buffer (20 mM sodium phosphate buffer, 320 mM sucrose, 1 mM EDTA, 0.2% Tween 20). The amount of proteins in cleared homogenates was determined using a Biorad D*c* Protein Assay (Biorad, USA). Dilutions of cleared homogenates (typically 100 µg of protein in 80µL) were mixed in a 96-well microtiter plate with 10 µl S-2288 (H-D-Ile-Pro-Arg-pNA), or S-2238 (D-Phe-Pip-Arg-pNA·2HCl) substrate (1.25 mg/ml in H_2O, Chromogenix, Sweden). The amidolytic activity was determined as described (Hengst et al., 2001). For each proteolytic assay experiment a separate set of mice was used.

Gel zymography

Gel zymography was adapted from the procedure previously described (Lantz and Ciborowski, 1994). Dilutions of cleared homogenates (5µg) and 30 µg of recombinant tPa (Genentech, USA) were separated by electrophoresis at 4°C on 10% polyacrylamide-SDS gels co-polymerized with casein (1 mg/ml; Sigma, USA) and plasminogen (2.5 U/ml; Chromogenix, Sweden). The lysis bands were visualized using coomassie blue staining.

In situ zymography

Zymography to detect tPA was performed essentially as described (Sappino et al., 1993). Mice were perfused with ice-cold PBS, the brains were rapidly dissected out, and cryostate sections were overlaid with a mixture containing 8% milk and plasminogen (2.5 U/ml; Chromogenix, Sweden), and allowed to develop at 37°C, until dark lysis zones appeared.

Immunoprecipitation

Synaptosomal-enriched plasma membrane was prepared as follows. Mice were perfused with ice-cold PBS and whole brains were homogenized in 10% sucrose in 5

mM Hepes buffer (pH 7.5) in the presence of a mixture of protease inhibitors with broad inhibitory specificity, inhibiting serine, cysteine, metalloproteases, as well as calpains (Complete Protein Inhibitor Cocktail Tablets, Roche, Switzerland) and centrifuged at 1000 g. The supernatants were further centrifuged at 12000 g. The supernatant was discarded and myelin was aspired from the pellet. The pellet was resuspended in 10% sucrose Hepes buffer with protease inhibitors. Immunoprecipitation was performed for 2 h at 4°C using 10 µl of goat anti-NMDARζ antibody (C-20) (Santa Cruz Biotechnologies, USA) recognizing the carboxy-terminus of the NR1 subunit, and the anti-tPA antibody (Molecular Innovations, USA), on 25 µg of plasma-membrane preparation in 1 mL of RIPA buffer (1% Nonidet P40, 0.5% sodium deoxycholate, 0.5% SDS in PBS) and was followed by adsorption to 20 µl of protein G-sepharose beads (Santa Cruz Biotechnologies, USA). Bound proteins were eluted in β-mercaptoethanol containing loading sample buffer, and boiled at 95°C. Then, a SDS-PAGE electrophoresis was performed before immunoblotting.

SDS-PAGE and immunoblot analysis

For the detection of PN-1 in whole brain homogenates prepared for the proteolytic assays, 5 µg of protein was separated on a 12% SDS-PAGE and transferred onto a Trans-Blot Transfer Medium nitrocellulose membrane (Bio-Rad Laboratories, USA). Membranes were blocked, and incubated with a PN-1 specific mouse monoclonal antibody (1:1000) (Meier et al., 1989) and with a mouse anti-actin antibody (1:1000, NeoMarkers, USA). For the analysis of the immunoprecipitation, membranes were blocked and incubated with the goat anti-NMDARζ antibody (C-20) (1:400, Santa Cruz Biotechnologies, USA) recognizing the carboxy-terminus of the NR1 subunit, and with the rabbit anti-tPA antibody (1:400, Molecular Innovations, USA).

Immunohistochemistry on sections

For the analysis in PN-1 KI mice, 50 μm thick cryostat sections were stained with X-gal as described, blocked for 1 h in 1.5% normal goat serum and incubated with the goat anti-tPA primary antibody (1:200, American Diagnostica, USA). The anti-tPa antibody was detected with the ABC kit (Santa Cruz Biotechnologies, USA) and visualized with diaminobenzidine. For detection of the NR1 subunit, brains from perfusion fixed wild-type and PN-$1^{-/-}$ littermates were postfixed for 2 h in ice-cold 4% PFA, equilibrated in 30% sucrose in PBS overnight and quickly frozen. Cryostat sections (12 μm) of wild-type and PN-$1^{-/-}$ littermates, collected side by side on the same slides, were blocked in 1.5% normal goat serum (Santa Cruz Biotechnologies, USA) for 1 h and incubated with the rabbit anti-NMDARζ antibody (H-300) (1:400) for 48 h at room temperature. For quantification, images were converted to grayscale and density was measured using Image-Pro Plus (Media Cybernetics, USA). The level of intensity in control animals was set to 1.

Epicranial sensory evoked-potential recordings

Epicranial recordings of evoked potentials, performed by Dr. Edgardo Troncoso from the University in Geneva, were done essentially as described (Troncoso et al., 2000). All mice (n=4 PN-$1^{-/-}$; n=4 PN-$1^{+/+}$; males, 4-6 months old) were singly housed for at least 2 months prior to the experiment. Recordings were performed under pentobarbital anesthesia (60 mg/kg i.p.). Briefly, 10 electromechanical stimuli driven by a computer-controlled signal were applied to whiskers with an inter-stimulus-interval of 3 s. An array of five electrodes (0.45 mm in external diameter and 2 cm length) was placed above the skull. The pertinent electrodes positioned in a row had the following coordinates relative to Bregma: AP –1/L 2.5, AP –2/L 3.0 and AP –3/L 3.5 (distances in mm). Signals were amplified (x10.000) and filtered (high pass 4 Hz, low pass 300 Hz), then hooked up and digitally converted (16 bits, 2 kHz with triggered scan) and stored for post-hoc analysis. For the activation, whiskers were stimulated unilaterally at 8 Hz during a period of 10 minutes. The responses after activation represent the mean of 3 series of 3 recordings at 10-minute intervals. For data analysis, the signal-processing technique of statistical outlier elimination

excluded samples fulfilling the rejection criteria (beyond 2 standard deviations from the original mean). The overall mean of the 3 recording series calculated after signal processing was taken as the result of each experimental condition (~20 responses). Then, the peak positive and negative values 10- 30 ms post-stimulus were measured. For the treatment with MK-801, different doses (0.3, 0.4, 0.5 and 0.6 mg/kg i.p.) were tested in a first group of 8 C57BL/6 mice to observe their tolerance with pentobarbital anesthesia. Two further groups of C57BL/6 mice (n=5) were compared, one of which received a dose of 0.3 mg/kg 30 minutes before pentobarbital anesthesia. This dose gave reproducible results and all animals survived and recovered normally. The effect lasted for more than 90 minutes before animals started to recover from anesthesia Recordings were obtained 60 minutes after induction of pentobarbital anesthesia, and 90 minutes after MK-801 was administered.

Measurement of synaptic currents

Measurements of NMDA-receptor dependent synaptic currents were performed by Dr. Carl Petersen from the EPFL in Lausanne. All mice (n=4 PN-$1^{-/-}$; n=4 PN-$1^{+/+}$; 4-6 months old) were singly housed for several weeks prior to the experiment. For the recordings the brains were removed and 300µm parasagittal slices of primary somatosensory barrel cortex were cut by a vibratome according to standard procedures (Petersen and Sakmann, 2000). The extracellular medium contained (in mM): 125 NaCl, 25 $NaHCO_3$, 25 glucose, 2.5 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$ and 1 $MgCl_2$ bubbled with 95% O_2 and 5% CO_2. All experimental procedures were carried out at 35°C. Barrels of the posterior medial barrel subfield were identified in bright field videomicroscopy at low magnification and a glass patch-pipette filled with extracellular solution served as the stimulation electrode and was placed in the center of a layer IV barrel. Stimulation was evoked by 200 µs current pulses of 10-30 µA. Whole-cell recordings were obtained from layer II/III pyramidal neurons lying in the stimulated barrel column, which were identified by video-enhanced infrared microscopy using a BX51WI (Olympus). The intracellular solution contained (in mM): 135 Cs-gluconate, 10 mM HEPES, 4 CsCl, 4 MgATP, 0.3 NaGTP (adjusted to

pH 7.2 with CsOH). Biocytin 2 mg/ml was included in the intracellular solution to allow post-recording morphological identification of the recorded neurons. Ionotropic GABAergic synaptic transmission was pharmacologically blocked by either 20 μM bicuculline or 100 μM picrotoxin and postsynaptic GABA-B responses were blocked in the recorded cell by the presence of Cs in the recording pipette. Synaptic currents evoked by electrical stimulation of layer 4 were recorded with the soma voltage-clamped switching between –60 mV and +40 mV (to relieve the voltage-dependent Mg-block of the NMDA receptor). The currents were recorded using Multiclamp 700 A amplifiers (Axon Instruments) filtered at 3 kHz and digitised at 10 kHz using an ITC-18 (Instrutech). EPSCs evoked at –60 mV were brief and entirely blocked by CNQX (10 μM), suggesting that they were mediated exclusively by AMPA/KA receptors. Thus the peak EPSC at –60 mV provided a measure of synaptically evoked AMPA/kainate currents. EPSCs recorded at +40 mV in general consisted of two components: a long-lasting NMDA-receptor dependent current which could be blocked by 100 μM APV leaving a brief EPSC with similar kinetics to those observed at –60 mV and which could be subsequently bocked by addition of CNQX (10 μM). The duration of the AMPA/kainate component was much less than 50 ms. The long-lasting NMDA component of the EPSC recorded at +40 mV was therefore quantified at 50 ms post-stimulus. Finally the ratio of NMDA to AMPA/KA currents was computed for each cell to allow statistical comparison between genotypes of cells measured from different brain slices and different animals.

Gap-crossing test

The effective use of whiskers of mice (n=12 PN-1$^{-/-}$; n=11 PN-1$^{+/+}$; females, 4-6 months old, single housed for at least 2 months) was tested in a modified gap-crossing paradigm (Barneoud et al., 1991b), performed by Marita Meins, Friedrich Miescher Institute, Basel. All training and testing was performed blind, by a single experimenter, during the dark cycle under dim illumination (10 lux), and the testing

was videotaped. Mice were trained 1 week before the start of the experiment to accustom to the apparatus [10 x 20 cm carrying box; a movable platform (6 x 6 cm) set against the escape tube (54 mm inner diameter, 12 cm in length, the first 6 cm transparent)] and to entering, crossing, and exiting the tube. The experiment consisted of 4 trials. For trial 1, each mouse was transferred to the platform placed 2 cm from the tube and re-tested after 120 s if it did not cross the gap. The gap was incrementally increased until the animal balked. Trial 2 was started after 1 day of rest. Animals were tested as above and the latency to jump was noted. Trial 3 was started at least 1 week later. The animals were tested as for trial 2 but were given a second chance at each distance if they did not jump by 15 s. On the day after trial 3, the mice were briefly anesthetized in metofane and their whiskers trimmed. Trial 4, a repeat of the trial 3, took place the following day. Performance was scored by the latency (the time to jump a particular distance with a maximal score of 15 s). The data from trial 2 were analyzed by two-way ANOVA [with distance as the within-subjects (repeated measure) variable and genotype as the between-subjects variable]; the data from trials 3 and 4 by the Wilcoxon signed rank test, to compare the change in distance crossed before and after cutting the whiskers. Locomotor activity was examined by placing mice individually in one corner of a perspex box (40 x 40 cm) with a total of 16 squares (10 x 10 cm). The number of squares crossed (at least two paws entering a square) and the number of rears made in 6 minutes were recorded. All analysis was performed using the Prism 3 (GraphPad, USA) statistical package.

4. Results

4.1.-4.3. Expression of PN-1 and changes in proteolytic activity controlled by neuronal activity

4.1. The PN-1 KI mouse

To investigate the activity-dependent expression of PN-1, a previously created reporter mouse was used (PN-1 KI mouse), with a construct containing a haemaglutinin-tagged PN-1 cDNA sequence followed by a LacZ marker gene (HAPN-1-IRES-LacZ) inserted in the PN-1 locus (Figure 12A). The purpose of this reporter system was the *in vivo* detection of PN-1 protein using the hemaglutinin (HA) tag, and of PN-1 transcription, following the expression of *LacZ*. *LacZ* (Kuby and Lardy, 1953) is a bacterial gene coding for the enzyme ß-galactosidase, which can hydrolyze a variety of substrates, producing colorful precipitates. Due to this feature, it has been extensively used for several decades as a cytochemical and histochemical marker (An et al., 1982). The insertion of *LacZ* into the coding exon of the *PN-1* gene placed it under the control of the *PN-1* promoter, thus allowing a faithful monitoring of the *in vivo* promoter activity through the expression of ß-galactosidase. The IRES (internal ribosome entry site) sequence between the HAPN-1 and the *LacZ* gene ensured independent translation of these two elements of the construct, resulting in two individual protein products, while the nuclear localization signal (NLS) in front of the *LacZ*, restricted the ß-galactosidase activity to the nucleus of cells expressing it. Most importantly, the presence of the HAPN-1 cDNA sequence in the construct ensured that these mice were not PN-$1^{-/-}$, but retained the expression of PN-1. The insertion of the construct in the correct location was verified using four PCR primers (designated HA15, HA23, HA25 and HA26), annealing within the genome, and the insert sequence (Figure 12B), allowing the distinction of homozygote and heterozygote mice.

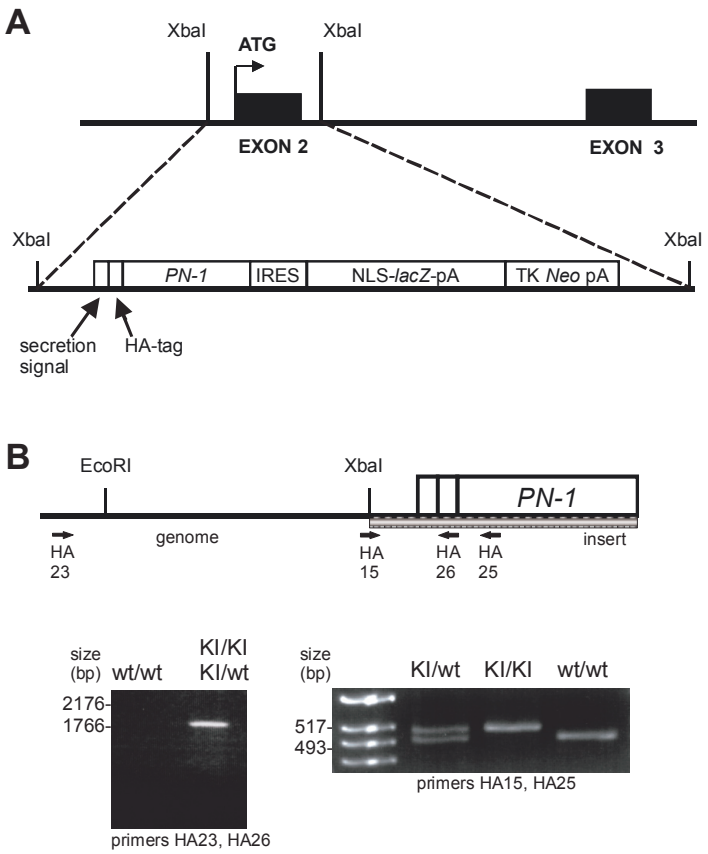

Figure 12. (A) Generation of PN-1 KI mice. Top: schematic representation of the PN-1 genomic locus with relevant restriction enzyme sites. The second and third exons of the mouse PN-1 gene are shown as black boxes, the translational start site as an arrow. Bottom: targeting vector. The elements of the transgene construct are shown as open boxes. (B) Top: schematic representation of the annealing locations of PCR primers used for genotyping PN-1 KI mice. The underlying shaded box indicates part of the insert; the directions of the primers are indicated by arrows. Bottom: PCR analysis of genomic DNA from a litter of a heterozygous cross, using primers HA23, HA26 (left), and HA15, HA25 (right) (see Materials and Methods).

4.2. PN-1 expression monitored by ß-galactosidase activity

As an initial step in characterizing the expression of PN-1, we examined the basal levels of ß-galactosidase activity in PN-1 KI mice. 400 µm thick saggital brain sections were stained with X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside), a commonly used chromogenic substrate that produces an intensive blue precipitate at the place of ß-galactosidase activity. Several distinct brain areas gave a strong signal (Figure 13). Structures such as the periglomerular cells in the olfactory bulb and inferior colliculus were particularly prominent. Parts of the visual system, e.g. the visual cortex and anterior pretectal nucleus, showed very strong X-gal labeling. ß-galactosidase activity was high in layer V of the cerebral cortex, some thalamic nuclei, the caudate putamen and several nuclei of the brain stem. In the cerebellum, PN-1 expression was almost completely confined to the Purkinje cells. Interestingly, the expression in the hippocampus was lower than in other brain regions. This expression profile was in accordance with *in situ* hybridization data (Mansuy et al., 1993; Reinhard et al., 1988), confirming that the insert was under the control of the PN-1 promoter.

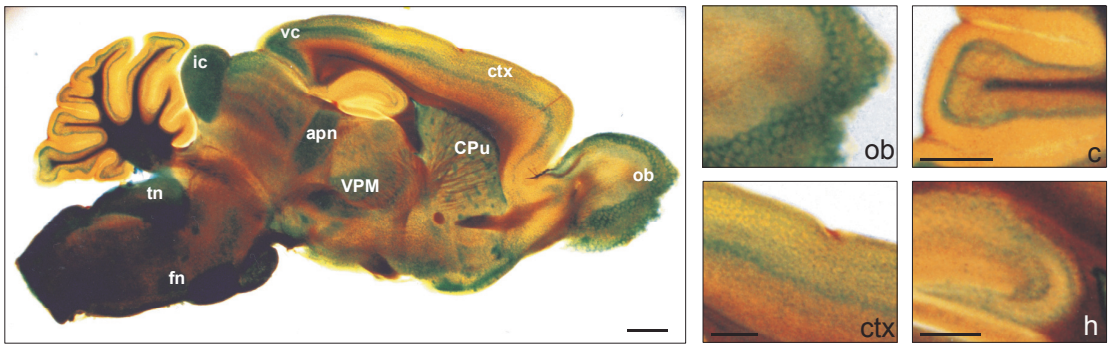

Figure 13. PN-1 expression in the adult mouse brain, demonstrated by the blue ß-galactosidase reaction product in a saggital section (left). Note the strong expression in brain regions such as the olfactory bulb (ob), cortical layers (ctx), visual cortex (vc), inferior colliculus (ic), caudate putamen (Cpu), ventral posteriomedial thalamic nucleus (VPM), anterior pretectal nucleus (apn), trigeminal nucleus (tn), and facial nucleus (fn). Enlargements (right) show the cellular localization of the expression in the olfactory bulb (ob), cerebellum (c), cortex (ctx), and hippocampus (h). Scale bar: left, 1 mm; right, ob, ctx (shown in ctx) 500 µm, c, h 500 µm.

4.3. Activity-dependent PN-1 expression and proteolytic activity regulation

As PN-1 appeared to be strongly expressed by structures involved in sensory processing, we investigated whether its expression can be modulated by sensory stimuli. We used short-term exposure to enriched environment, known to induce neuronal activity and widespread plastic responses (van Praag et al., 2000) as a stimulation paradigm. PN-1 KI mice were exposed to 24 hours of enriched environment, and X-gal staining was performed on saggital brain sections. A strong upregulation in ß-galactosidase activity was found in several brain areas, compared to control mice single housed in standard cages (Figure 14A).

