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Review

Trypsin and trypsin-like proteases in the brain: Proteolysis and cellular functions

Y. Wang[†], W. Luo[†] and G. Reiser[*]

Institut für Neurobiochemie, Medizinische Fakultät, Otto-von-Guericke-Universität Magdeburg, Leipziger Str. 44, 39120 Magdeburg (Germany), Fax: +49-391-6713097, e-mail: georg.reiser@med.ovgu.de

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Abstract. Several serine proteases including thrombin, tissue-type plasminogen activator and urokinase-type plasminogen activator have been well characterized in the brain. In this article, we review the brain-related trypsin and trypsin-like serine proteases. Accumulating evidence demonstrates that trypsin and trypsin-like serine proteases play very important roles in neural development, plasticity, neurodegeneration and neuroregeneration in the brain. Neuropsin is able to hydrolyze the extracellular matrix components by its active site serine, and regulates learning and memory in normal brain. The mutant neurotrypsin contributes to mental retardation in children. Neurosin seems to be involved in the pathogenesis of neurodegenerative disorders, like Alzheimer's disease, Parkinson's disease or multiple sclerosis. Although mesotrypsin/trypsin IV is also implicated in neurodegeneration, its functional significance still remains largely unknown. Particularly, mesotrypsin/trypsin IV, P22 and neurosin exert their physiological and pathological functions through activation of certain protease-activated receptors (PARs). In the brain, the presence of serpins controls the activity of serine proteases. Therefore, understanding the interaction among brain trypsin, serpins and PARs will provide invaluable tools for regulating normal brain functions and for the clinical treatment of neural disorders.

Keywords. Serine protease, central nervous system, proteolytic activity, plasticity, neurodegeneration.

Introduction

Serine proteases, including thrombin, trypsin, trypsin-like proteases, and many others, are ubiquitously distributed throughout the human body and play pivotal roles in many important physiological and pathological processes like food digestion, homeostasis, reproduction, immune response, as well as signal transductions. In the central nervous system (CNS), the importance of several well-known extracellular serine proteases, such as thrombin, tissue-type plasminogen activator (tPA), urokinase-type plasminogen activator (uPA) and plasmin, has received great attention. These proteases are expressed in the brain, and have functional roles in regulating the consequences of ischemic stroke, synaptic plasticity, neurodegeneration and neuroregeneration [1, 2].

Thrombin can be produced locally in the brain tissue associated with neurofibrillary tangles in Alzheimer's disease and parkinsonism-dementia complex of Guam [3]. Alternatively, thrombin can also diffuse into the brain upon disruption of the blood-brain barrier under certain pathophysiological conditions, like trauma and ischemia [4]. This enzyme has been shown impressively to induce numerous cellular

† These authors made equal contributions.
* Corresponding author.

Table 1. Trypsin and trypsin-like serine proteases in the brain.

Protease name (alternative names)	Functions	Expression in brain
Trypsin IV (Mesotrypsin)	Activation of PAR-1 and calcium mobilization [37] PAR-dependent neurogenic inflammation and pain [38] Hydrolysis of myelin basic protein [42] Up-regulation of GFAP in astrocytes and βA4 staining in neurons in the transgenic mouse brain [23]	Wide in the brain including neurons and glial cells, predominantly in astrocytes [12, 19]
P22	Activation of PAR-2 and calcium mobilization [13] Degradation of laminin and type IV collagen [13]	Local in the brain [13]
Neurosin (Protease M/ Zyme/ MSP/ Kallikrein 6/ PRSS9)	Activation of PAR-2 and calcium mobilization [60] Aβ production [54, 57] Degradation of ECM proteins, laminin, fibronectin and collagen [128] Degradation of α-synuclein, resulting in the pathogenesis of synucleinopathies [61] Demyelination [129] Maintenance of myelination [64]	In the brain, mainly in oligodendrocytes [53, 130]
Neuropsin (Kallikrein 8 /BSP1/ Ovasin/ PRSS19)	LTP [79] Degradation of ECM proteins, fibronectin and presynaptic adhesion molecule L1, regulating hippocampal plasticity [72, 73] Synaptogenesis/maturation of synaptic structures [80] Neurite outgrowth and fasciculation of hippocampal neurons [85] Demyelination, oligodendroglial cell death, and pathogenesis of EAE and spinal cord injury [87, 88] Epilepsy [83, 84] Unclear whether PARs are activated or inactivated	Predominant in the hippocampal pyramidal neurons in mice [15] Broad in human brain, mainly in CNPase-positive oligodendrocytes [62, 70]
Neurotrypsin (Motopsin/ PRSS12)	Cleavage of proteoglycan agrin [94] Mental retardation by enzyme deficiency [96] Long-term memory formation in *Drosophila* [97] Regeneration of facial motor neurons [98] Unclear whether PARs are activated or inactivated	Cerebral cortex and Limbic system [16]
PRSS20 (TLSP)	Unknown	Hippocampal pyramidal neuron in human brain [131] Preferentially in the mouse fetal brain [132]