To verify that enhanced ß-galactosidase activity reflected an increase in PN-1 protein, wild-type C57BL/6 mice were exposed to 24 hours of enriched environment and a western blot against PN-1 was performed on whole brain homogenates. A strong increase in PN-1 protein was detected in mice housed in the enriched environment (Figure 14B), compared to control-housed littermates.

As PN-1 is a strong inhibitor of serine proteases, we investigated whether the increase in PN-1 protein levels changed the proteolytic activity in the brain. The proteolytic activity in brain homogenates from C57BL/6 mice was detected using a broad-spectrum chromogenic substrate S-2288. Strikingly, housing mice for 24 hours in enriched environment was sufficient to induce a strong decrease in proteolytic activity, as shown by measurements of the rate of hydrolysis of the chromogenic substrate by endogenous proteases (mOD_{405}/min) (0.104 ± 0.0084 vs 0.051 ± 0.0035, $^{**}P$=0.0043 Student's t-test) (Figure 14C). The same but smaller effect was observed if mice were housed for 72 hours in the enriched environment (0.1075 ± 0.0045 vs 0.069 ± 0.0038, $^{**}P$=0.008 Student's t-test).

PN-1 *in vitro* inhibits serine proteases such as tPA (Scott et al., 1985), uPA (Crisp et al., 2002), and thrombin (Scott et al., 1985). As no change in thrombin proteolytic activity was detected using a thrombin-specific chromogenic substrate S-2238 (Figure 14D), we analyzed our samples by tPA and uPA-specific gel zymography (Lantz and Ciborowski, 1994). Only one band of proteolytic activity was visible, corresponding to the size of recombinant tPA (Figure 14E). Samples from mice exposed to enriched environment gave a weaker signal, compared to control animals. Thus, tPA inhibition was stronger in mice exposed to the enriched environment.

PN-1$^{-/-}$ mice housed under control conditions showed a higher level of brain proteolytic activity than their wild-type littermates (0.148 ± 0.062 vs 0.344 ± 0.068, **P*=0.0303 Student's t-test) (Figure 14F), which is consistent with a lack of inhibitor. This increased proteolytic activity could be inhibited to wild-type levels using 0.25 mM tPA-STOP, a synthetic tPA inhibitor (data not shown), strongly suggesting that this proteolytic activity was due to tPA. To further examine the link between PN-1 and the activity-dependent regulation of proteolytic activity, we exposed PN-1$^{-/-}$ mice to enriched environment for 24 hours; compared with PN-1$^{-/-}$ mice housed under control conditions a marked increase in proteolytic activity was detected (0.053 ± 0.009 vs 0.124 ± 0.019, **P*=0.0154 Student's t-test) (Figure 14G). The observed variation in the absolute values of proteolytic activity in different experiments was most likely due to factors such as differences in batches of chromogenic substrates used for the measurements, or changes in the external temperature, which can strongly influence the kinetics of serine protease activity. To further assess the enhanced proteolytic activity in PN-1$^{-/-}$ mice, tPA-specific *in situ* gel zymography assays were performed on cortical sections from PN-1$^{-/-}$ mice housed under control conditions and after enrichment. Compared with their wild-type littermates, they showed increased proteolytic activity, visible as dark lytic zones, which was even more prominent upon enrichment (Figure 14H, I). These data are in line with the fact that the expression and release of serine proteases can be enhanced by neuronal activity (Pawlak et al., 2003; Seeds et al., 1995). They clearly indicate that PN-1$^{-/-}$

mice have a perturbed control of activity-dependent brain extracellular proteolytic activity and that other inhibitors did not compensate for the lack of this control.

In summary, we found that housing in the enriched environment induced strong changes in the overall brain proteolytic activity, which has to be tightly controlled, and we identified PN-1 as a major determinant of the balance between serine proteases and their inhibitors in the adult brain.

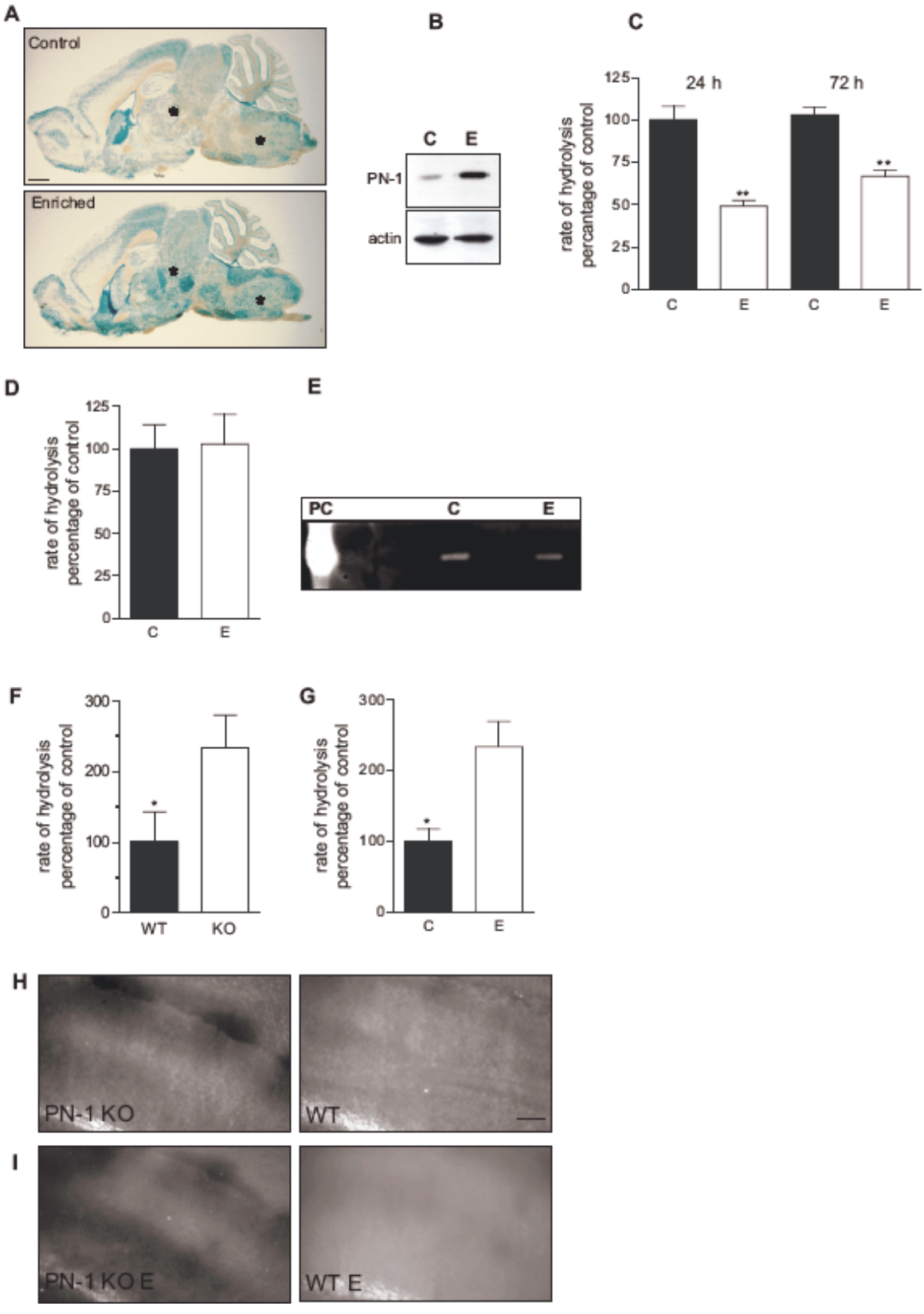
A
Control
Enriched
B
C E
PN-1
actin
C
24 h
72 h
rate of hydrolysis percentage of control
125
100
75
50
25
0
**
**
C E C E
D
rate of hydrolysis percentage of control
125
100
75
50
25
0
C E
E
PC C E
F
rate of hydrolysis percentage of control
300
200
100
0
*
WT KO
G
rate of hydrolysis percentage of control
300
200
100
0
*
C E
H
PN-1 KO
WT
I
PN-1 KO E
WT E

Figure 14. PN-1 expression and proteolytic activity in the brain upon exposure to enriched environment. (A) Saggital sections showing upregulation of ß-galactosidase activity in PN-1 KI mice after 24 h of enriched environment. Asterisks indicate regions of strongest upregulation. (B) Western blot against PN-1 on brain homogenates from C57BL/6 mice housed in the enriched environment for 24 h shows increased PN-1 protein levels. (C) The proteolytic activity, measured using the broad-spectrum chromogenic substrate S-2288, is significantly decreased after 24 and 72 hours of housing in the enriched environment (E24 n=3, E72 n=3, C n=3). Equal amounts of protein were tested and the rate of hydrolysis measured in the homogenates from control mice was set to 100%. (D) After 24 hours of enriched environment thrombin activity is not altered. (E) A gel zymography showing tPA activity in brain homogenates from mice housed for 72 hours in the enriched environment (E), and from control mice (C). PC, positive control (purified tPA). A representative of three zymographies is shown. (F) PN-1$^{-/-}$ mice (KO, n=5) housed under control conditions have increased brain proteolytic activity, as compared with wild-type littermates (WT, n=5). (G) When exposed for 24 hours to enriched environment, PN-1$^{-/-}$ mice (E, n=4) show increased brain proteolytic activity, as compared with control housed PN-1$^{-/-}$ mice (C, n=4). (H, I) Gel zymography on cortical sections showing enhanced proteolytic activity (dark lysis zones) in PN-1$^{-/-}$ mice housed under standard conditions, and after enriched environment. Scale bar: (A) 1 mm; (H, I) (shown in H) 150 µm.

4.4. Changes in the NR1 subunit of the NMDA receptor in PN-1$^{-/-}$ mice

As tPA-dependent cleavage of the extracellular part of the NR1 subunit of the NMDA receptor has been shown *in vitro* (Nicole et al., 2001), and decreased LTP due to reduced NMDA receptor-mediated EPSCs was previously detected in hippocampal slices from PN-1$^{-/-}$ mice (Luthi et al., 1997), we investigated whether increased

proteolytic activity in these mice may lead to changes in the NR1 subunit. Immunoprecipitations and western-blot analysis with an antibody against the C-terminus of NR1 revealed decreased levels of NR1 in synaptosomal fractions from PN-1$^{-/-}$ mice, as compared to WT littermates (Figure 15A). To determine in which parts of the brain these changes occurred, immunohistochemistry on sections from PN-1$^{-/-}$ and WT mice was performed using an antibody directed against the N-terminal extracellular domain of the NR1 subunit. A marked reduction in immunoreactivity was revealed in the somatosensory cortex (Figure 15B, quantification in Figure 15G), and in the hippocampus of PN-1$^{-/-}$ mice. To further test the link between decreased NR1 immunoreactivity and proteolytic activity, we exposed PN-1$^{-/-}$ mice for 24 hours to enriched environment, knowing that such exposure leads to increased brain proteolytic activity. Analysis of synaptosomal fractions revealed a further decrease in NR1 immunoreactivity, compared with PN-1$^{-/-}$ mice housed under control conditions (Figure 15C), while no decrease was observed when wild-type mice were exposed to enriched environment (Figure 15D). This decrease was also found in somatosensory cortex sections (Figure 15E, quantification in Figure 15H). Compared to sections from wild-type littermates housed under control conditions, NR1 immunoreactivity was slightly increased in wild-type mice exposed to enriched environment (Figure 15F, quantification in Figure 15I).

As a direct interaction between tPA and NR1 has been previously reported (Nicole et al., 2001), we performed immunoprecipitations against NR1 followed by western-blot analysis with an anti-tPA antibody. As expected, more tPA was co-immunoprecipitated with NR1 in wild-type mice (Figure 15J). Next, we performed immunoprecipitations against tPA, followed by western-blot analysis with an anti-NR1 antibody. In PN-1$^{-/-}$ mice, which have decreased levels of NR1, more NR1 was co-immunoprecipitated together with tPA then in their wild-type littermates (Figure 15K), indicating an increased interaction between tPA and NR1 due to the presence of more active tPA.

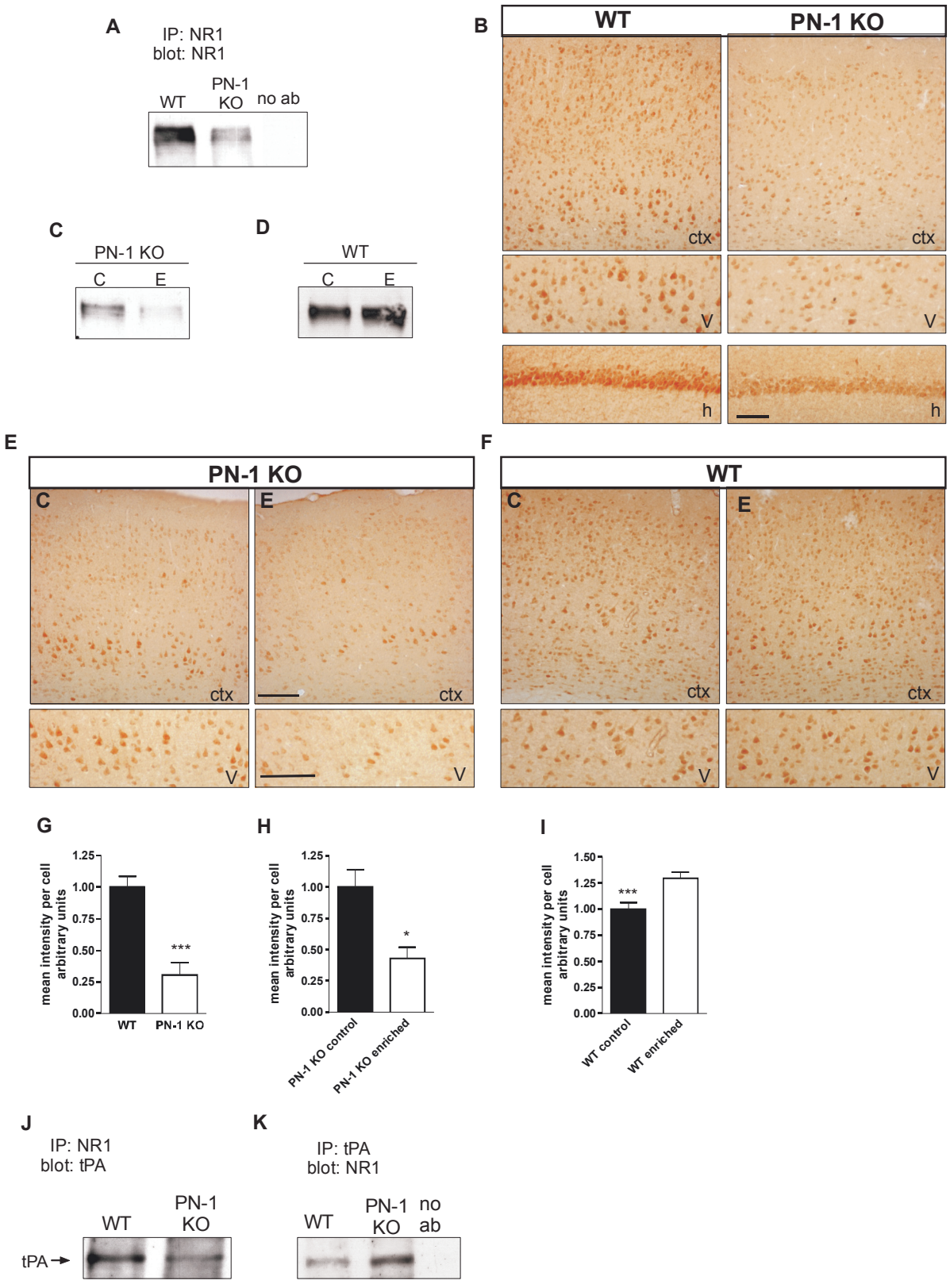
A
IP: NR1
blot: NR1
WT
PN-1 KO
no ab
B
WT
PN-1 KO
ctx
V
h
C
PN-1 KO
C
E
D
WT
C
E
E
PN-1 KO
C
E
ctx
V
F
WT
C
E
ctx
V
G
mean intensity per cell arbitrary units
1.25
1.00
0.75
0.50
0.25
0.00
***
WT
PN-1 KO
H
mean intensity per cell arbitrary units
*
PN-1 KO control
PN-1 KO enriched
I
mean intensity per cell arbitrary units
1.50
1.25
1.00
0.75
0.50
0.25
0.00
***
WT control
WT enriched
J
IP: NR1
blot: tPA
WT
PN-1 KO
tPA
K
IP: tPA
blot: NR1
WT
PN-1 KO
no ab

Figure 15. NR1 subunit immunoreactivity in PN-1$^{-/-}$ mice. (A) Decreased NR1 subunit immunoreactivity in synaptosomal fractions from PN-1$^{-/-}$ mice. (B) Coronal cortical sections stained with the anti-NR1 antibody show a prominent decrease in immunoreactivity in PN-1$^{-/-}$ mice (Quantification is in (G), ***P=0.0003). (C) An immunoblot analysis indicated a further decrease in NR1 subunit immunoreactivity in synaptosomal fractions from PN$^{-/-}$ mice housed in the enriched environment, compared with control-housed PN-1$^{-/-}$ mice. (D) In wild-type mice enrichment does not induce a decrease in immunoreactivity. A similar result was obtained by immunoistochemistry on sections [(E) and (F), quantifications are shown in (H), PN-1$^{-/-}$ *P=0.0168, and (I), WT ***P=0.0006]. (J) Anti-NR1 immunoprecipitation probed with an anti-tPA antibody. (K) Co-immunoprecipitation against tPA precipitates larger amounts of NR1 in PN-1$^{-/-}$ mice. Each result is a representative of three independent experiments. Scale bars: (shown in E) 100 µm; h, 100 µm. ctx, cortex; II/III, V, cortical layers; h, hippocampus.

4.5 PN-1 expression after sensory experience in the whisker pathway

Given the activity-dependent expression of PN-1 and the proteolytic modification of NMDA receptors in the somatosensory cortex, we focused on the whisker-to-barrel pathway, which allows studies of the effects of non-intrusive and specific modifications of the sensory input (Fox, 2002; Barth et al., 2000; Knott et al., 2002). In the cortex, each whisker is anatomically and functionally represented by a unique group of neurons, called a “barrel” (Woolsey and Van der Loos, 1970), providing numerous advantages for histological and electrophysiological studies.

First, we determined the effects of changed sensory experience on the expression of PN-1 in this pathway. PN-1 KI mice, unilaterally deprived of all but the D1 whisker, were placed in the enriched environment to encourage whisker-dependent

exploration. This paradigm has been used to study the activity-dependent expression of a number of molecules (Barth et al., 2000; Staiger et al., 2000). After 24 hours of single whisker experience, whole mount X-gal staining of the brain revealed strong localized upregulation of ß-galactosidase in the barrel cortex contralateral to the spared whisker (Figure 16A, arrows). X-gal histochemistry on coronal sections revealed an area of strong ß-galactosidase activity within layers II/III and Vb of the contralateral barrel cortex (Figure 16B-D, con, arrow). The upregulation of PN-1 in layer II/III of the stimulated barrel cortex could be attributed to a distinct population of cells (Figure 16D). The ipsilateral side showed a pattern of PN-1-expressing cells similar to that in the barrel cortex of non-stimulated animals (Figure 16B-D, ips), indicating that changed sensory experience triggered expression of PN-1 in previously non-expressing cells.

72 hours of single whisker experience induced even stronger upregulation of PN-1 and spreading of the signal to other cortical layers and neighboring barrels (Figure 16E, arrow). The upregulation was particularly prominent in layers IV and Vb (Figure 16F, arrow). We also found PN-1 upregulation in several thalamic nuclei belonging to the major projections of the whisker pathway (Figure 16E).

To find out whether enriched environment is required for the upregulation of PN-1 expression we unilaterally deprived mice of all but the D1 whisker and returned them to their home cages. After 24 hours, an upregulation of ß-galactosidase was also found in the contralateral barrel cortex (Figure 16G, arrow), showing that expression of PN-1 in barrel-related columns can be as well triggered under control normal housing conditions.

A modified paradigm of sensory deprivation was used to determine whether brain stem structures of the whisker pathway exhibited activity-dependent PN-1 expression. All whiskers on one side of the head were removed and the mice placed in the enriched environment for 72 hours. A strong unilateral upregulation of PN-1 was detected in the facial nucleus, in the spinal trigeminal nucleus oralis (Figure 16H, left,

arrow, respectively arrowhead) and in the spinal trigeminal nucleus interpolaris (Figure 16I, left, arrowhead). The upregulation was ipsilateral to the side with intact whiskers. No unilateral upregulation was observed in control mice housed in the enriched environment without whisker removal (Figure 16H, I, right).

Single-whisker experience has been shown to induce plasticity in the whisker pathway (Fox, 2002; Barth et al., 2000). Here we found that expression of PN-1 was induced by neuronal activity using the same experimental paradigm.

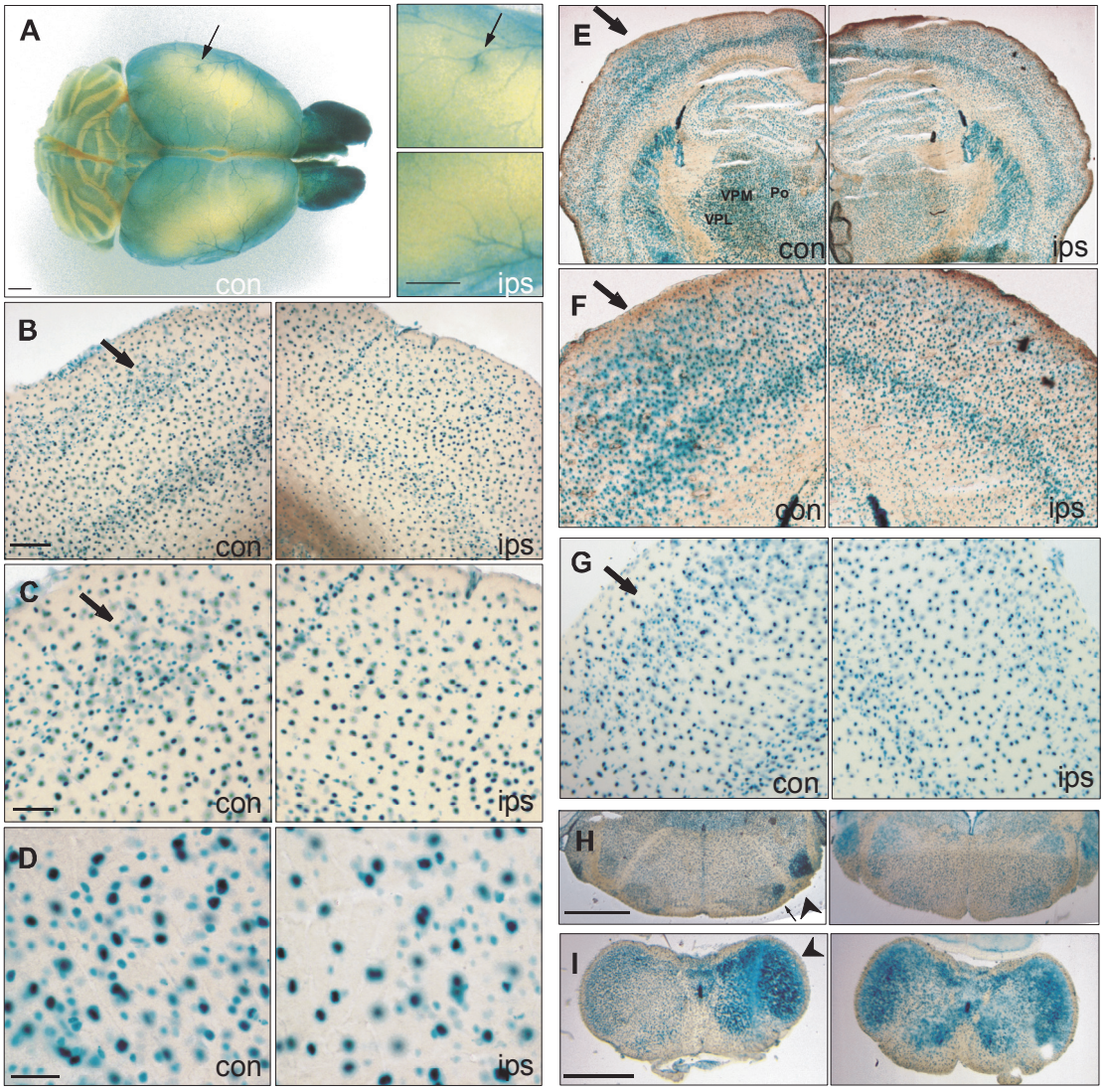

Figure 16. PN-1 transcription is upregulated in the whisker pathway after modified sensory experience. (A) Whole mount X-gal staining of a PN-1 KI mouse brain after 24 hours of single-whisker experience in the enriched environment. The ß-galactosidase upregulation is visible as a blue dot on the contralateral cortical surface (arrow). The enlargements show the ipsi- and contralaral cortical surfaces. (B-D) Coronal sections showing the location of the upregulation of PN-1 expression in the barrel cortex. The upregulation is found only on the side contralateral to the spared whisker in layers II/III and V (B, C, arrow; con, contralateral; ips, ipsilateral). (D) PN-1 is upregulated in a specific

population of cells, which do not express PN-1 under control conditions. (E) After 72 hours of single whisker experience the upregulation of PN-1 expression is found throughout the barrel cortex (arrow) and in nuclei of the contralateral thalamus [contralateral posterior nucleus (Po), ventral posterior medial nucleus (VPM), ventral posterior lateral thalamic nucleus (VPL)]. (F) Several cortical layers, including layer IV, show prominent PN-1 upregulation. (G) Single-whisker experience for 24 hours in control housing conditions also leads to specific upregulation of PN-1 transcription in the contralateral barrel cortex (arrowhead). (H, I) In the brainstem, 72 hours of unilateral whisker deprivation leads to an ipsilateral upregulation of PN-1 expression (arrow and arrowhead). Scale bars: (A), 1 mm, (B, F) (shown in B) 250 µm; (C, G) (shown in C) 125 µm; (D) 100 µm; (E) 400 µm; (H) 300 µm; (I) 1.5 mm.

4.6. tPA expression after single-whisker experience

As our previous results showed that PN-1 primarily inhibits tPA proteolytic activity, we investigated whether cells upregulating PN-1 upon changed sensory experience also express tPA. The vast majority of cells upregulating PN-1 in layers II/III were positive for tPA (Figure 17A, B, filled arrowheads) and some pyramidal-shaped cells in layer V also co-expressed PN-1 and tPA (Figure 17C, arrowheads). Interestingly, cells with PN-1 and tPA co-localization were also found in the retrosplenial cortex (Figure 17D) and the hippocampus (Figure 17E), where we observed activity-dependent alteration of the NR1 subunit.

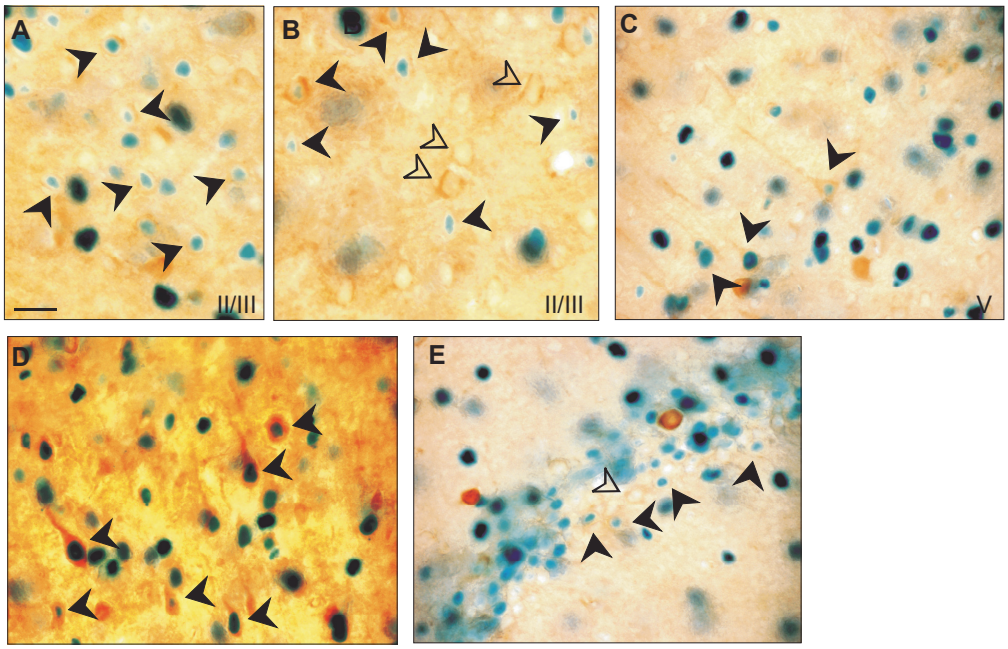

Figure 17. tPA immunoreactivity in cells upregulating PN-1. (A, B) Combined X-gal histochemistry and tPA immunohistochemistry reveal a colocalization in layer II/III cells upregulating PN-1 after 24 hours of single-whisker experience (filled arrowheads). PN-1 upregulating cells are distinguished by their small, light blue nuclei, and tPA immunoreactivity is visible as brown membrane staining. Some cells of layer II/III express tPA but not PN-1 (empty arrowheads). (C) In layer V, some, but not all, cells expressing PN-1 also express tPA. PN-1 and tPA co-expressing cells are found also in the retrosplenial cortex (D) and in the hippocampus (E). Scale bar, A-E (shown in A) 50 µm.

4.7. Immunohistochemical characterization of PN-1 upregulating cells

Aiming at the identification of the cells upregulating PN-1 after single-whisker experience in the barrel cortex, we performed X-gal histochemistry together with immunohistochemistry against cell-type specific markers. SMI32, an antibody against the nonphosphorylated heavy subunit of neurofilament, was used to identify cortical

pyramidal neurons in layers III and Vb. The double labeling confirmed that cells upregulating PN-1, which could be distinguished by their small, light blue nuclei, were restricted to layer II/III (Figure 18A, arrowheads), but were not pyramidal neurons (Figure 18B, empty arrowheads). Interestingly, most of the SMI32-positive cells in layer V expressed PN-1 (Figure 18C, arrowheads). Anti-GAD immunohistochemistry showed that GABA-ergic inhibitory neurons in layers III and IV did not express PN-1 (Figure 18D, empty arrowheads). Most GFAP-positive glial cells all expressed PN-1 (Figure 18E, arrowheads); however, PN-1 upregulating cells in layer II/III had no GFAP immunoreactivity (Figure 18F, empty arrowheads). Interestingly, some PN-1 upregulating cells expressed NG2, a chondroitin sulphate proteoglycan used as a marker for oligodendrocyte precursors (Figure 18G, arrowheads). Few other cells also co-expressed NG2 and PN-1 but appeared not to be cells upregulating PN-1 (Figure 18G, arrows).

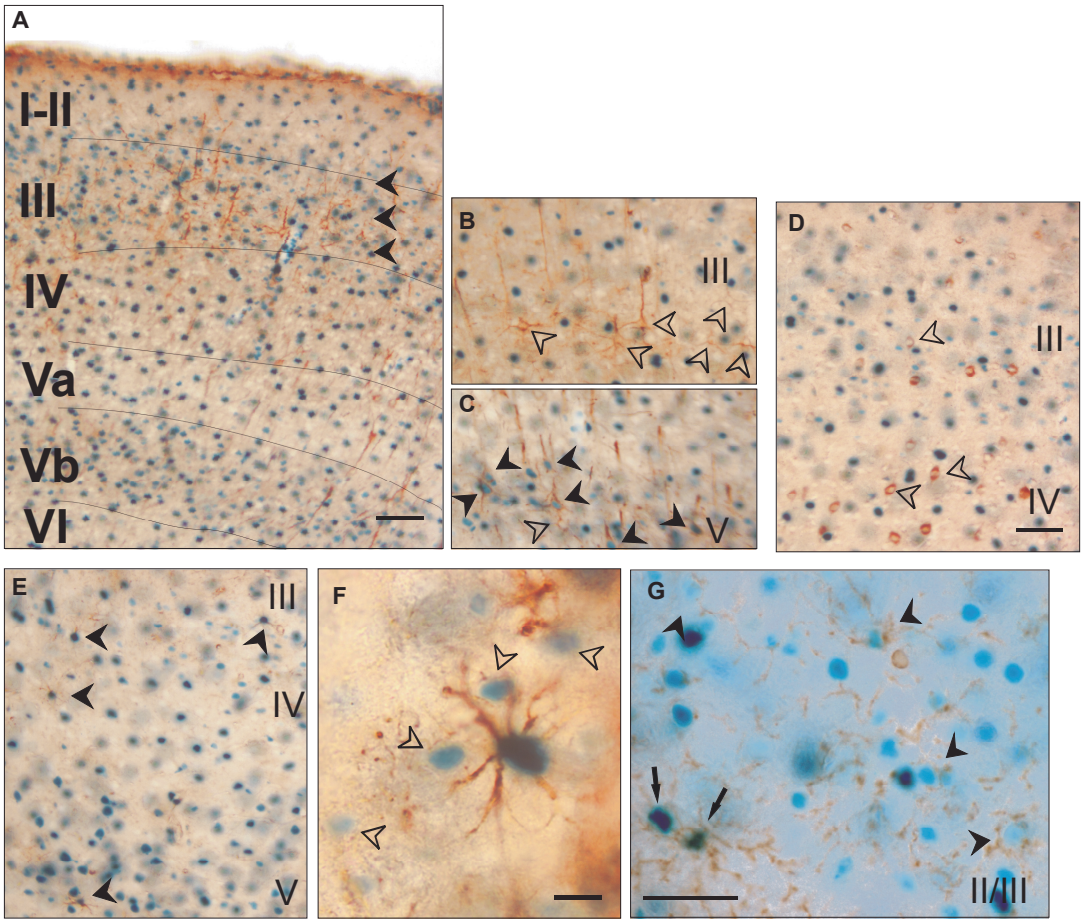

Figure 18. (A) Distribution of PN-1 upregulating cells upon single whisker experience. PN-1 upregulating cells could be distinguished by their small, light blue nuclei. SMI32 antibody (brown), specific for layers III and V pyramidal neurons was used to distinguish cortical layers. Note that cells upregulating PN-1 are found mainly in the in layer II/III (arrowheads). (B) In layer III, SMI32 is not expressed by PN-1 upregulating cells (empty arrowheads), whereas in layer V most of the SMI32 positive cells express PN-1 (arrowheads) (C). (D) No inhibitory neurons in layers III or IV, detected with an anti-GAD antibody are X-gal positive (empty arrowheads). (E, F) Most of the GFAP-positive glial cells express PN-1 (arrowheads), but these are not the cells upregulating it upon sensory experience (empty arrowheads in (F)). (G) Some PN-1 upregulating cells co-express NG2, a chondroitin sulphate proteoglycan (arrowheads). Other cells

are immunoreactive for NG2 and express PN-1 but appear to be different from those upregulating it upon sensory experience (arrows). Scale bar, (A) 100 mm; (B-E), (shown in D) 50 mm; F), 10 mm, (G), 100 mm.

4.8 NMDA receptor-dependent synaptic currents in the barrel cortex of PN-1$^{-/-}$ mice

(Contribution by Dr. Carl Petersen, EPFL, Lausanne)

To find out if the decrease in NMDA receptors in the somatosensory cortex of PN-1$^{-/-}$ mice leads to a deficiency in NMDA receptor function, NMDA receptor-dependent synaptic currents were measured in the barrel cortex of PN-1$^{-/-}$ mice and their wild-type littermates (Figure 19 A, B). An extracellular stimulation electrode was placed in the center of a layer IV barrel and evoked EPSCs were recorded in layer II/III pyramidal neurons. EPSCs recorded at –60 mV were dominated by AMPA/KA-mediated currents which could be completely blocked by 10 μM CNQX, and the peak EPSC provided a measure of AMPA/KA-receptor component of the EPSC. The EPSC at +40 mV had an early component mediated by AMPA/KA-receptors, and a late component reflecting NMDA receptor-dependent currents. Quantified at 50 ms post-stimulus, the EPSC was entirely dependent upon currents through NMDA-receptors (they could be blocked by 50 μM D-APV) and this provided a measure of the NMDA-receptor component of the evoked EPSC. The membrane potential was alternated between –60 mV and +40 mV, and the ratio of NMDA- to AMPA/KA-receptor dependent current was calculated for each experiment. Figure 18A shows an experiment of EPSCs recorded in a PN-1$^{+/+}$ (top), and in a PN-1$^{-/-}$ mouse (bottom). In the wild-type the EPSC recorded at -60 mV lasts a few tens of milliseconds and is mediated by AMPA/KA receptors. The EPSC recorded at +40 mV is much longer lasting and the late component (>40 ms) is mediated almost exclusively by NMDA receptors. In PN-1$^{-/-}$ mice the EPSC recorded at –60 mV was similar to WT animals, showing rapid kinetics and complete dependence upon AMPA/KA receptors. The

EPSC kinetics recorded in these animals at +40 mV were similar to those recorded at –60 mV, in contrast to WT mice which showed a prominent long-lasting NMDA receptor-dependent component at +40 mV. The ratios between PN-$1^{-/-}$ and PN-$1^{+/+}$ mice were statistically different (Figure 18B) (Student's t-test $P<0.01$), with the PN-$1^{+/+}$ having an NMDA/AMPA ratio of 0.91± 0.18, and the PN-$1^{-/-}$ having a ratio of 0.32 ± 0.07. Thus the data further support that the $PN1^{-/-}$ mice are deficient in functional NMDA receptors at excitatory synapses of the barrel cortex.

4.9 Sensory evoked potential recordings in the somatosensory cortex

(Contribution by Dr. Edgardo Troncoso, University of Geneva)

To find out whether the changes in NMDA receptors in the somatosensory cortex of PN-$1^{-/-}$ mice had an impact on cortical physiology, we measured cortical responses to whisker stimulation. Barrel field cytoarchitecture, revealed in tangential sections through layer IV by cytochrome oxidase staining, appeared normal in PN-$1^{-/-}$ mice (data not shown).