responses through proteolytic activation of cell surface receptors, so-called protease-activated receptors (PARs). Preclinical studies demonstrate that thrombin at low concentrations protects neurons from damage by ischemic injury, whereas at higher concentrations, thrombin causes neurodegeneration and brain insults [1, 5]. tPA and uPA are distributed broadly in the brain and exert a variety of functions during development as well as in the adult nervous system by mediating neural plasticity [6]. It was reported that overexpression of tPA in neurons could enhance long-term potentiation (LTP) and thereby improve learning and memory [7]. In the clinic, this serine protease has been developed as an important therapeutic target. Recombinant tPA has been used to treat ischemic stroke patients, since it is involved in degrading fibrin clots and thereby improves patients' outcomes after ischemic stroke [8]. Although tPA is beneficial in the acute treatment of stroke, some studies have also demonstrated that tPA activates microglia cells, and exogenous administration of recombinant tPA into mice exacerbates injury in several ischemic models [2]. Similarly, plasmin detected in the brain contributes to excitotoxic neurodegeneration and modulates the outcome of an ischemic stroke [2].

Trypsin, another important serine protease, was initially isolated from the pancreatic juice of animals, but it was later identified in many different tissues [9–11]. Trypsin plays pivotal roles in food digestion as well as in cellular signal transduction mediated through proteolytic activation of PARs. In the brain, the extrapancreatic trypsin and trypsin-like serine proteases have been detected [12–16]. They are involved in neural development, plasticity, neurodegeneration and neuroregeneration in the brain (Table 1). The importance of these serine proteases in the brain tissue has attracted much scientific attention. Here, we review the recent advances in this field.

Trypsin IV and mesotrypsin

Human pancreas secretes three trypsinogen isoforms: mainly cationic trypsinogen [PRSS1 (protease, serine), p*I* 4.9] and anionic trypsinogen (PRSS2, p*I* 6.2), and as a minor constituent mesotrypsinogen (PRSS3, p*I* 5.7). The latter accounts for 3–10% of the total trypsinogen content in normal pancreatic juice. These trypsinogens are activated by enterokinase in the small intestine [17, 18]. Trypsinogen IV, a tissue-specific alternatively spliced form of mesotrypsinogen, was originally identified in human brain [12]. It has been shown that trypsinogen IV is widely distributed in the human brain [19]. Immunohistochemistry demonstrates that human trypsinogen IV is expressed in neurons and glial cells, predominantly in astrocytes. Thus, trypsinogen IV was accepted as a brain trypsinogen.

The *TRYPSINOGEN IV* gene is formed by segmental duplications derived from the *LOC120224* gene at chromosome 11q24 and mesotrypsinogen exons at chromosome 7q35 [20]. Sequence analysis revealed that trypsinogen IV contains two isoforms, isoform A and isoform B [12]. Isoform A encompasses a traditional AUG (methionine) start codon and a 72-residue N-terminal leader peptide, whereas isoform B possesses a 28-residue N-terminal leader peptide, and it can be derived from isoform A *via* unconventional translation initiation from an internal CUG (leucine) start codon (Fig. 1a). It was shown that isoform B is the predominant form of the enzyme in the brain [21]. Genetic studies identified a variant of trypsinogen IV cDNA in primates. It has a deletion of three nucleotides (GAG), resulting in a lack of Glu^{32} residue in the deduced amino acid sequence (marked in orange, Fig. 1b) [12]. Through analysis of allelic frequency, it was found that this deletion mutation is a common genetic polymorphism in human [22]. Biochemical analysis indicated that the mutant variant of trypsin IV fully functions *in vitro,* as does the wild-type enzyme. However, the functional significance of the polymorphism of trypsin IV is still unclear.

Structurally, trypsinogen IV (GenBank accession number: P35030 or CAH69873) and mesotrypsinogen (GenBank accession number: NP_002762) have a common C terminus of about 234 amino acids, but the amino acid sequences of the N terminus encoded by the alternative exon 1 are completely different (Fig. 1a, b) [12, 18]. Therefore, trypsinogen IV lacks a recognizable signal sequence and is likely to be a cytoplasmic protein [23]. However, it was supposed that trypsinogen IV might be a secreted enzyme, because it contains three potential furin-processing sites (Arg-X-X-Arg↓). The sequences are indicated by underlinings in Figure 1b, as outlined in the figure legend [12]. This assumption has been supported by a recent study showing that trypsinogen IV is detected in the extracellular matrix (ECM) in the brain [19]. A low level of expression of enterokinase has been detected previously in the brain, although its exact functional significance remains unclear [24]. Presumably, brain enterokinase is able to convert trypsinogen IV into mature trypsin IV. After enterokinase cleavage (↓) at DDDDK↓I (Fig. 1b), the active trypsin IV displays exactly the same amino acid sequence as mature mesotrypsin. However, the precise mechanisms of secretion and/or activation of trypsinogen IV in the brain tissue still remain obscure, which needs further investigation.

The most intriguing property of mesotrypsin/trypsin IV is its resistance to polypeptide trypsin inhibitors, such as the Kunitz-type soybean trypsin inhibitor, Kunitz-type bovine pancreatic trypsin inhibitor, the Kazal-type pancreatic secretory trypsin inhibitor (serine protease inhibitor, Kazal type 1 OMIM 167790), lima bean inhibitor, canine submandibular gland inhibitor, chicken ovomucoid, Alzheimer precursor protein (APP) trypsin inhibitor domain, ecotin and the serum serpin α-1 antitrypsin [10, 18, 25, 26]. These protease inhibitors are unable to form a tight complex with mesotrypsin/trypsin IV. Analysis of the crystal structure of mesotrypsin/trypsin IV indicates that the presence of an arginine residue in place of the highly conserved Gly^{198} (Gly^{193} in the chymotrypsinogen numbering system) found in the two other trypsinogen isoforms contributes to the peculiar inhibitor resistance and stabilization of mesotrypsin [26]. Arg^{198} in mesotrypsin/trypsin IV occupies the S2' subsite and exerts a steric clash with inhibitor's P2' side chain. Furthermore, Arg^{198} contributes to an unusually strong clustering of positive charges around the primary specificity pocket of mesotrypsin/trypsin IV, which may also influence inhibitor binding. However, mesotrypsin/trypsin IV still retains low but significant affinity towards trypsin inhibitors [27]. As demonstrated in a recent study, after cleavage of the Arg^{358}-Ser^{359} reactive-site peptide bond of a natural Pittsburgh variant of α-1 antitrypsin by mesotrypsin/trypsin IV, mesotrypsin/trypsin IV forms a stable covalent complex with α-1 antitrypsin Pittsburgh, which leads to inactive mesotrypsin/trypsin IV [28]. There are several natural trypsin inhibitors with the P1 Arg residue, such as SERPINB12, in the brain [29], suggesting that SERPINB12 might be another potential inhibitor of mesotrypsin/trypsin IV. Therefore, these findings refute the notion that mesotrypsin/trypsin IV can act in an uncontrolled manner in the presence of inhibitors.

Although mesotrypsin/trypsin IV is completely resistant to the serpin-type inhibitor α-1 antitrypsin, it