Primary sensory evoked potentials (SEP) were simultaneously recorded over the contralateral anterior and posterior barrel cortex and the auditory cortex using a non-invasive epicranial array of three electrodes (Troncoso et al., 2000). For the analysis, we focused on the early positive and negative responses obtained ~13 and ~25 ms after stimulation. A typical waveform of SEP recorded at –1 mm behind Bregma (over the anterior barrel cortex) is shown in Figure 19C. The amplitude of field macro-potentials in PN-$1^{-/-}$ mice was lower than in their wild-type littermates, while the latencies were not different. Slopes (μV/ms) measured from peak positive (P) and negative (N) values revealed that sensory activity in PN-$1^{-/-}$ mice was significantly decreased over the barrel cortex (at –2 mm behind Bregma 40.8 ± 2.9 vs 28.7 ± 3.4, $*P=0.034$, at –1 mm behind Bregma 30.4 ± 1.9 vs 11.8 ± 1.1, $**P= 0.002$) (Figure

19D). Responses obtained at –3 mm were similar in PN-1$^{-/-}$ and PN-1$^{+/+}$ mice. These were considered as auditory responses because of their anatomical location (Franklin and Paxinos, 1996) and because they were recorded in response to the stimulator click, even without whisker deflection (data not shown).

A partial deficiency of NMDA receptor transmission, induced by a proteolytic modification of the NR1 subunit, could explain the lower SEPs recorded in PN-1$^{-/-}$ mice. To test this hypothesis we recorded the sensory evoked potentials in two groups of wild-type C57BL/6 mice. One group received MK-801, an irreversible, high-affinity, non-competitive NMDA receptor antagonist (Loscher et al., 1991). The inhibition of the NMDA receptor resulted in a flattening of SEP values (Figure 19E), and slopes (μV/ms) measured from peak positive and negative values revealed that sensory activity in treated mice was significantly decreased (28.6 ±1.9 vs 18.7 ± 2.8, **P*<0.01) (Figure 19 F). Furthermore, ketamine, another specific non-competitive NMDA receptor blocker with a reversible inhibitory effect, produced an even more pronounced flattening of SEP values at sub-anesthetic doses (data not shown). Thus, pharmacological blockade of NMDA receptors caused a decrease in sensory evoked potentials in wild-type mice, similar to the reduced SEPs recorded in PN-1$^{-/-}$ mice. The smaller SEPs recorded in PN-1$^{-/-}$ mice might therefore reflect a deficiency in NMDA receptor-dependent synaptic transmission.

4.10 Whisker-dependent sensory motor function in PN-1$^{-/-}$ mice

(Contribution by Marita Meins, Friedrich Miescher Institute, Basel)

Since the barrel cortex is crucial for whisker function (Hutson and Masterton, 1986), we tested whether performance in whisker-dependent tasks is modified in PN-1$^{-/-}$ mice. The ability of mice to cross a gap of increasing width was tested (Jenkinson and Glickstein, 2000) (Figure 19G). All mice displayed a distance-dependent delay in

crossing the gap ($F(1,20)=21.58$, $P<0.0001$). Compared with their wild-type littermates, PN-1$^{-/-}$ mice needed more time to perform the task ($F(1,20)=8.51$, $P<0.005$; posttest, $t=2.84$, $^{*}P < 0.05$) (Figure 19H). The slope of the performance curves changed noticeably after 5 cm, in line with the idea that parameters such as whisker input influencing performance come into play with increasing distance (Barneoud et al., 1991a).

To find out how much PN-1$^{-/-}$ mice rely on their whiskers we tested their ability, and the ability of their wild-type littermates, to carry out the task after complete whisker trimming. As expected, wild-type mice were strongly impaired (0.318 ± 0.076, $^{*}P = 0.0156$) but, strikingly, the performance of PN-1$^{-/-}$ mice did not change (0.125 ± 0.109, $P=0.1875$) (Figure 19I), indicating that the latter depend on whisker input less than wild-type mice. No obvious difference in approaching the task was observed namely, animals of both groups thrust forward their snouts, explored (or attempted to) with their whiskers, used either their paws or nose to first make contact but jumped only after the nose contacted the far edge. These changes in performance did not appear to result from differences in motor activity or general motility, as PN-1$^{-/-}$ mice did not differ from their wild-type littermates in an open-field paradigm (Figure 19J). To exclude the possibility that the impaired performance of PN-1$^{-/-}$ mice was due to problems with balance or coordination of movement, a beam balance test, in which a mouse learns to cross a narrow beam to reach a platform (Meins et al., 2001), was performed. This test did not show any difference between PN-1$^{-/-}$ mice and their wild-type littermates (data not shown). Thus, we conclude that the absence of PN-1 is accompanied by NMDA receptor-dependent impairment of the whisker-to-barrel sensory system.

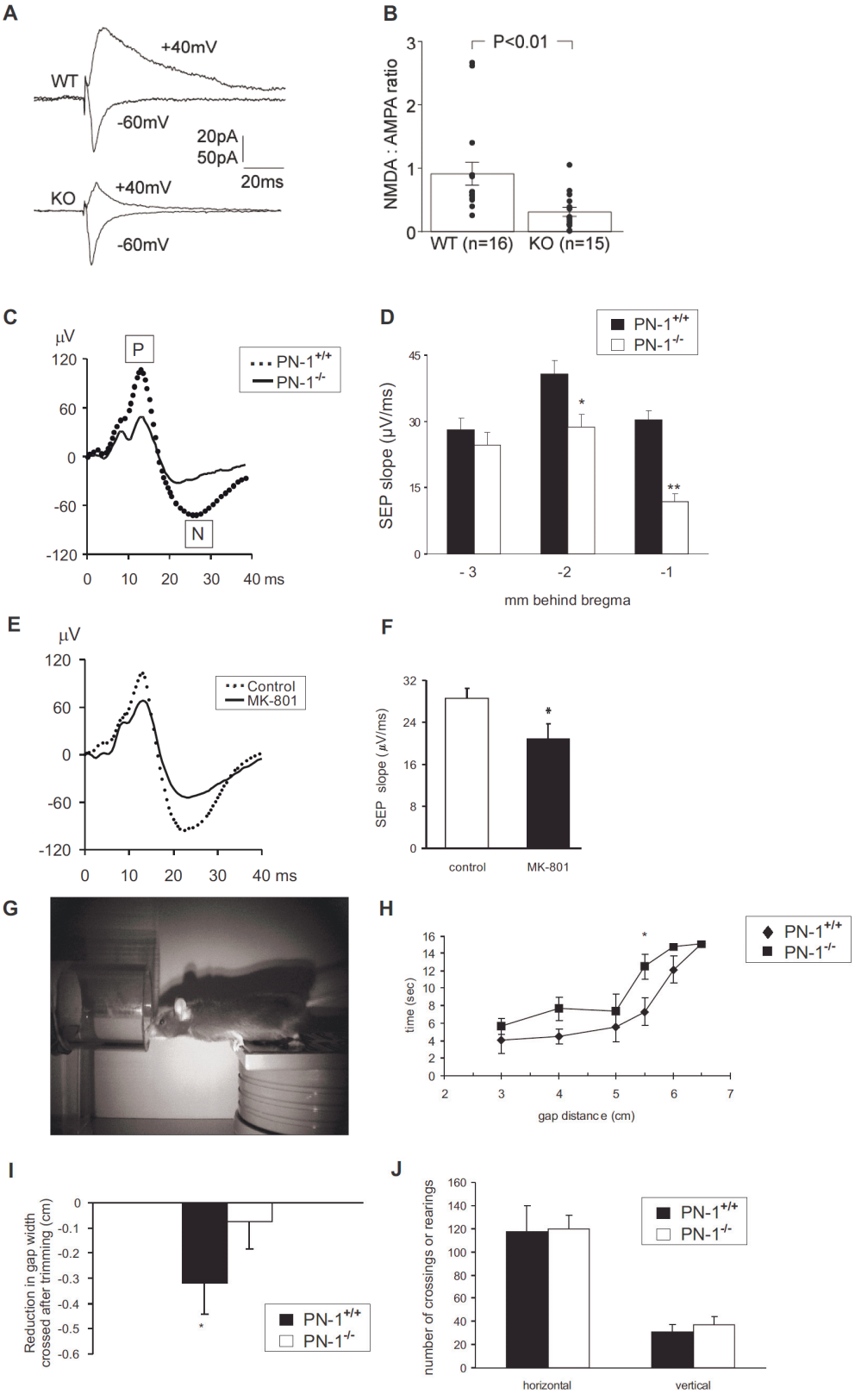
A
+40mV
WT
-60mV
20pA
50pA
20ms
KO
+40mV
-60mV
B
P<0.01
NMDA : AMPA ratio
WT (n=16)
KO (n=15)
C
µV
P
N
PN-1+/+
PN-1-/-
ms
D
PN-1+/+
PN-1-/-
SEP slope (µV/ms)
mm behind bregma
E
µV
Control
MK-801
ms
F
SEP slope (µV/ms)
control
MK-801
G
H
PN-1+/+
PN-1-/-
time (sec)
gap distance (cm)
I
Reduction in gap width crossed after trimming (cm)
PN-1+/+
PN-1-/-
J
number of crossings or rearings
PN-1+/+
PN-1-/-
horizontal
vertical

Figure 19. Decreased NMDA receptor-dependent synaptic currents, decreased sensory evoked potentials and impaired whisker-dependent sensory motor function in PN-1$^{-/-}$ mice. (A) An example of a synaptic current measurement in a wild-type (top), and a PN-1$^{-/-}$ animal (bottom). Note the strong reduction in the EPSCs recorded at +40 mV in the PN-1$^{-/-}$ mouse. (B) Reduced NMDA receptor-dependent synaptic currents, relative to AMPA/kainate receptor-dependent currents, in PN-1$^{-/-}$ mice. Each point represents an individual cell, n=16 WT; n=15 PN-1$^{-/-}$. (C) Typical waveform of sensory evoked potentials induced by unilateral whisker stimulation recorded at -1 mm behind Bregma, showing lower amplitude of field macro-potentials in PN-1$^{-/-}$ mice. Latencies were not different. (D) Evoked sensory potentials in the posterior (-2 mm behind Bregma) and anterior (-1 mm behind Bregma) barrel cortex of PN-1$^{-/-}$ mice (n=4) were significantly decreased compared with PN-1$^{+/+}$ mice (n=4). Sensory potentials at -3 mm behind Bregma were considered as auditory responses and were not different. (E) Effect of partial blocking of NMDA receptor in wild-type mice with MK-801 on the amplitude of sensory evoked potentials in the barrel cortex. Note the lower amplitude in treated mice. (F) Decreased sensory evoked potentials in mice treated with MK-801 (n=5), compared with control mice (n=5). (G) A gap-crossing test revealed that PN-1$^{-/-}$ mice (n=11) need significantly more time to perform this whisker-dependent task than their wild-type littermates (n=11) (H); values are the mean crossing time (up to 15 seconds) ± SEM. (I) When tested for the maximal distance crossed before and after whisker trimming, wild-type littermates (n=11); but not PN-1$^{-/-}$ mice (n=12) crossed shorter distances when deprived of whiskers; values are the difference in the mean distance crossed within 15 seconds before and after the trim ± SEM. (J) No differences were found in open-field activity between PN-1$^{-/-}$ mice (n=11) and their wild-type littermates (n=11); values are mean ± SEM.

5. Discussion and Outlook

By its nature, neuronal plasticity requires ongoing changes in cell morphology and cell-to-cell interactions. Thus, proteolytic modifications of proteins are exquisitely suited to serve a plasticity function. In this study we have demonstrated the importance of a tight control of proteolytic modifications in the brain through the activity-dependent expression of a serine protease inhibitor. Control of extracellular proteolysis appears to be essential for the proper functioning of a sensory pathway.

The finding that neuronal activity leads to the upregulation of PN-1 and modifies the brain proteolytic activity suggests that experience-dependent expression of serine protease inhibitors, as the only known elements that can limit the effects of proteases, may be crucial for the fine-tuning of plastic changes and prevention of excessive proteolysis. Serine proteases can affect a broad range of target molecules and induce modifications in cellular processes such as receptor signaling (Nicole et al., 2001; Gingrich et al., 2000; Zhuo et al., 2000), dendritic arborization (Hirata et al., 2001), axonal outgrowth and synaptic connectivity (Baranes et al., 1998; Wu et al., 2000), indicating that they can contribute to the multiple effects seen during plasticity. Several studies suggested that increased levels of extracellular proteolytic activity can have an effect on brain functions such as LTP and learning (Madani et al., 1999; Baranes et al., 1998; Meiri et al., 1994). However, the activity-dependent expression of serine proteases (Gualandris et al., 1996; Pawlak et al., 2003; Seeds et al., 1995; Parmer et al., 1997) and the data presented here, suggest that plasticity may not only depend on the amount of extracellular proteolysis, but rather on the dynamic and precise orchestration of its activity. While their quick and localized release may be crucial during intensive synaptic remodeling, it appears that their activity has to be also strictly controlled to prevent excessive degradation of target molecules.

Exposure to enriched environment has been shown to elicit various plastic responses to which serine proteases could contribute, such as increased dendrite branching (Holloway, Jr., 1966a), changes in synapse formation (Rampon et al., 2000b;

Greenough et al., 1985), altered pre- and postsynaptic responses and LTP (van Praag et al., 1999a; Green and Greenough, 1986). Similarly, modifications of sensory input in the barrel cortex lead to potentiation of neuronal responses and to neuronal plasticity (Diamond et al., 1994; Fox, 2002; Barth et al., 2000; Simons and Land, 1987; Fox, 1992), as well as to structural changes (Erchova et al., 2003; Knott et al., 2002). The column-specific upregulation of PN-1 upon single-whisker experience is reminiscent of the upregulation of other inducible molecules such as transcription factors (Herdegen and Leah, 1998; Staiger et al., 2000), BDNF (Rocamora et al., 1996), zinc (Brown and Dyck, 2002) and the cAMP response element (Barth et al., 2000). However, it is interesting to note that the majority of these molecules were upregulated in the barrel, located in layer IV, while PN-1 was found upregulated primarily in layer II/III cells. The reason for this specific localization is not clear, but it suggests a tight correlation with neuronal potentiation. It has been previously shown that within the first few hours of single-whisker experience only a subgroup of neurons in layer II/III undergoes potentiation, which subsequently spreads to other cortical layers (Barth et al., 2000; Diamond et al., 1994). Furthermore, cells in these layers are involved in the sensory responses and plastic changes upon modified whisker experience (Petersen et al., 2003; Fox, 2002). Similarly, the spreading of PN-1 upregulation after 72 hours of single-whisker experience possibly reflects the spreading of potentiation through the barrel cortex (Armstrong-James et al., 1992).

The identification of PN-1 upregulating cells in layer II/III may further specify the role of PN-1 in this system. However, immunohistochemical labeling with general markers for cell types such as pyramidal neurons, inhibitory interneurons and glial cells yielded negative results. This is not surprising, as the cell populations in the cortex remain largely undefined, and previous electrophysiological and immunohistochemical studies in the somatosensory cortex have shown the existence of several distinct subtypes of pyramidal, non-pyramidal and inhibitory neurons, as well as glial cells (Ren et al., 1992; Ren et al., 1994; Lubke et al., 2003; van Brederode et al., 2000). Thus, PN-1 upregulating cells could belong to a specific

subpopulation of one of these categories, or to some other, still undefined, cell-type. In any case, these cells appear to be part of a homogenous population, as judged by the specific and virtually identical shape of their nuclei, revealed by the X-gal staining. However, the issue of their identity and its possible functional significance is further complicated by the fact that PN-1 is a secreted molecule (Halfter et al., 1989; Farrell et al., 1988), meaning that it can affect cells expressing it, as well as neighboring cells. Furthermore, at present we were only able to identify the cells expressing PN-1, while the location of the PN-1 protein is not yet known. As it is the case for neuroserpin (Osterwalder et al., 1996), PN-1 could be transported along axons of cells expressing it, and released away from the actual place of production.

It is interesting to speculate about the factors regulating the activity-dependent expression of PN-1 and serine proteases. PN-1 gene expression was *in vitro* shown to be dependent on the growth factor FGF-2 (Kury et al., 1997), while the expression, activity and release of tPA was upregulated by BDNF (Fiumelli et al., 1999). Interestingly, both of these factors are regulated by activity; FGF-2 is upregulated by physical activity and spatial learning (Gomez-Pinilla et al., 1999; Gomez-Pinilla et al., 1998), while neuronal activity elevates the expression of BDNF (Falkenberg et al., 1992; Rocamora et al., 1996). FGF-2 has been implicated in plastic changes (Simonato et al., 1998), while BDNF is one of the key regulators of neuronal development and synaptic plasticity (Teng and Hempstead, 2004; Kang and Schuman, 1995). Thus, some of the effects elicited by activity-dependent expression of growth factors may be mediated through the action of serine proteases and their inhibitors. This hypothesis could be tested by examining the levels and the activity-dependent expression of serine proteases and their inhibitors in mice with reduced levels of these growth factors.

Uncontrolled proteolytic activity can cause increased degradation of target molecules leading to pathological consequences, as it has been described for the reproductive (Murer et al., 2001), respiratory (Barnes et al., 2003; Carden et al., 1998) and digestive system (Naruse et al., 1999). Creation of transgenic mouse models

overexpressing tPA (Madani et al., 1999) and uPA (Meiri et al., 1994) in the brain indicated that non physiological levels of proteolytic activity can affect hippocampal LTP and learning. Similarly, overexpression of PN-1 in postmitotic neurons also lead to changes in LTP, showing that a balance between proteases and inhibitors is crucial for the functional integrity of the nervous system. In PN-$1^{-/-}$ mice elevated levels of tPA activity are correlated with decreased NMDA receptor function in the cortex and hippocampus (Luthi et al., 1997), deficiencies in the electrophysiological performance of the somatosensory cortex and the whisker-dependent motor function. At present, two serine proteases are known to affect NMDA receptors *in vitro*, through the cleavage of the NR1 subunit. Thrombin can cleave its C-terminal, intracellular part (Gingrich et al., 2000), while tPA can bind and cleave at its N-terminus and induce changes in receptor signaling (Nicole et al., 2001). The physiological relevance of the thrombin-induced cleavage is not clear, because active thrombin is not present in the brain, except in pathological situations (Sinnreich et al., 2004). Furthermore, extracellular serine proteases such as thrombin cannot, under physiological conditions, come in contact with the intracellular part of the NR1 subunit. In contrast, the extracellular, tPA-dependent cleavage can be relevant both pathological situations when large amounts of blood-derived tPA are present, but also as a potential physiological modulator of NMDA receptor signaling.

Our data indicate that proteolysis may decrease the availability of functional NMDA receptors, leading to a partial loss of their function in some brain structures. In this respect it is interesting to comment on the fact that *in vitro* cleavage of NMDA receptors was correlated with an NMDA-dependent calcium influx (Nicole et al., 2001), suggesting their potentiation, while we found a decrease in their signaling. This could be due to the differences between the *in vitro* and *in vivo* situation, or the amount and length of exposure to proteolysis. Interestingly, one study reported that increased tPA levels can *in vitro* induce a rapid potentiation of LTP through LRP receptors (Zhuo et al., 2000), while another one showed that activation of LRPs can lead to a potentiation of NMDA receptor function (Bacskai et al., 2000). Therefore, it

is possible that the potentiation of NMDA receptors in the presence of tPA reported by Nicole et al. was related to its binding to the LRP receptor, rather then its cleavage. *In vivo,* and over longer periods of time, the proteolyitic cleavage of NR1 could lead to NMDA receptor destabilization and internalization. However, at present, there is no available information on the longer-term *in vitro* effects of tPA on NMDA receptor stability and signaling, which could help clarifying this issue.

The NR1 subunit is an essential part of functional NMDA receptors. The discovery that NR1 null mice die shortly after birth (Forrest et al., 1994) prompted conditional deletion mutant approaches, which revealed that a cortex-restricted disruption of NR1 impairs neuronal patterning, leading to the absence of barrels (Erzurumlu and Kind, 2001; Iwasato et al., 2000). In this respect it is interesting to comment on the fact that PN-1$^{-/-}$ mice, which have strongly reduced NMDA receptor activity in the cortex, appear to have a normal organization of the barrels (data not shown). This could be explained in several ways. First, NMDA receptor-dependent thalamocortical patterning occurs during early postnatal development, possibly suggesting that during this period NMDA-receptor signaling is not impaired in PN-1$^{-/-}$ mice. Secondly, it is not clear which amount of NMDA receptor activity is needed for thalamocortical patterning, and whether this threshold is achieved in PN-1$^{-/-}$ mice. In this respect, examination and comparison with patterning in transgenic mice expressing only 5-10% of the normal levels of NR1 (Mohn et al., 1999) may be of interest. Finally, it is still possible that PN-1$^{-/-}$ mice have small alterations in the barrel cortex, which could not be detected by cytochrome oxidase staining. Thus, more detailed examination of the barrel cortex structure by immunohistochemical markers and electron microscopy may be necessary in order to address this issue.

The decreased sensory evoked potentials and whisker impairment of PN-1$^{-/-}$ mice are in line with studies showing that NMDA receptors contribute significantly to the sensory responses of the adult barrel cortex (Armstrong-James et al., 1993). However, NMDA receptors are also crucial for adult experience-dependent plasticity (Rema et al., 1998; Jablonska et al., 1999). While the decreased SEP in PN-1$^{-/-}$ mice appear to

be due to decreased NMDA receptor signaling, it is not clear to which degree the plasticity of their barrel cortex is impaired. It would be of major interest to explore this topic, considering the large plastic potential of this sensory pathway. This could be done by an electrophysiological characterization of plastic changes induced by whisker manipulation (Rema et al., 1998; Fox, 2002), or by assessing the potential for activity-dependent structural changes such as synapse formation (Genoud et al., 2004; Knott et al., 2002). Furthermore, it would be worthwhile to examine the functional representation (Huntley, 1997; Polley et al., 1999) of whiskers in the barrel cortex of PN-1$^{-/-}$ mice to find out if experience-dependent restructuring of neuronal maps is changed. Notably, it has been recently found that the plasticity of the barrel cortex is impaired if whiskers are not used early in life (Rema et al., 2003). Considering the functional whisker deficit of PN-1$^{-/-}$ mice, this adds an additional level of complexity to the analysis of their barrel cortex plasticity.

The impairment of whisker-related sensory function in PN-1$^{-/-}$ mice is of special interest considering that the whisker pathway is a major source of sensory information for rodents and very important for their behavior. This is illustrated by the cortical whisker representation which is disproportionately large, compared to representations of other body parts (Woolsey and Van der Loos, 1970). Rodents whisk, or rhythmically palpate objects with their whiskers during exploratory behavior and texture discrimination (Carvell and Simons, 1990), and whiskers have been shown to be important for a variety of activities, including maze learning and spatial orientation (Riesenfeld, 1979), swimming (Ahl, 1986) and depth perception (Schiffman et al., 1970). As PN-1$^{-/-}$ mice appear to have a serious deficit in their performance of whisker-dependent tasks, it would be interesting to examine in more details the nature of this deficiency. It is most likely that the sensory deficit originates in the barrel cortex, as a consequence of decreased NMDA receptor signaling. The barrel cortex is crucial for whisker function. Ablations of barrels impair roughness discrimination (Guic-Robles et al., 1992) and performance in tasks depending on the active movement of the whiskers (Hutson and Masterton, 1986). Furthermore, the fact

that the activation of these receptors appears to be crucial for the sensory activity of the barrel cortex (Rema et al., 1998), indirectly suggests that they may also influence whisker-related sensory performance. In addition, even though we observed activity-dependent PN-1 expression in thalamic nuclei and the brainstem, the latencies of signals upon whisker stimulation were not changed, possibly suggesting that these connections were not impaired. This is in line with preliminary observations indicating that the whisking patterns of PN-1$^{-/-}$ mice were not different from their wild-type littermates, and suggesting that the whisking pattern generator, shown to be located in the brainstem (Hattox et al., 2003), was not affected. However, it would be of interest to assess this issue in more details, and explore the performance of other whisker-related brain structures. Assessment of some more specific whisker functions, such as the capability of roughness discrimination (Guic-Robles et al., 1992; Cybulska-Klosowicz and Kossut, 2001) could provide additional information about the degree and nature of the whisker impairment on PN-1$^{-/-}$ mice. Interestingly, PN-1$^{-/-}$ mice didn't show any obvious difference in their approach to the gap-crossing task, suggesting that they have developed mechanisms to partially adapt to decreased whisker function, possibly by relying on other sensory systems. Such compensatory mechanisms have been previously proposed for whisker models of a sensory deficit (Symons and Tees, 1990; Volgyi et al., 1993). However, keeping in mind the importance of whiskers as a source of sensory information for rodents, it would be worthwhile to examine whether such lack of whisker function induced some broader behavioral deficits. It would be also interesting to find out whether the cortical and whisker phenotype appears early in development, or only during adulthood. Experimental evidence suggests the involvement of tPA in the control of plasticity of the developing visual system (Mataga et al., 2002; Muller and Griesinger, 1998), and preliminary data showed increased NMDA receptor signaling in the barrel cortex of P14 PN-1$^{-/-}$ mice (Carl Petersen, unpublished data), indicating dynamic NMDA receptor regulation. Thus, along with post-developmental effects related to activity-dependent changes in proteolytic activity, PN-1 may participate in the development, through the control of NMDA receptor signaling or other substrates.

It is possible that some other proteolysis-induced modifications contribute to the decreased sensory evoked potentials and the whisker-dependent sensory deficit of PN-1$^{-/-}$ mice. The modification of NMDA receptor signaling could represent one out of many effects that excessive proteolytic activity could have on the brain. This is suggested by the increased *in vitro* epileptiform activity and susceptibility to kainic acid-induced seizures in PN-1$^{-/-}$ mice (Luthi et al., 1997), indicating enhanced CNS excitability, which cannot be explained by reduced NMDA receptor signaling. What are the other possible targets of enhanced proteolysis? tPA can convert plasminogen into plasmin, a broad–spectrum protease that can degrade components of the extracellular matrix such as laminin (Chen and Strickland, 1997), NCAM (Endo et al., 1998) and cell-surface proteoglycanes (Wu et al., 2000). Plasmin-induced laminin degradation can *in vitro* lead to LTP disruption (Nakagami et al., 2000). Similarly, increased levels of proteolytic activity upon excitotoxic injections in the hippocampus can promote neuronal death through laminin degradation (Chen and Strickland, 1997). Therefore, changes in extracellular matrix composition may lead to decreased LTP and other electrophysiological deficits. Furthermore, interactions between tPA and the LRP receptor *in vitro* lead to LTP potentiation (Zhuo et al., 2000). Both PN-1 and tPA can bind to LRP receptors, alone or in complex (Zhang et al., 1998; Kasza et al., 1997). Thus, a disrupted protease-inhibitor balance could affect LRP-mediated control of LTP. Finally, there is evidence that some growth factors such as hepatocyte growth factor (HGF) and BDNF require proteolytic activation (Mars et al., 1993; Mowla et al., 2001; Meyer et al., 1996). It would be interesting to find out if their activation and activity is changed in PN-1$^{-/-}$ mice, and what could be the consequences of such a change.

Altogether, the effects of enhanced proteolysis and absence of PN-1 in the brain may be cumulative, or they may interfere with each other, thus creating a complex phenotype (Figure 20). A systematic immunohistochemical investigation could provide more insight in the status of these molecules in PN-1$^{-/-}$ mice, while an electrophysiological *in vitro* approach could be used to find out whether other cell-

surface receptors and channels are affected. Additionally, a proteomic analysis of PN-$1^{-/-}$ brain tissue could reveal still unknown targets of proteolytic activity.

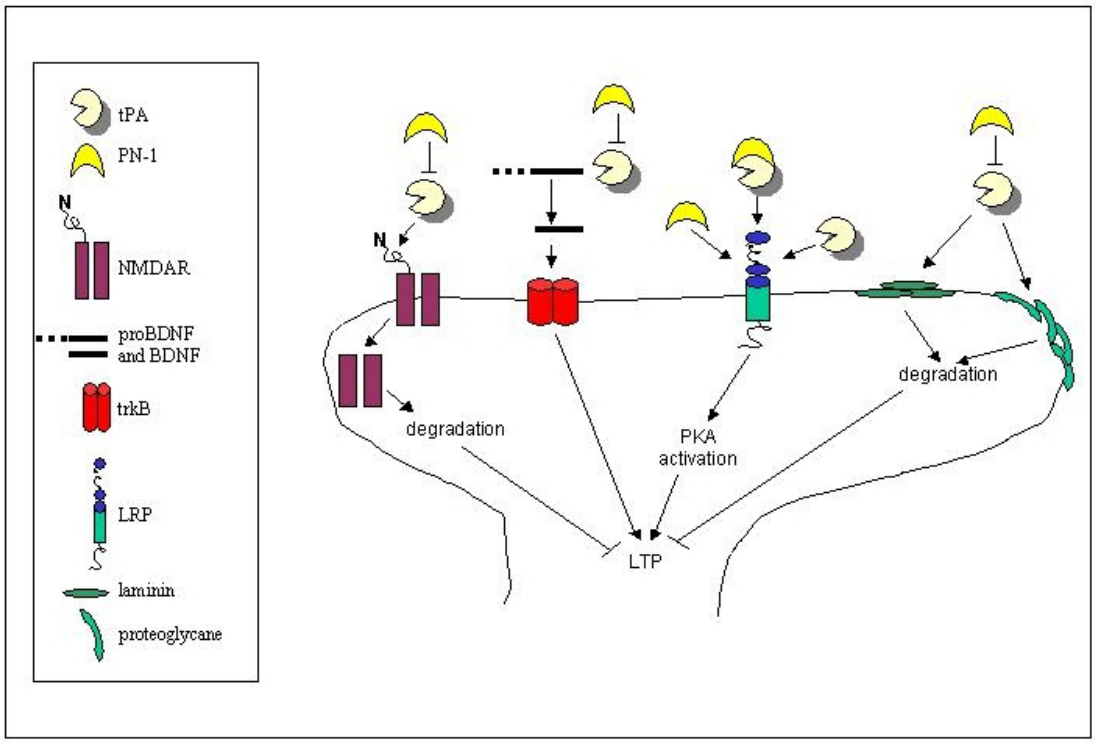

Figure 20. Schematic model of the putative effects of serine proteases and their inhibitors on brain plasticity. Several potential targets of extracellular proteolysis in the CNS are depicted, among which are cell-surface receptors (NMDA and LRP receptors), growth factors (BDNF), and extracellular matrix molecules (laminin and proteoglycanes). Their degradation or modified interaction with proteases and their inhibitors could lead to changes in intracellular signaling, and ultimatively affect neuronal plasticity.

NMDA receptors are particularly interesting targets of extracellular proteolysis because of their multiple functions. The modulation of their signaling by serine proteases represents a very attractive mechanism for plasticity-related effects of serine proteases in the CNS. The *in vivo* cleavage of the NR1 subunit has been previously postulated mainly for pathological states during which large amounts of

blood-derived tPA cross the blood-brain barrier (Nicole et al., 2001; Fernandez-Monreal et al., 2004). However, such a modification could also have a role in normal brain functions. Proteolytic degradation of NMDA receptors could represent a homeostatic mechanism for their rapid removal from the cell membrane. Recently, it has been found that NMDA receptors undergo constitutive and rapid internalization (Nong et al., 2004), and the mechanisms triggering this process are not yet fully understood. Thus, they may also involve cleavage by serine proteases. Alternatively, the cleavage could induce modifications of NMDA receptors and change their signaling properties. These hypothesis are supported by a behavioral study suggesting a change in NMDA receptor-dependent function in tPA deficient mice (Horwood et al., 2004). It has been shown in the past that visual experience alters the properties of NMDA receptors through changes in subunit composition (Philpot et al., 2001; Quinlan et al., 1999). It is, thus, an intriguing possibility that proteolytic cleavage induced by activity-dependent local upregulation of serine proteases could represent an alternative regulation mechanism of NMDA receptor signaling. Clearly, such a phenomenon would require a tight control of proteolytic activity, through the upregulation of appropriate inhibitors, such as PN-1.

In summary, this study presents the first example of extracellular proteases downregulating a cell-surface neurotransmitter receptor *in vivo*, and shows that PN-1-mediated control of extracellular proteolysis is essential for the proper functioning of sensory pathways. The importance of the proteolytic regulation of the NR1 subunit is highlighted by earlier publications showing the crucial role of NMDA receptors in plasticity and disease. Inappropriate NMDA receptor signaling has been implicated in several disorders. Mice with strongly reduced NMDA function display behaviors related to schizophrenia (Mohn et al., 1999), and the lack of NR1 in the CA1 hippocampal structure leads to impaired object recognition and associative memories (Nakazawa et al., 2002; Rampon et al., 2000b). NMDA receptors also appear to be important for the functioning of memory storage circuits in the cortex (Cui et al., 2004). Furthermore, the modification of anxiety-like behavior (Pawlak et al., 2003)

and memory (Madani et al., 1999) in tPA deficient and overexpressing mice, may be mediated through NMDA receptor cleavage. All these findings open a broad perspective for the further characterization of PN-$1^{-/-}$ mice, indicating they may also represent valuable models for human pathologies. Consequently, we provide a model not only for *in vivo* molecular changes triggered by neuronal activity-dependent proteolytic events, but also for studying other normal or pathological brain functions in which such modifications could play a role.

6. References

Ahl,A.S. (1986). The role of vibrissae in behavior: a status review. Vet. Res. Commun. *10*, 245-268.

Altman,J. and Das,G.D. (1964). AUTORADIOGRAPHIC EXAMINATION OF THE EFFECTS OF ENRICHED ENVIRONMENT ON THE RATE OF GLIAL MULTIPLICATION IN THE ADULT RAT BRAIN. Nature *204*, 1161-1163.

An,G., Hidaka,K., and Siminovitch,L. (1982). Expression of bacterial beta-galactosidase in animal cells. Mol. Cell Biol. *2*, 1628-1632.

Andres,F.L. and Van der Loos H. (1985). From sensory periphery to cortex: the architecture of the barrelfield as modified by various early manipulations of the mouse whiskerpad. Anat. Embryol. (Berl) *172*, 11-20.

Armstrong-James,M. (1975). The functional status and columnar organization of single cells responding to cutaneous stimulation in neonatal rat somatosensory cortex S1. J. Physiol *246*, 501-538.

Armstrong-James,M., Fox,K., and Das-Gupta,A. (1992). Flow of excitation within rat barrel cortex on striking a single vibrissa. J. Neurophysiol. *68*, 1345-1358.

Armstrong-James,M., Welker,E., and Callahan,C.A. (1993). The contribution of NMDA and non-NMDA receptors to fast and slow transmission of sensory information in the rat SI barrel cortex. J. Neurosci. *13*, 2149-2160.

Bacskai,B.J., Xia,M.Q., Strickland,D.K., Rebeck,G.W., and Hyman,B.T. (2000). The endocytic receptor protein LRP also mediates neuronal calcium signaling via N-methyl-D-aspartate receptors. Proc. Natl. Acad. Sci. U. S. A *97*, 11551-11556.

Baranes,D., Lederfein,D., Huang,Y.Y., Chen,M., Bailey,C.H., and Kandel,E.R. (1998). Tissue plasminogen activator contributes to the late phase of LTP and to synaptic growth in the hippocampal mossy fiber pathway. Neuron *21*, 813-825.

Barneoud,P., Gyger,M., Andres,F., and Van der Loos,H. (1991a). Vibrissa-related behavior in mice: transient effect of ablation of the barrel cortex. Behav. Brain Res. *44*, 87-99.

Barneoud,P., Gyger,M., Andres,F., and Van der,L.H. (1991b). Vibrissa-related behavior in mice: transient effect of ablation of the barrel cortex. Behav. Brain Res. *44*, 87-99.

Barnes,P.J., Shapiro,S.D., and Pauwels,R.A. (2003). Chronic obstructive pulmonary disease: molecular and cellular mechanisms. Eur. Respir. J. *22*, 672-688.

Barrett,A.J. and Salvesen,G.S. (1986). Proteinase inhibitors.

Barros,C., Crosby,J.A., and Moreno,R.D. (1996). Early steps of sperm-egg interactions during mammalian fertilization. Cell Biol. Int. *20*, 33-39.

Barth,A.L., McKenna,M., Glazewski,S., Hill,P., Impey,S., Storm,D., and Fox,K. (2000). Upregulation of cAMP response element-mediated gene expression during experience-dependent plasticity in adult neocortex. J. Neurosci. *20*, 4206-4216.

Basham,M.E. and Seeds,N.W. (2001). Plasminogen expression in the neonatal and adult mouse brain. J. Neurochem. *77*, 318-325.

Belin,D., Wohlwend,A., Schleuning,W.D., Kruithof,E.K., and Vassalli,J.D. (1989). Facultative polypeptide translocation allows a single mRNA to encode the secreted and cytosolic forms of plasminogen activators inhibitor 2. EMBO J. *8*, 3287-3294.

Bennett,E.L., Rosenzweig,M.R., Morimoto,H., and Hebert,M. (1979). Maze training alters brain weights and cortical RDA/DNA ratios. Behav. Neural Biol. *26*, 1-22.

Benveniste,M. and Mayer,M.L. (1991). Kinetic analysis of antagonist action at N-methyl-D-aspartic acid receptors. Two binding sites each for glutamate and glycine. Biophys. J. *59*, 560-573.

Bi,G. and Poo,M. (2001). Synaptic modification by correlated activity: Hebb's postulate revisited. Annu. Rev. Neurosci. *24*, 139-166.

Bleuel,A., de Gasparo,M., Whitebread,S., Puttner,I., and Monard,D. (1995). Regulation of protease nexin-1 expression in cultured Schwann cells is mediated by angiotensin II receptors. J. Neurosci. *15*, 750-761.

Bleuel,A. and Monard,D. (1995). Regulation of protease nexin-1 and angiotensin II receptor subtype 1 expression: inverse relationship in experimental models of nerve injury. J. Neurosci. Res. *42*, 562-570.

Bliss,T.V. and Collingridge,G.L. (1993). A synaptic model of memory: long-term potentiation in the hippocampus. Nature *361*, 31-39.

Blow,D.M. (1968). Chymotrypsin: tertiary structure and enzymic activity. Biochem. J. *110*, 2P.

Bode,W. and Renatus,M. (1997). Tissue-type plasminogen activator: variants and crystal/solution structures demarcate structural determinants of function. Curr. Opin. Struct. Biol. *7*, 865-872.