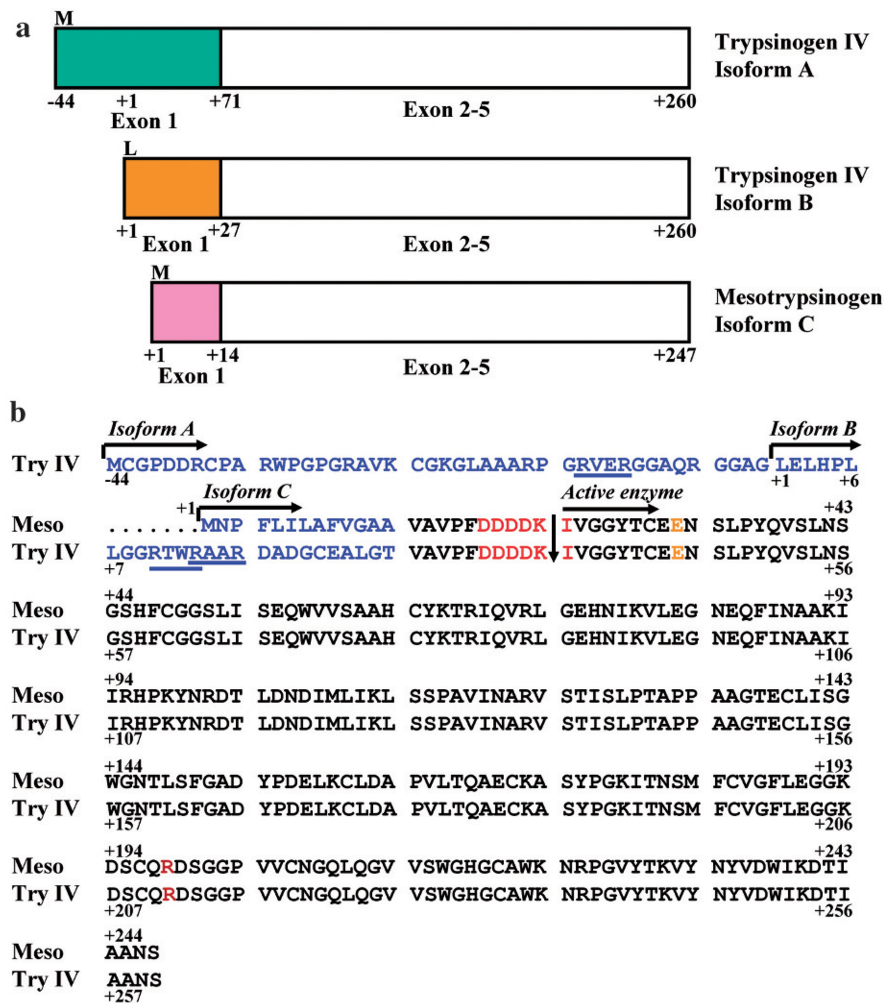

Figure 1. Three isoforms encoded by the *PRSS3* gene. (*a*) Structural scheme of human trypsinogen IV (isoforms A and B) and mesotrypsinogen (isoform C). The three isoforms of PRSS3 share exon 2–5, but differ in exon 1 at the N terminus, as a result of alternative splicing. Isoforms A and C contain a traditional translation initiation AUG (Met, M) codon, whereas isoform B possesses an unconventional translation initiation CUG (Leu, L) codon. The amino acid residues of all three isoforms are numbered. (*b*) Alignment of the deduced amino acid sequence of human mesotrypsinogen (Meso) with trypsinogen IV (Try IV). Mesotrypsinogen and trypsinogen IV have a completely different N terminus encoded by the alternative exon 1 (marked in blue). Trypsinogen IV lacks a leader peptide that is important for secretion, but it contains three furin-processing sites ($^{-13}$RVER^{-10}; ^{10}RTWR^{13}; ^{13}RAAR^{16}, marked by underlinings, as indicated in [99]), which may be responsible for enzyme secretion. Through sequence analysis, a fourth potential furin-processing site ($^{-10}$RPGR^{-13}) is also identified in the exon 1 of trypsinogen IV. The putative endoproteolytic domain is shown in red. The vertical arrow gives the cleavage site. The Glu^{32} residue marked in orange designates the site of polymorphism of mesotrypsinogen/trypsinogen IV. Arginine (R) at 198, marked in red, is responsible for inhibitor resistance of mesotrypsin/trypsin IV. The number of amino acid residues starts from translation initiation codon.