Brown,C.E. and Dyck,R.H. (2002). Rapid, experience-dependent changes in levels of synaptic zinc in primary somatosensory cortex of the adult mouse. J. Neurosci. *22*, 2617-2625.

Buonomano,D.V. and Merzenich,M.M. (1998). Cortical plasticity: from synapses to maps. Annu. Rev. Neurosci. *21*, 149-186.

Calabresi,P., Napolitano,M., Centonze,D., Marfia,G.A., Gubellini,P., Teule,M.A., Berretta,N., Bernardi,G., Frati,L., Tolu,M., and Gulino,A. (2000). Tissue plasminogen activator controls multiple forms of synaptic plasticity and memory. Eur. J. Neurosci. *12*, 1002-1012.

Carden,D., Xiao,F., Moak,C., Willis,B.H., Robinson-Jackson,S., and Alexander,S. (1998). Neutrophil elastase promotes lung microvascular injury and proteolysis of endothelial cadherins. Am. J. Physiol *275*, H385-H392.

Carrell,R.W. and Boswell,D.R. (1986). Serpins: the superfamily of plasma serine proteinase inhibitors.

Carrell,R.W., Evans,D.L., and Stein,P.E. (1991). Mobile reactive centre of serpins and the control of thrombosis. Nature *353*, 576-578.

Carvell,G.E. and Simons,D.J. (1990). Biometric analyses of vibrissal tactile discrimination in the rat. J. Neurosci. *10*, 2638-2648.

Cavallaro,S., Gibbs,C.J., and Pergami,P. (1999). Early induction of protein nexin-I in scrapie. Neuroreport *10*, 1677-1681.

Centonze,D., Napolitano,M., Saulle,E., Gubellini,P., Picconi,B., Martorana,A., Pisani,A., Gulino,A., Bernardi,G., and Calabresi,P. (2002). Tissue plasminogen activator is required for corticostriatal long-term potentiation. Eur. J. Neurosci. *16*, 713-721.

Chen,C. and Tonegawa,S. (1997). Molecular genetic analysis of synaptic plasticity, activity-dependent neural development, learning, and memory in the mammalian brain. Annu. Rev. Neurosci. *20*, 157-184.

Chen,Z.L. and Strickland,S. (1997). Neuronal death in the hippocampus is promoted by plasmin-catalyzed degradation of laminin. Cell *91*, 917-925.

Choi,B.H., Kim,R.C., Vaughan,P.J., Lau,A., Van Nostrand,W.E., Cotman,C.W., and Cunningham,D.D. (1995). Decreases in protease nexins in Alzheimer's disease brain. Neurobiol. Aging *16*, 557-562.

Choi,S.H., Lee,d.Y., Ryu,J.K., Kim,J., Joe,E.H., and Jin,B.K. (2003). Thrombin induces nigral dopaminergic neurodegeneration in vivo by altering expression of death-related proteins. Neurobiol. Dis. *14*, 181-193.

Collen,D. and Wiman,B. (1979). Turnover of antiplasmin, the fast-acting plasmin inhibitor of plasma. Blood *53*, 313-324.

Conese,M., Nykjaer,A., Petersen,C.M., Cremona,O., Pardi,R., Andreasen,P.A., Gliemann,J., Christensen,E.I., and Blasi,F. (1995). alpha-2 Macroglobulin receptor/Ldl receptor-related protein(Lrp)- dependent internalization of the urokinase receptor. J. Cell Biol. *131*, 1609-1622.

Constantine-Paton,M. and Cline,H.T. (1998). LTP and activity-dependent synaptogenesis: the more alike they are, the more different they become. Curr. Opin. Neurobiol. *8*, 139-148.

Coughlin,S.R. (2000). Thrombin signalling and protease-activated receptors. Nature *407*, 258-264.

Crisp,R.J., Knauer,M.F., and Knauer,D.J. (2002). Protease nexin 1 is a potent urinary plasminogen activator inhibitor in the presence of collagen type IV. J. Biol. Chem. *277*, 47285-47291.

Cui,Z., Wang,H., Tan,Y., Zaia,K.A., Zhang,S., and Tsien,J.Z. (2004). Inducible and reversible NR1 knockout reveals crucial role of the NMDA receptor in preserving remote memories in the brain. Neuron *41*, 781-793.

Cull-Candy,S., Brickley,S., and Farrant,M. (2001). NMDA receptor subunits: diversity, development and disease. Curr. Opin. Neurobiol. *11*, 327-335.

Cybulska-Klosowicz,A. and Kossut,M. (2001). Mice can learn roughness discrimination with vibrissae in a jump stand apparatus. Acta Neurobiol. Exp. (Wars.) *61*, 73-76.

Das,S., Sasaki,Y.F., Rothe,T., Premkumar,L.S., Takasu,M., Crandall,J.E., Dikkes,P., Conner,D.A., Rayudu,P.V., Cheung,W., Chen,H.S., Lipton,S.A., and Nakanishi,N. (1998). Increased NMDA current and spine density in mice lacking the NMDA receptor subunit NR3A. Nature *393*, 377-381.

Diamond,M.C., Law,F., Rhodes,H., Lindner,B., Rosenzweig,M.R., Krech,D., and Bennett,E.L. (1966). Increases in cortical depth and glia numbers in rats subjected to enriched environment. J. Comp Neurol. *128*, 117-126.

Diamond,M.C., Lindner,B., Johnson,R., Bennett,E.L., and Rosenzweig,M.R. (1975). Differences in occipital cortical synapses from environmentally enriched, impoverished, and standard colony rats. J. Neurosci. Res. *1*, 109-119.

Diamond,M.E., Huang,W., and Ebner,F.F. (1994). Laminar comparison of somatosensory cortical plasticity. Science *265*, 1885-1888.

Dingledine,R., Borges,K., Bowie,D., and Traynelis,S.F. (1999). The glutamate receptor ion channels. Pharmacol. Rev. *51*, 7-61.

Dityatev,A. and Schachner,M. (2003). Extracellular matrix molecules and synaptic plasticity. Nat. Rev. Neurosci. *4*, 456-468.

Donovan,F.M., Vaughan,P.J., and Cunningham,D.D. (1994). Regulation of protease nexin-1 target protease specificity by collagen type IV. J. Biol. Chem. *269*, 17199-17205.

Endo,A., Nagai,N., Urano,T., Ihara,H., Takada,Y., Hashimoto,K., and Takada,A. (1998). Proteolysis of highly polysialylated NCAM by the tissue plasminogen activator-plasmin system in rats. Neurosci. Lett. *246*, 37-40.

Engineer,N.D., Percaccio,C.R., Pandya,P.K., Moucha,R., Rathbun,D.L., and Kilgard,M.P. (2004). Environmental Enrichment Improves Response Strength, Threshold, Selectivity, and Latency of Auditory Cortex Neurons. J. Neurophysiol.

Erchova,I.A., Petersen,R.S., and Diamond,M.E. (2003). Effect of developmental sensory and motor deprivation on the functional organization of adult rat somatosensory cortex. Brain Res. Bull. *60*, 373-386.

Erno,H., Kury,P., Botteri,F.M., and Monard,D. (1996a). A Krox binding site regulates protease nexin-1 promoter activity in embryonic heart, cartilage and parts of the nervous system. Mech. Dev. *60*, 139-150.

Erno,H., Kury,P., Nitsch,C., Jost,J.P., and Monard,D. (1996b). Excitotoxic brain lesion modifies binding to a USF binding site acting as a negative regulatory element in the Protease nexin-1 promoter. Mol. Cell Neurosci. *8*, 28-37.

Erno,H. and Monard,D. (1993). Molecular organization of the rat glia-derived nexin/protease nexin-1 promoter. Gene Expr. *3*, 163-174.

Erzurumlu,R.S. and Kind,P.C. (2001). Neural activity: sculptor of 'barrels' in the neocortex. Trends Neurosci. *24*, 589-595.

Falkenberg,T., Mohammed,A.K., Henriksson,B., Persson,H., Winblad,B., and Lindefors,N. (1992). Increased expression of brain-derived neurotrophic factor mRNA in rat hippocampus is associated with improved spatial memory and enriched environment. Neurosci. Lett. *138*, 153-156.

Farrant,M., Feldmeyer,D., Takahashi,T., and Cull-Candy,S.G. (1994). NMDA-receptor channel diversity in the developing cerebellum. Nature *368*, 335-339.

Farrell,D.H., Wagner,S.L., Yuan,R.H., and Cunningham,D.D. (1988). Localization of protease nexin-1 on the fibroblast extracellular matrix. J. Cell Physiol. *134*, 179-188.

Fernandez-Monreal,M., Lopez-Atalaya,J.P., Benchenane,K., Leveille,F., Cacquevel,M., Plawinski,L., MacKenzie,E.T., Bu,G., Buisson,A., and Vivien,D. (2004). Is tissue-type plasminogen activator a neuromodulator? Mol. Cell Neurosci. *25*, 594-601.

Fiumelli,H., Jabaudon,D., Magistretti,P.J., and Martin,J.L. (1999). BDNF stimulates expression, activity and release of tissue-type plasminogen activator in mouse cortical neurons. Eur. J. Neurosci. *11*, 1639-1646.

Flint,A.C., Maisch,U.S., Weishaupt,J.H., Kriegstein,A.R., and Monyer,H. (1997). NR2A subunit expression shortens NMDA receptor synaptic currents in developing neocortex. J. Neurosci. *17*, 2469-2476.

Forrest,D., Yuzaki,M., Soares,H.D., Ng,L., Luk,D.C., Sheng,M., Stewart,C.L., Morgan,J.I., Connor,J.A., and Curran,T. (1994). Targeted disruption of NMDA receptor 1 gene abolishes NMDA response and results in neonatal death. Neuron *13*, 325-338.

Foster,T.C., Fugger,H.N., and Cunningham,S.G. (2000). Receptor blockade reveals a correspondence between hippocampal- dependent behavior and experience-dependent synaptic enhancement. Brain Res. *871*, 39-43.

Fox,K. (1992). A critical period for experience-dependent synaptic plasticity in rat barrel cortex. J. Neurosci. *12*, 1826-1838.

Fox,K. (2002). Anatomical pathways and molecular mechanisms for plasticity in the barrel cortex. Neuroscience *111*, 799-814.

Franklin,K.B.J. and Paxinos,G. (1996). The Mouse Brain in Stereotaxic Coordinates. (San Diego: Academic Press).

Frey,U., Muller,M., and Kuhl,D. (1996). A different form of long-lasting potentiation revealed in tissue plasminogen activator mutant mice. J. Neurosci. *16*, 2057-2063.

Garraghty,P.E. and Kaas,J.H. (1992). Dynamic features of sensory and motor maps. Curr. Opin. Neurobiol. *2*, 522-527.

Genoud,C., Knott,G.W., Sakata,K., Lu,B., and Welker,E. (2004). Altered synapse formation in the adult somatosensory cortex of brain-derived neurotrophic factor heterozygote mice. J. Neurosci. *24*, 2394-2400.

Gettins,P.G. (2002). Serpin structure, mechanism, and function. Chem. Rev. *102*, 4751-4804.

Gingrich,M.B., Junge,C.E., Lyuboslavsky,P., and Traynelis,S.F. (2000). Potentiation of NMDA receptor function by the serine protease thrombin. J. Neurosci. *20*, 4582-4595.

Gingrich,M.B. and Traynelis,S.F. (2000). Serine proteases and brain damage - is there a link? Trends Neurosci. *23*, 399-407.

Globus,A., Rosenzweig,M.R., Bennett,E.L., and Diamond,M.C. (1973). Effects of differential experience on dendritic spine counts in rat cerebral cortex. J. Comp Physiol Psychol. *82*, 175-181.

Gloor,S., Odink,K., Guenther,J., Nick,H., and Monard,D. (1986). A glia-derived neurite promoting factor with protease inhibitory activity belongs to the protease nexins. Cell *47*, 687-693.

Gomez-Pinilla,F., Choi,J., and Ryba,E.A. (1999). Visual input regulates the expression of basic fibroblast growth factor and its receptor. Neuroscience *88*, 1051-1058.

Gomez-Pinilla,F., So,V., and Kesslak,J.P. (1998). Spatial learning and physical activity contribute to the induction of fibroblast growth factor: neural substrates for increased cognition associated with exercise. Neuroscience *85*, 53-61.

Gottfried,J.A. and Chesler,M. (1994). Endogenous H+ modulation of NMDA receptor-mediated EPSCs revealed by carbonic anhydrase inhibition in rat hippocampus. J. Physiol *478 Pt 3*, 373-378.

Green,E.J. and Greenough,W.T. (1986). Altered synaptic transmission in dentate gyrus of rats reared in complex environments: evidence from hippocampal slices maintained in vitro. J. Neurophysiol. *55*, 739-750.

Greenough,W.T., Hwang,H.M., and Gorman,C. (1985). Evidence for active synapse formation or altered postsynaptic metabolism in visual cortex of rats reared in complex environments. Proc. Natl. Acad. Sci. U. S. A *82*, 4549-4552.

Greenough,W.T., West,R.W., and DeVoogd,T.J. (1978). Subsynaptic plate perforations: changes with age and experience in the rat. Science *202*, 1096-1098.

Gschwend,T.P., Krueger,S.R., Kozlov,S.V., Wolfer,D.P., and Sonderegger,P. (1997). Neurotrypsin, a novel multidomain serine protease expressed in the nervous system. Mol. Cell Neurosci. *9*, 207-219.

Gualandris,A., Jones,T.E., Strickland,S., and Tsirka,S.E. (1996). Membrane depolarization induces calcium-dependent secretion of tissue plasminogen activator. J. Neurosci. *16*, 2220-2225.

Guenther,J., Nick,H., and Monard,D. (1985). A glia-derived neurite-promoting factor with protease inhibitory activity. EMBO J. *4*, 1963-1966.

Guic-Robles,E., Jenkins,W.M., and Bravo,H. (1992). Vibrissal roughness discrimination is barrelcortex-dependent. Behav. Brain Res. *48*, 145-152.

Guttridge,D.C., Lau,A.L., and Cunningham,D.D. (1993). Protease nexin-1, a thrombin inhibitor, is regulated by interleukin-1 and dexamethasone in normal human fibroblasts. J. Biol. Chem. *268*, 18966-18974.

Halfter,W., Reinhard,E., Liverani,D., Ortman,R., and Monard,D. (1989). Immunocytochemical Localization of Glia-Derived Nexin, Laminin and Fibronectin on the Surface or Extracellular Matrix of C6 Rat Glioma Cells, Astrocytes and Fibroblasts. Eur. J. Neurosci. *1*, 297-308.

Hardingham,G.E. and Bading,H. (2003). The Yin and Yang of NMDA receptor signalling. Trends Neurosci. *26*, 81-89.

Hattox,A., Li,Y., and Keller,A. (2003). Serotonin regulates rhythmic whisking. Neuron *39*, 343-352.

Hedstrom,L. (2002). Serine protease mechanism and specificity. Chem. Rev. *102*, 4501-4524.

Hengst,U., Albrecht,H., Hess,D., and Monard,D. (2001). The phosphatidylethanolamine-binding protein is the prototype of a novel family of serine protease inhibitors. J. Biol. Chem. *276*, 535-540.

Herdegen,T. and Leah,J.D. (1998). Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expression by Jun, Fos and Krox, and CREB/ATF proteins. Brain Res. Brain Res. Rev. *28*, 370-490.

Herndon,M.E., Stipp,C.S., and Lander,A.D. (1999). Interactions of neural glycosaminoglycans and proteoglycans with protein ligands: assessment of selectivity, heterogeneity and the participation of core proteins in binding. Glycobiology *9*, 143-155.

Herz,J. and Strickland,D.K. (2001). LRP: a multifunctional scavenger and signaling receptor. J. Clin. Invest *108*, 779-784.

Hirata,A., Yoshida,S., Inoue,N., Matsumoto-Miyai,K., Ninomiya,A., Taniguchi,M., Matsuyama,T., Kato,K., Iizasa,H., Kataoka,Y., Yoshida,N., and Shiosaka,S. (2001). Abnormalities of synapses and neurons in the hippocampus of neuropsin- deficient mice. Mol. Cell Neurosci. *17*, 600-610.

Hockly,E., Cordery,P.M., Woodman,B., Mahal,A., Van Dellen,A., Blakemore,C., Lewis,C.M., Hannan,A.J., and Bates,G.P. (2002). Environmental enrichment slows disease progression in R6/2 Huntington's disease mice. Ann. Neurol. *51*, 235-242.

Hoffmann,M.C., Nitsch,C., Scotti,A.L., Reinhard,E., and Monard,D. (1992). The prolonged presence of glia-derived nexin, an endogenous protease inhibitor, in the hippocampus after ischemia-induced delayed neuronal death. Neuroscience *49*, 397-408.

Holloway,R.L., Jr. (1966b). Dendritic branching: some preliminary results of training and complexity in rat visual cortex. Brain Res. *2*, 393-396.

Holloway,R.L., Jr. (1966a). Dendritic branching: some preliminary results of training and complexity in rat visual cortex. Brain Res. *2*, 393-396.

Horwood,J.M., Ripley,T.L., and Stephens,D.N. (2004). Evidence for disrupted NMDA receptor function in tissue plasminogen activator knockout mice. Behav. Brain Res. *150*, 127-138.

Hoyte,L., Barber,P.A., Buchan,A.M., and Hill,M.D. (2004). The Rise and Fall of NMDA Antagonists for Ischemic Stroke. Curr. Mol. Med. *4*, 131-136.

Huang,Y.Y., Bach,M.E., Lipp,H.P., Zhuo,M., Wolfer,D.P., Hawkins,R.D., Schoonjans,L., Kandel,E.R., Godfraind,J.M., Mulligan,R., Collen,D., and Carmeliet,P. (1996). Mice lacking the gene encoding tissue-type plasminogen activator show a selective interference with late-phase long-term potentiation in both Schaffer collateral and mossy fiber pathways. Proc. Natl. Acad. Sci. U. S. A *93*, 8699-8704.

Huntley,G.W. (1997). Differential effects of abnormal tactile experience on shaping representation patterns in developing and adult motor cortex. J. Neurosci. *17*, 9220-9232.

Hutson,K.A. and Masterton,R.B. (1986). The sensory contribution of a single vibrissa's cortical barrel. J. Neurophysiol. *56*, 1196-1223.

Indyk,J.A., Chen,Z.L., Tsirka,S.E., and Strickland,S. (2003). Laminin chain expression suggests that laminin-10 is a major isoform in the mouse hippocampus and is degraded by the tissue plasminogen activator/plasmin protease cascade during excitotoxic injury. Neuroscience *116*, 359-371.

Irving,J.A., Pike,R.N., Lesk,A.M., and Whisstock,J.C. (2000). Phylogeny of the serpin superfamily: implications of patterns of amino acid conservation for structure and function. Genome Res. *10*, 1845-1864.

Irving,J.A., Steenbakkers,P.J., Lesk,A.M., Op den Camp,H.J., Pike,R.N., and Whisstock,J.C. (2002). Serpins in prokaryotes. Mol. Biol. Evol. *19*, 1881-1890.

Iwasato,T., Datwani,A., Wolf,A.M., Nishiyama,H., Taguchi,Y., Tonegawa,S., Knopfel,T., Erzurumlu,R.S., and Itohara,S. (2000). Cortex-restricted disruption of NMDAR1 impairs neuronal patterns in the barrel cortex. Nature *406*, 726-731.