exhibits a relatively high proteolytic specificity for Lys/Arg-Ser/Thr peptide bonds [28]. Compared with the residue Thr or Ser at the P1’ position, the residue Gly or Met inhibits the cleavage 13- and 25-fold, respectively, whereas substitution with Asn, Asp, Ile, Phe or Tyr results in 100–200-fold diminished rates of proteolysis, and the residue Pro completely abolishes proteolytic activity of mesotrypsin/trypsin IV. In addition, mesotrypsin/trypsin IV is also able to degrade other trypsin inhibitors like soybean trypsin inhibitor (Kunitz) and human pancreatic secretory trypsin inhibitor [27]. Therefore, mesotrypsin/trypsin IV is not a defective protease on polypeptide substrates. The preferential cleavage of mesotrypsin/trypsin IV on Lys/Arg-Ser/Thr peptide bonds suggests that mesotrypsin/trypsin IV might activate certain PARs (see cleavage sites in the tethered ligand in Fig. 2). PARs, a family of G protein-coupled receptors (GPCRs), are widely expressed in the CNS, including neurons, microglia, astrocytes, and oligodendrocytes [30–35]. Four members (PAR-1, -2, -3 and -4) of the PAR family have been identified. They are activated by proteolytic cleavage of their N termini by serine proteases, such as thrombin and trypsin. After pro-

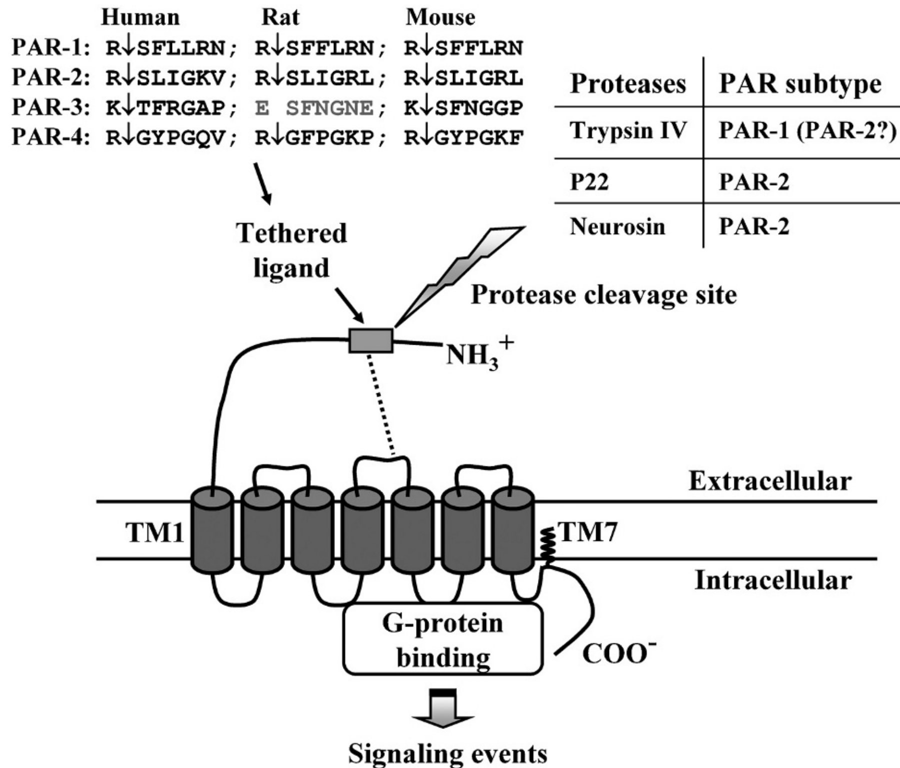

Figure 2. Structure and activation mechanism of protease-activated receptors (PARs) by brain trypsins (modified from [4]). PAR, a distinct subfamily of G protein-coupled receptor (GPCR), has seven transmembrane (TM) helix bundles and an additional eighth intracellular C-terminal helix anchored by Cys-palmitoylate lipids to the inner leaflet of the lipid bilayer [133]. Following irreversible proteolytic cleavage of PAR by serine proteases, such as trypsin IV, P22 and neurosin, a new N terminus is unmasked acting as a tethered ligand (boxed segment). The tethered ligand domain sequences for the four different PARs in human, rat and mouse are shown on the left. The vertical arrow shows the cleavage site. However, the tethered ligand of rat PAR-3 is still unknown (gray sequence). Based on human and mouse PAR-3 domains, the corresponding sequence of rat PAR-3 is shown in gray, which cannot be cleaved by thrombin. The exposed tethered ligand can interact with the second extracellular loop of the receptor (indicated by dotted line). Afterwards, the conformation of the receptor is changed, facilitating G protein coupling with the PAR at the intracellular loops 2, 3 and the eighth helix. Consequently, G proteins transmit brain trypsin signals to cellular downstream effectors [134].

teolytic cleavage, a new N terminus is unmasked acting as a tethered ligand, which can interact with the second extracellular loop of the receptor, initiating multiple signal transductions (Fig. 2).