Jablonska,B., Gierdalski,M., Kossut,M., and Skangiel-Kramska,J. (1999). Partial blocking of NMDA receptors reduces plastic changes induced by short-lasting classical conditioning in the SI barrel cortex of adult mice. Cereb. Cortex *9*, 222-231.

Jeanmonod,D., Rice,F.L., and Van der Loos H. (1981). Mouse somatosensory cortex: alterations in the barrelfield following receptor injury at different early postnatal ages. Neuroscience *6*, 1503-1535.

Jenkinson,E.W. and Glickstein,M. (2000). Whiskers, barrels, and cortical efferent pathways in gap crossing by rats. J. Neurophysiol. *84*, 1781-1789.

Johnson,D.E. (2000). Noncaspase proteases in apoptosis. Leukemia *14*, 1695-1703.

Jones,F.S. and Jones,P.L. (2000). The tenascin family of ECM glycoproteins: structure, function, and regulation during embryonic development and tissue remodeling. Dev. Dyn. *218*, 235-259.

Kang,H. and Schuman,E.M. (1995). Long-lasting neurotrophin-induced enhancement of synaptic transmission in the adult hippocampus. Science *267*, 1658-1662.

Kasza,A., Kowanetz,M., Poslednik,K., Witek,B., Kordula,T., and Koj,A. (2001). Epidermal growth factor and pro-inflammatory cytokines regulate the expression of

components of plasminogen activation system in U373-MG astrocytoma cells. Cytokine *16*, 187-190.

Kasza,A., Petersen,H.H., Heegaard,C.W., Oka,K., Christensen,A., Dubin,A., Chan,L., and Andreasen,P.A. (1997). Specificity of serine proteinase/serpin complex binding to very-low- density lipoprotein receptor and alpha2-macroglobulin receptor/low-density-lipoprotein-receptor-related protein. Eur. J. Biochem. *248*, 270-281.

Kempermann,G., Brandon,E.P., and Gage,F.H. (1998). Environmental stimulation of 129/SvJ mice causes increased cell proliferation and neurogenesis in the adult dentate gyrus. Curr. Biol. *8*, 939-942.

Knauer,M.F., Crisp,R.J., Kridel,S.J., and Knauer,D.J. (1999). Analysis of a structural determinant in thrombin-protease nexin 1 complexes that mediates clearance by the low density lipoprotein receptor-related protein. J. Biol. Chem. *274*, 275-281.

Knauer,M.F., Kridel,S.J., Hawley,S.B., and Knauer,D.J. (1997). The efficient catabolism of thrombin-protease nexin 1 complexes is a synergistic mechanism that requires both the LDL receptor-related protein and cell surface heparins. J. Biol. Chem. *272*, 29039-29045.

Knott,G.W., Quairiaux,C., Genoud,C., and Welker,E. (2002). Formation of dendritic spines with GABAergic synapses induced by whisker stimulation in adult mice. Neuron *34*, 265-273.

Kossut,M. (1992). Plasticity of the barrel cortex neurons. Prog. Neurobiol. *39*, 389-422.

Krueger,S.R., Ghisu,G.P., Cinelli,P., Gschwend,T.P., Osterwalder,T., Wolfer,D.P., and Sonderegger,P. (1997). Expression of neuroserpin, an inhibitor of tissue plasminogen activator, in the developing and adult nervous system of the mouse. J. Neurosci. *17*, 8984-8996.

Krystosek,A. and Seeds,N.W. (1981). Plasminogen activator release at the neuronal growth cone. Science *213*, 1532-1534.

Kuby,S.A. and Lardy,H.A. (1953). Purification and kinetics of beta-D-galactosidase from Escherichia coli, strain K-12. J. Am. Chem. Soc. *75*.

Kury,P., Schaeren-Wiemers,N., and Monard,D. (1997). Protease nexin-1 is expressed at the mouse met-/mesencephalic junction and FGF signaling regulates its promoter activity in primary met- /mesencephalic cells. Development *124*, 1251-1262.

Lantz,M.S. and Ciborowski,P. (1994). Zymographic techniques for detection and characterization of microbial proteases. Methods Enzymol. *235*, 563-594.

Laube,B., Hirai,H., Sturgess,M., Betz,H., and Kuhse,J. (1997). Molecular determinants of agonist discrimination by NMDA receptor subunits: analysis of the glutamate binding site on the NR2B subunit. Neuron *18*, 493-503.

LeMosy,E.K., Hong,C.C., and Hashimoto,C. (1999). Signal transduction by a protease cascade. Trends Cell Biol. *9*, 102-107.

Li,W.Y., Chong,S.S., Huang,E.Y., and Tuan,T.L. (2003). Plasminogen activator/plasmin system: a major player in wound healing? Wound. Repair Regen. *11*, 239-247.

Lisman,J.E. and McIntyre,C.C. (2001). Synaptic plasticity: a molecular memory switch. Curr. Biol. *11*, R788-R791.

Lochner,J.E., Kingma,M., Kuhn,S., Meliza,C.D., Cutler,B., and Scalettar,B.A. (1998). Real-time imaging of the axonal transport of granules containing a tissue plasminogen activator/green fluorescent protein hybrid. Mol. Biol. Cell *9*, 2463-2476.

Loopuijt,L.D. and Schmidt,W.J. (1998). The role of NMDA receptors in the slow neuronal degeneration of Parkinson's disease. Amino. Acids *14*, 17-23.

Loscher,W., Fredow,G., and Ganter,M. (1991). Comparison of pharmacodynamic effects of the non-competitive NMDA receptor antagonists MK-801 and ketamine in pigs. Eur. J. Pharmacol. *192*, 377-382.

Lubke,J., Roth,A., Feldmeyer,D., and Sakmann,B. (2003). Morphometric analysis of the columnar innervation domain of neurons connecting layer 4 and layer 2/3 of juvenile rat barrel cortex. Cereb. Cortex *13*, 1051-1063.

Luthi,A., Putten,H., Botteri,F.M., Mansuy,I.M., Meins,M., Frey,U., Sansig,G., Portet,C., Schmutz,M., Schroder,M., Nitsch,C., Laurent,J.P., and Monard,D. (1997). Endogenous serine protease inhibitor modulates epileptic activity and hippocampal long-term potentiation. J. Neurosci. *17*, 4688-4699.

Madani,R., Hulo,S., Toni,N., Madani,H., Steimer,T., Muller,D., and Vassalli,J.D. (1999). Enhanced hippocampal long-term potentiation and learning by increased neuronal expression of tissue-type plasminogen activator in transgenic mice. EMBO J. *18*, 3007-3012.

Magarinos,A.M., Verdugo,J.M., and McEwen,B.S. (1997). Chronic stress alters synaptic terminal structure in hippocampus. Proc. Natl. Acad. Sci. U. S. A. *94*, 14002-14008.

Mansuy,I.M., van der,P.H., Schmid,P., Meins,M., Botteri,F.M., and Monard,D. (1993). Variable and multiple expression of Protease Nexin-1 during mouse organogenesis and nervous system development. Development *119*, 1119-1134.

Mars,W.M., Zarnegar,R., and Michalopoulos,G.K. (1993). Activation of hepatocyte growth factor by the plasminogen activators uPA and tPA. Am. J. Pathol. *143*, 949-958.

Martin,S.J., Grimwood,P.D., and Morris,R.G. (2000). Synaptic plasticity and memory: an evaluation of the hypothesis. Annu. Rev. Neurosci. *23*, 649-711.

Mataga,N., Nagai,N., and Hensch,T.K. (2002). Permissive proteolytic activity for visual cortical plasticity. Proc. Natl. Acad. Sci. U. S. A *99*, 7717-7721.

Meier,R., Spreyer,P., Ortmann,R., Harel,A., and Monard,D. (1989). Induction of glia-derived nexin after lesion of a peripheral nerve. Nature *342*, 548-550.

Meins,M., Piosik,P., Schaeren-Wiemers,N., Franzoni,S., Troncoso,E., Kiss,J.Z., Brosamle,C., Schwab,M.E., Molnar,Z., and Monard,D. (2001). Progressive neuronal and motor dysfunction in mice overexpressing the serine protease inhibitor protease nexin-1 in postmitotic neurons. J. Neurosci. *21*, 8830-8841.

Meiri,N., Masos,T., Rosenblum,K., Miskin,R., and Dudai,Y. (1994). Overexpression of urokinase-type plasminogen activator in transgenic mice is correlated with impaired learning. Proc. Natl. Acad. Sci. U. S. A *91*, 3196-3200.

Meyer,A., Chretien,P., Massicotte,G., Sargent,C., Chretien,M., and Marcinkiewicz,M. (1996). Kainic acid increases the expression of the prohormone convertases furin and PC1 in the mouse hippocampus. Brain Res. *732*, 121-132.

Mihailescu,D., Kury,P., and Monard,D. (1999). An octamer-binding site is crucial for the activity of an enhancer active at the embryonic met-/mesencephalic junction. Mech. Dev. *84*, 55-67.

Mikus,P., Urano,T., Liljestrom,P., and Ny,T. (1993). Plasminogen-activator inhibitor type 2 (PAI-2) is a spontaneously polymerising SERPIN. Biochemical characterisation of the recombinant intracellular and extracellular forms. Eur. J. Biochem. *218*, 1071-1082.

Miyazaki,J., Nakanishi,S., and Jingami,H. (1999). Expression and characterization of a glycine-binding fragment of the N-methyl-D-aspartate receptor subunit NR1. Biochem. J. *340 (Pt 3)*, 687-692.

Mohn,A.R., Gainetdinov,R.R., Caron,M.G., and Koller,B.H. (1999). Mice with reduced NMDA receptor expression display behaviors related to schizophrenia. Cell *98*, 427-436.

Molinari,F., Rio,M., Meskenaite,V., Encha-Razavi,F., Auge,J., Bacq,D., Briault,S., Vekemans,M., Munnich,A., Attie-Bitach,T., Sonderegger,P., and Colleaux,L. (2002). Truncating neurotrypsin mutation in autosomal recessive nonsyndromic mental retardation. Science *298*, 1779-1781.

Monard,D., Solomon,F., Rentsch,M., and Gysin,R. (1973). Glia-induced morphological differentiation in neuroblastoma cells. Proc. Natl. Acad. Sci. U. S. A *70*, 1894-1897.

Monyer,H., Burnashev,N., Laurie,D.J., Sakmann,B., and Seeburg,P.H. (1994). Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. Neuron *12*, 529-540.

Mori,H. and Mishina,M. (1995). Structure and function of the NMDA receptor channel. Neuropharmacology *34*, 1219-1237.

Moriyoshi,K., Masu,M., Ishii,T., Shigemoto,R., Mizuno,N., and Nakanishi,S. (1991). Molecular cloning and characterization of the rat NMDA receptor. Nature *354*, 31-37.

Mowla,S.J., Farhadi,H.F., Pareek,S., Atwal,J.K., Morris,S.J., Seidah,N.G., and Murphy,R.A. (2001). Biosynthesis and post-translational processing of the precursor to brain-derived neurotrophic factor. J. Biol. Chem. *276*, 12660-12666.

Muller,C.M. and Griesinger,C.B. (1998). Tissue plasminogen activator mediates reverse occlusion plasticity in visual cortex. Nat. Neurosci. *1*, 47-53.

Murer,V., Spetz,J.F., Hengst,U., Altrogge,L.M., de Agostini,A., and Monard,D. (2001). Male fertility defects in mice lacking the serine protease inhibitor protease nexin-1. Proc. Natl. Acad. Sci. U. S. A *98*, 3029-3033.

Nakagami,Y., Abe,K., Nishiyama,N., and Matsuki,N. (2000). Laminin degradation by plasmin regulates long-term potentiation. J. Neurosci. *20*, 2003-2010.

Nakazawa,K., Quirk,M.C., Chitwood,R.A., Watanabe,M., Yeckel,M.F., Sun,L.D., Kato,A., Carr,C.A., Johnston,D., Wilson,M.A., and Tonegawa,S. (2002). Requirement for hippocampal CA3 NMDA receptors in associative memory recall. Science *297*, 211-218.

Naruse,S., Kitagawa,M., and Ishiguro,H. (1999). Molecular understanding of chronic pancreatitis: a perspective on the future. Mol. Med. Today *5*, 493-499.

Neurath,H. (1984). Evolution of proteolytic enzymes. Science *224*, 350-357.

Niclou,S.P., Suidan,H.S., Pavlik,A., Vejsada,R., and Monard,D. (1998). Changes in the expression of protease-activated receptor 1 and protease nexin-1 mRNA during rat nervous system development and after nerve lesion. Eur. J. Neurosci. *10*, 1590-1607.

Nicole,O., Docagne,F., Ali,C., Margaill,I., Carmeliet,P., MacKenzie,E.T., Vivien,D., and Buisson,A. (2001). The proteolytic activity of tissue-plasminogen activator enhances NMDA receptor-mediated signaling. Nat. Med. *7*, 59-64.

Nishio,T., Kawaguchi,S., Iseda,T., Kawasaki,T., and Hase,T. (2003). Secretion of tenascin-C by cultured astrocytes: regulation of cell proliferation and process elongation. Brain Res. *990*, 129-140.

Nong,Y., Huang,Y., and Salter,M.W. (2004). NMDA receptors are movin'in. Curr. Opin. Neurobiol. *14*.

Ohlsson,K. and Laurell,C.B. (1976). The disappearance of enzyme-inhibitor complexes from the circulation of man. Clin. Sci. Mol. Med. *51*, 87-92.

Osterwalder,T., Contartese,J., Stoeckli,E.T., Kuhn,T.B., and Sonderegger,P. (1996). Neuroserpin, an axonally secreted serine protease inhibitor. EMBO J. *15*, 2944-2953.

Ozawa,S., Kamiya,H., and Tsuzuki,K. (1998). Glutamate receptors in the mammalian central nervous system. Prog. Neurobiol. *54*, 581-618.

Pacteau,C., Einon,D., and Sinden,J. (1989). Early rearing environment and dorsal hippocampal ibotenic acid lesions: long-term influences on spatial learning and alternation in the rat. Behav. Brain Res. *34*, 79-96.

Parmer,R.J., Mahata,M., Mahata,S., Sebald,M.T., O'Connor,D.T., and Miles,L.A. (1997). Tissue plasminogen activator (t-PA) is targeted to the regulated secretory pathway. Catecholamine storage vesicles as a reservoir for the rapid release of t-PA. J. Biol. Chem. *272*, 1976-1982.

Parsons,C.G. (2001). NMDA receptors as targets for drug action in neuropathic pain. Eur. J. Pharmacol. *429*, 71-78.

Pasternak,J.R. and Woolsey,T.A. (1975). The number, size and spatial distribution of neurons in lamina IV of the mouse SmI neocortex. J. Comp Neurol. *160*, 291-306.

Patthy,L. (1985). Evolution of the proteases of blood coagulation and fibrinolysis by assembly from modules. Cell *41*, 657-663.

Patthy,L. (1993). Modular design of proteases of coagulation, fibrinolysis, and complement activation: implications for protein engineering and structure-function studies. Methods Enzymol. *222*, 10-21.

Pawlak,R., Magarinos,A.M., Melchor,J., McEwen,B., and Strickland,S. (2003). Tissue plasminogen activator in the amygdala is critical for stress- induced anxiety-like behavior. Nat. Neurosci. *6*, 168-174.

Paxinos G. (1995). The rat nervous system.

Perona,J.J. and Craik,C.S. (1995). Structural basis of substrate specificity in the serine proteases. Protein Sci. *4*, 337-360.

Pesold,C., Liu,W.S., Guidotti,A., Costa,E., and Caruncho,H.J. (1999). Cortical bitufted, horizontal, and Martinotti cells preferentially express and secrete reelin into perineuronal nets, nonsynaptically modulating gene expression. Proc. Natl. Acad. Sci. U. S. A *96*, 3217-3222.

Petersen,C.C., Grinvald,A., and Sakmann,B. (2003). Spatiotemporal dynamics of sensory responses in layer 2/3 of rat barrel cortex measured in vivo by voltage-sensitive dye imaging combined with whole-cell voltage recordings and neuron reconstructions. J. Neurosci. *23*, 1298-1309.

Petersen,C.C. and Sakmann,B. (2000). The excitatory neuronal network of rat layer 4 barrel cortex. J. Neurosci. *20*, 7579-7586.

Petralia,R.S., Wang,Y.X., and Wenthold,R.J. (1994). The NMDA receptor subunits NR2A and NR2B show histological and ultrastructural localization patterns similar to those of NR1. J. Neurosci. *14*, 6102-6120.

Philpot,B.D., Sekhar,A.K., Shouval,H.Z., and Bear,M.F. (2001). Visual experience and deprivation bidirectionally modify the composition and function of NMDA receptors in visual cortex. Neuron *29*, 157-169.

Pinaud,R., Tremere,L.A., Penner,M.R., Hess,F.F., Robertson,H.A., and Currie,R.W. (2002). Complexity of sensory environment drives the expression of candidate-plasticity gene, nerve growth factor induced-A. Neuroscience *112*, 573-582.

Pizzorusso,T., Medini,P., Berardi,N., Chierzi,S., Fawcett,J.W., and Maffei,L. (2002). Reactivation of ocular dominance plasticity in the adult visual cortex. Science *298*, 1248-1251.

Polley,D.B., Chen-Bee,C.H., and Frostig,R.D. (1999). Two directions of plasticity in the sensory-deprived adult cortex. Neuron *24*, 623-637.

Por,S.B., Bennett,E.L., and Bondy,S.C. (1982). Environmental enrichment and neurotransmitter receptors. Behav. Neural Biol. *34*, 132-140.

Qian,Z., Gilbert,M.E., Colicos,M.A., Kandel,E.R., and Kuhl,D. (1993). Tissue-plasminogen activator is induced as an immediate-early gene during seizure, kindling and long-term potentiation. Nature *361*, 453-457.

Quinlan,E.M., Philpot,B.D., Huganir,R.L., and Bear,M.F. (1999). Rapid, experience-dependent expression of synaptic NMDA receptors in visual cortex in vivo. Nat. Neurosci. *2*, 352-357.

Rampon,C., Jiang,C.H., Dong,H., Tang,Y.P., Lockhart,D.J., Schultz,P.G., Tsien,J.Z., and Hu,Y. (2000a). Effects of environmental enrichment on gene expression in the brain. Proc. Natl. Acad. Sci. U. S. A *97*, 12880-12884.

Rampon,C., Tang,Y.P., Goodhouse,J., Shimizu,E., Kyin,M., and Tsien,J.Z. (2000b). Enrichment induces structural changes and recovery from nonspatial memory deficits in CA1 NMDAR1-knockout mice. Nat. Neurosci. *3*, 238-244.

Rasmuson,S., Olsson,T., Henriksson,B.G., Kelly,P.A., Holmes,M.C., Seckl,J.R., and Mohammed,A.H. (1998). Environmental enrichment selectively increases 5-HT1A receptor mRNA expression and binding in the rat hippocampus. Brain Res. Mol. Brain Res. *53*, 285-290.

Rawlings,N.D., Tolle,D.P., and Barrett,A.J. (2004). MEROPS: the peptidase database. Nucleic Acids Res. *32 Database issue*, D160-D164.

Reinhard,E., Meier,M., Halfter,W., Rovelli ,G., and Monard,D. (1988). Detection of glia-derived nexin in the olfactory system of the rat. Neuron *1*, 387-394.

Reinhard,E., Suidan,H.S., Pavlik,A., and Monard,D. (1994). Glia-derived nexin/protease nexin-1 is expressed by a subset of neurons in the rat brain. J. Neurosci. Res. *37*, 256-270.

Rema,V., Armstrong-James,M., and Ebner,F.F. (1998). Experience-dependent plasticity of adult rat S1 cortex requires local NMDA receptor activation. J. Neurosci. *18*, 10196-10206.

Rema,V., Armstrong-James,M., and Ebner,F.F. (2003). Experience-dependent plasticity is impaired in adult rat barrel cortex after whiskers are unused in early postnatal life. J. Neurosci. *23*, 358-366.

Rema,V. and Ebner,F.F. (1999). Effect of enriched environment rearing on impairments in cortical excitability and plasticity after prenatal alcohol exposure. J. Neurosci. *19*, 10993-11006.

Ren,J.Q., Aika,Y., Heizmann,C.W., and Kosaka,T. (1992). Quantitative analysis of neurons and glial cells in the rat somatosensory cortex, with special reference to GABAergic neurons and parvalbumin-containing neurons. Exp. Brain Res. *92*, 1-14.

Ren,J.Q., Heizmann,C.W., and Kosaka,T. (1994). Regional difference in the distribution of parvalbumin-containing neurons immunoreactive for monoclonal antibody HNK-1 in the mouse cerebral cortex. Neurosci. Lett. *166*, 221-225.

Riesenfeld,A. (1979). Vibrissae removal and the phenomenon of unexplained sudden death. Acta Anat. (Basel) *105*, 47-49.

Rocamora,N., Welker,E., Pascual,M., and Soriano,E. (1996). Upregulation of BDNF mRNA expression in the barrel cortex of adult mice after sensory stimulation. J. Neurosci. *16*, 4411-4419.

Rock,D.M. and Macdonald,R.L. (1995). Polyamine regulation of N-methyl-D-aspartate receptor channels. Annu. Rev. Pharmacol. Toxicol. *35*, 463-482.

Rogers,M. and Dani,J.A. (1995). Comparison of quantitative calcium flux through NMDA, ATP, and ACh receptor channels. Biophys. J. *68*, 501-506.

Rosenzweig,M.R. (1966). Environmental complexity, cerebral change, and behavior. Am. Psychol. *21*, 321-332.

Rosenzweig,M.R. (2003). Effects of differential experience on the brain and behavior. Dev. Neuropsychol. *24*, 523-540.

Rosenzweig,M.R. and Bennett,E.L. (1969). Effects of differential environments on brain weights and enzyme activities in gerbils, rats, and mice. Dev. Psychobiol. *2*, 87-95.

Rosenzweig,M.R., Bennett,E.L., and Diamond,M.C. (1967). Effects of differential environments on brain anatomy and brain chemistry. Proc. Annu. Meet. Am. Psychopathol. Assoc. *56*, 45-56.

Rosenzweig,M.R., Krech,D., Bennett,E.L., and Diamond,M.C. (1962). Effects of environmental complexity and training on brain chemistry and anatomy: a replication and extension. J. Comp Physiol Psychol. *55*, 429-437.

Rovelli,G., Stone,S.R., Preissner,K.T., and Monard,D. (1990). Specific interaction of vitronectin with the cell-secreted protease inhibitor glia-derived nexin and its thrombin complex. Eur. J. Biochem. *192*, 797-803.

Ruoslahti,E. (1996). Brain extracellular matrix. Glycobiology *6*, 489-492.

Salles,F.J. and Strickland,S. (2002). Localization and regulation of the tissue plasminogen activator-plasmin system in the hippocampus. J. Neurosci. *22*, 2125-2134.

Sappino,A.P., Madani,R., Huarte,J., Belin,D., Kiss,J.Z., Wohlwend,A., and Vassalli,J.D. (1993). Extracellular proteolysis in the adult murine brain. J. Clin. Invest. *92*, 679-685.

Schechter,I. and Berger,A. (1967). On the size of the active site in proteases. I. Papain. Biochem. Biophys. Res. Commun. *27*, 157-162.

Schiffman,H.R., Lore,R., Passafiume,J., and Neeb,R. (1970). Role of vibrissae for depth perception in the rat (Rattus norvegicus). Anim Behav. *18*, 290-292.

Scott,R.W., Bergman,B.L., Bajpai,A., Hersh,R.T., Rodriguez,H., Jones,B.N., Barreda,C., Watts,S., and Baker,J.B. (1985). Protease nexin. Properties and a modified purification procedure. J. Biol. Chem. *260*, 7029-7034.

Scotti,A.L., Monard,D., and Nitsch,C. (1994). Re-expression of glia-derived nexin/protease nexin 1 depends on mode of lesion-induction or terminal degeneration: observations after excitotoxin or 6-hydroxydopamine lesions of rat substantia nigra. J. Neurosci. Res. *37*, 155-168.

Seeds,N.W., Basham,M.E., and Ferguson,J.E. (2003). Absence of tissue plasminogen activator gene or activity impairs mouse cerebellar motor learning. J. Neurosci. *23*, 7368-7375.

Seeds,N.W., Williams,B.L., and Bickford,P.C. (1995). Tissue plasminogen activator induction in Purkinje neurons after cerebellar motor learning. Science *270*, 1992-1994.

Selvarajan,S., Lund,L.R., Takeuchi,T., Craik,C.S., and Werb,Z. (2001). A plasma kallikrein-dependent plasminogen cascade required for adipocyte differentiation. Nat. Cell Biol. *3*, 267-275.

Sharon,R., Abramovitz,R., and Miskin,R. (2002). Plasminogen mRNA induction in the mouse brain after kainate excitation: codistribution with plasminogen activator inhibitor-2 (PAI-2) mRNA. Brain Res. Mol. Brain Res. *104*, 170-175.

Sharp,P.E., McNaughton,B.L., and Barnes,C.A. (1985). Enhancement of hippocampal field potentials in rats exposed to a novel, complex environment. Brain Res. *339*, 361-365.

Sheng,M. and Pak,D.T. (2000). Ligand-gated ion channel interactions with cytoskeletal and signaling proteins. Annu. Rev. Physiol *62*, 755-778.

Siao,C.J., Fernandez,S.R., and Tsirka,S.E. (2003). Cell type-specific roles for tissue plasminogen activator released by neurons or microglia after excitotoxic injury. J. Neurosci. *23*, 3234-3242.

Siao,C.J. and Tsirka,S.E. (2002). Extracellular proteases and neuronal cell death. Cell Mol. Biol. (Noisy. -le-grand) *48*, 151-161.

Silva,A.J., Kogan,J.H., Frankland,P.W., and Kida,S. (1998). CREB and memory. Annu. Rev. Neurosci. *21*, 127-148.

Silverman,G.A., Bird,P.I., Carrell,R.W., Church,F.C., Coughlin,P.B., Gettins,P.G., Irving,J.A., Lomas,D.A., Luke,C.J., Moyer,R.W., Pemberton,P.A., Remold-O'Donnell,E., Salvesen,G.S., Travis,J., and Whisstock,J.C. (2001). The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Evolution, mechanism of inhibition, novel functions, and a revised nomenclature. J. Biol. Chem. *276*, 33293-33296.

Sim,R.B. and Laich,A. (2000). Serine proteases of the complement system. Biochem. Soc. Trans. *28*, 545-550.

Simonato,M., Molteni,R., Bregola,G., Muzzolini,A., Piffanelli,M., Beani,L., Racagni,G., and Riva,M. (1998). Different patterns of induction of FGF-2, FGF-1 and BDNF mRNAs during kindling epileptogenesis in the rat. Eur. J. Neurosci. *10*, 955-963.

Simons,D.J. and Land,P.W. (1987). Early experience of tactile stimulation influences organization of somatic sensory cortex. Nature *326*, 694-697.

Sinnreich,M., Meins,M., Niclou,S.P., Suidan,H.S., and Monard,D. (2004). Prothrombin overexpressed in post-natal neurones requires blood factors for activation in the mouse brain. J. Neurochem. *88*, 1380-1388.

Smart,T.G., Xie,X., and Krishek,B.J. (1994). Modulation of inhibitory and excitatory amino acid receptor ion channels by zinc. Prog. Neurobiol. *42*, 393-41.

Sommer,J., Gloor,S.M., Rovelli,G.F., Hofsteenge,J., Nick,H., Meier,R., and Monard,D. (1987). cDNA sequence coding for a rat glia-derived nexin and its homology to members of the serpin superfamily. Biochemistry *26*, 6407-6410.

Staiger,J.F., Bisler,S., Schleicher,A., Gass,P., Stehle,J.H., and Zilles,K. (2000). Exploration of a novel environment leads to the expression of inducible transcription factors in barrel-related columns. Neuroscience *99*, 7-16.

Stone,S.R., Nick,H., Hofsteenge,J., and Monard,D. (1987). Glial-derived neurite-promoting factor is a slow-binding inhibitor of trypsin, thrombin, and urokinase. Arch. Biochem. Biophys. *252*, 237-244.

Strickland,D.K., Ashcom,J.D., Williams,S., Burgess,W.H., Migliorini,M., and Argraves,W.S. (1990). Sequence identity between the alpha 2-macroglobulin receptor and low density lipoprotein receptor-related protein suggests that this molecule is a multifunctional receptor. J. Biol. Chem. *265*, 17401-17404.

Stubbs,M.T., Renatus,M., and Bode,W. (1998). An active zymogen: unravelling the mystery of tissue-type plasminogen activator. Biol. Chem. *379*, 95-103.

Sugihara,H., Moriyoshi,K., Ishii,T., Masu,M., and Nakanishi,S. (1992). Structures and properties of seven isoforms of the NMDA receptor generated by alternative splicing. Biochem. Biophys. Res. Commun. *185*, 826-832.

Symons,L.A. and Tees,R.C. (1990). An examination of the intramodal and intermodal behavioral consequences of long-term vibrissae removal in rats. Dev. Psychobiol. *23*, 849-867.

Tang,Y.P., Shimizu,E., Dube,G.R., Rampon,C., Kerchner,G.A., Zhuo,M., Liu,G., and Tsien,J.Z. (1999). Genetic enhancement of learning and memory in mice. Nature *401*, 63-69.

Teng,K.K. and Hempstead,B.L. (2004). Neurotrophins and their receptors: signaling trios in complex biological systems. Cell Mol. Life Sci. *61*, 35-48.

Trachtenberg,J.T., Chen,B.E., Knott,G.W., Feng,G., Sanes,J.R., Welker,E., and Svoboda,K. (2002). Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex. Nature *420*, 788-794.

Troncoso,E., Muller,D., Czellar,S., and Zoltan,K.J. (2000). Epicranial sensory evoked potential recordings for repeated assessment of cortical functions in mice. J. Neurosci. Methods *97*, 51-58.

van Brederode,J.F., Foehring,R.C., and Spain,W.J. (2000). Morphological and electrophysiological properties of atypically oriented layer 2 pyramidal cells of the juvenile rat neocortex. Neuroscience *101*, 851-861.

Van den Steen,P.E., Opdenakker,G., Wormald,M.R., Dwek,R.A., and Rudd,P.M. (2001). Matrix remodelling enzymes, the protease cascade and glycosylation. Biochim. Biophys. Acta *1528*, 61-73.

Van der,L.H. (1977a). Structural changes in the cerebral cortex upon modification of the periphery: barrels in somatosensory cortex. Philos. Trans. R. Soc. Lond B Biol. Sci. *278*, 373-376.

Van der,L.H. (1977b). Structural changes in the cerebral cortex upon modification of the periphery: barrels in somatosensory cortex. Philos. Trans. R. Soc. Lond B Biol. Sci. *278*, 373-376.

van Praag,H., Christie,B.R., Sejnowski,T.J., and Gage,F.H. (1999a). Running enhances neurogenesis, learning, and long-term potentiation in mice. Proc. Natl. Acad. Sci. U. S. A *96*, 13427-13431.

van Praag,H., Kempermann,G., and Gage,F.H. (1999b). Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. Nat. Neurosci. *2*, 266-270.

van Praag,H., Kempermann,G., and Gage,F.H. (2000). Neural consequences of environmental enrichment. Nat. Rev. Neurosci. *1*, 191-198.

Vaughan,P.J. and Cunningham,D.D. (1993). Regulation of protease nexin-1 synthesis and secretion in cultured brain cells by injury-related factors. J. Biol. Chem. *268*, 3720-3727.

Vaughan,P.J., Su,J., Cotman,C.W., and Cunningham,D.D. (1994). Protease nexin-1, a potent thrombin inhibitor, is reduced around cerebral blood vessels in Alzheimer's disease. Brain Res. *668*, 160-170.

Volgyi,B., Farkas,T., and Toldi,J. (1993). Compensation of a sensory deficit inflicted upon newborn and adult animals. A behavioural study. Neuroreport *4*, 827-829.

Wainwright,P.E., Levesque,S., Krempulec,L., Bulman-Fleming,B., and McCutcheon,D. (1993). Effects of environmental enrichment on cortical depth and Morris-maze performance in B6D2F2 mice exposed prenatally to ethanol. Neurotoxicol. Teratol. *15*, 11-20.

Wang,X., Lee,S.R., Arai,K., Lee,S.R., Tsuji,K., Rebeck,G.W., and Lo,E.H. (2003). Lipoprotein receptor-mediated induction of matrix metalloproteinase by tissue plasminogen activator. Nat. Med. *9*, 1313-1317.

Ware,J.H., DiBenedetto,A.J., and Pittman,R.N. (1995). Localization of tissue plasminogen activator mRNA in adult rat brain. Brain Res. Bull. *37*, 275-281.

Weinberger,N.M. (1995). Dynamic regulation of receptive fields and maps in the adult sensory cortex. Annu. Rev. Neurosci. *18*, 129-158.

Welker,E. and Van der Loos H. (1986). Quantitative correlation between barrel-field size and the sensory innervation of the whiskerpad: a comparative study in six strains of mice bred for different patterns of mystacial vibrissae. J. Neurosci. *6*, 3355-3373.

White,E.L. (1976). Ultrastructure and synaptic contacts in barrels of mouse SI cortex. Brain Res. *105*, 229-251.

Wood,M.W., VanDongen,H.M., and VanDongen,A.M. (1995). Structural conservation of ion conduction pathways in K channels and glutamate receptors. Proc. Natl. Acad. Sci. U. S. A *92*, 4882-4886.

Woolsey,T.A., Dierker,M.L., and Wann,D.F. (1975). Mouse SmI cortex: qualitative and quantitative classification of golgi-impregnated barrel neurons. Proc. Natl. Acad. Sci. U. S. A *72*, 2165-2169.

Woolsey,T.A. and Van der Loos,H. (1970). The structural organization of layer IV in the somatosensory region (SI) of mouse cerebral cortex. The description of a cortical field composed of discrete cytoarchitectonic units. Brain Res. *17*, 205-242.

Wright,J.W., Reichert,J.R., Davis,C.J., and Harding,J.W. (2002). Neural plasticity and the brain renin-angiotensin system. Neurosci. Biobehav. Rev. *26*, 529-552.

Wu,Y.P., Siao,C.J., Lu,W., Sung,T.C., Frohman,M.A., Milev,P., Bugge,T.H., Degen,J.L., Levine,J.M., Margolis,R.U., and Tsirka,S.E. (2000). The tissue plasminogen activator (tPA)/plasmin extracellular proteolytic system regulates seizure-induced hippocampal mossy fiber outgrowth through a proteoglycan substrate. J. Cell Biol. *148*, 1295-1304.

Wyllie,D.J., Behe,P., and Colquhoun,D. (1998). Single-channel activations and concentration jumps: comparison of recombinant NR1a/NR2A and NR1a/NR2D NMDA receptors. J. Physiol *510 (Pt 1)*, 1-18.

Ye,S. and Goldsmith,E.J. (2001). Serpins and other covalent protease inhibitors. Curr. Opin. Struct. Biol. *11*, 740-745.

Yepes,M., Sandkvist,M., Coleman,T.A., Moore,E., Wu,J.Y., Mitola,D., Bugge,T.H., and Lawrence,D.A. (2002). Regulation of seizure spreading by neuroserpin and tissue-type plasminogen activator is plasminogen-independent. J. Clin. Invest *109*, 1571-1578.

Yepes,M., Sandkvist,M., Moore,E.G., Bugge,T.H., Strickland,D.K., and Lawrence,D.A. (2003). Tissue-type plasminogen activator induces opening of the blood-brain barrier via the LDL receptor-related protein. J. Clin. Invest *112*, 1533-1540.

Young,D., Lawlor,P.A., Leone,P., Dragunow,M., and During,M.J. (1999). Environmental enrichment inhibits spontaneous apoptosis, prevents seizures and is neuroprotective. Nat. Med. *5*, 448-453.

Yuste,R. and Bonhoeffer,T. (2001). Morphological changes in dendritic spines associated with long-term synaptic plasticity. Annu. Rev. Neurosci. *24*, 1071-1089.

Zhang,J.C., Sakthivel,R., Kniss,D., Graham,C.H., Strickland,D.K., and McCrae,K.R. (1998). The low density lipoprotein receptor-related protein/alpha2-macroglobulin receptor regulates cell surface plasminogen activator activity on human trophoblast cells. J. Biol. Chem. *273*, 32273-32280.

Zhuo,M., Holtzman,D.M., Li,Y., Osaka,H., DeMaro,J., Jacquin,M., and Bu,G. (2000). Role of tissue plasminogen activator receptor LRP in hippocampal long- term potentiation. J. Neurosci. *20*, 542-549.

Note

All the experiments presented in this thesis were performed by myself with the exeption of the experiments displayed in Figures 14C, 18A-F and Figure 19, which were contributed by the collaborators.

Curriculum Vitae

Name: Mirna Kvajo

Date and place of birth: 10.05.1976, Koper, Slovenia

Nationality: Croatian

Education:

2000-2004 Postgraduate study in Neuroscience

University of Basel, Switzerland

Friedrich Miescher Institute

Ph.D. thesis supervisor: Prof. Dr. Denis Monard

1994-1999 Molecular biology study

University of Zagreb, Croatia

Degree: diploma in molecular biology

Diploma thesis supervisor: Dr. Maja Osmak

1990-1994 Gymnasium “Vladimir Gortan”, Buje, Croatia

Publications

Osmak,M., Bordukalo,T., Kosmrlj,J., Kvajo,M., Marijanovic,Z., Eljuga,D., and Polanc,S. (1999). Diazenes: modificators of tumor cell resistance to cisplatin. Neoplasma *46*, 201-206.

Kvajo,M., Albrecht H., Meins M., Hengst U., Troncoso E., Lefort S., Kiss J., Petersen C., Monard D. Regulation of brain proteolytic activity controls the function of NMDA receptors (J Neurosci).

Patent

H. Albrecht, M. Kvajo, D. Monard (Inventors). "Screening method for compounds that modulate neuronal activity", WO03046552.

Talks

Barrel cortex meeting 2004, Lausanne, Switzerland.

FMI Conference on: Formation and function of neuronal circuits, 2003, Ascona, Switzerland.

FMI & Novartis Neuroscience Meeting 2003, Basel, Switzerland.

Annual Meeting of the Friedrich Miescher Institute, 2003, Pontresina, Switzerland.

Annual Meeting of the Friedrich Miescher Institute, 2001, St. Moritz, Switzerland.