Our recent work demonstrates that both cationic and anionic trypsin, but not mesotrypsin/trypsin IV, activate PAR-2 in human bronchial epithelial (HBE), human embryonic kidney (HEK)-293 and A549 (human pulmonary epithelial) cells [36]. However, even at high concentrations human recombinant mesotrypsin/trypsin IV fails to activate PARs in these various human epithelial cell lines (HBE, A549 and HEK-293 cells). All these cell lines abundantly express functional PAR-2, and to a much lesser extent PAR-1. In contrast, mesotrypsin/trypsin IV at high concentration weakly activates PAR-1 in human astrocytoma (1321N1) cells, which express only PAR-1 and PAR-3 [36].

Our further study revealed that mesotrypsin/trypsin IV (70 nM) remarkably and selectively activates PAR-1, but not PAR-2 in primary rat astrocytes and rat retinal ganglion cells RGC-5 [37]. The maximal effect of mesotrypsin/trypsin IV on intracellular calcium mobilization in rat astrocytes is much higher than that observed in the human astrocytoma 1321N1 cells. This suggests that the activity of mesotrypsin/trypsin IV is species specific. In addition, the possible involvement of PAR-4 activation by mesotrypsin/trypsin IV in astrocytes is also suggested [37]. Alternatively, it has been reported that trypsin IV is able to activate PAR-2 and PAR-4 in human epithelial cells [11]. Recently, the same research group further demonstrated that human mesotrypsin/trypsin IV, at micromolar concentrations, induces activation of PAR-1 and PAR-2 in transfected cells and rat dorsal root ganglion neurons [38]. Therefore, the capacity of mesotrypsin/trypsin IV to activate PARs may depend on the preparation of the enzyme, and cell type- and species-specific PAR expression.

Although it is still controversial which PAR is sensitive to mesotrypsin/trypsin IV, PAR activation by mesotrypsin/trypsin IV might play an important

role in the brain. Recent data from our laboratory have shown that activation of either PAR-1 or PAR-2 in the brain can protect astrocytes and cortical neurons from toxic insults [39–41]. Thus, we suppose that mesotrypsin/trypsin IV, *via* activation of PAR-1 or PAR-2, might contribute to neuroprotection in the brain. Emerging evidence also suggests that brain trypsin IV is involved in neurodegenerative diseases. Intraplantar injection of mesotrypsin/trypsin IV causes edema and granulocyte infiltration in mice paw, which are diminished in PAR-1- or PAR-2-deficient mice [38]. Similarly, mesotrypsin/trypsin IV induces PAR-2-dependent hyperalgesia to thermal and mechanical stimuli in mice paw. Thus, mesotrypsin/trypsin IV contributes to neurogenic inflammation and pain. In addition, trypsin IV has been proposed to enhance expression of glial fibrillary acidic protein (GFAP) in astrocytes and to process the APP in neurons in transgenic mice [23]. Recently, Medveczk et al. reported that human trypsin IV selectively cleaves the Arg^{79}-Thr^{80} and Arg^{97}-Thr^{98} peptide bonds in the lipid-bound form of myelin basic protein [42]. Myelin basic protein, an autoantigen in multiple sclerosis, is involved in neuroinflammation and is associated with multiple sclerosis [43]. Therefore, mesotrypsin/trypsin IV might play a role in the pathogenesis of multiple sclerosis.

It is still unclear whether or not mesotrypsin/trypsin IV might be unique to humans. So far, mesotrypsin in other animals remains unknown. Although no inhibitor-resistant trypsin activity was found in pancreatic extracts from dog, cow, pig, rat, mouse and hamster [10], it seems that mesotrypsin-like enzymes are widespread in the animal kingdom [27]. Mesotrypsin-like enzymes in animals can be divided into structural orthologs, which carry the mesotrypsin signature mutation of Gly^{198} (chymotrypsinogen numbering Gly^{193}), and functional orthologs, which exhibit inhibitor resistance. Rat trypsinogen V, which was detected at the protein level [44], was expected to exhibit mesotrypsin-like inhibitor-resistance and inhibitor-degrading properties [18]. In addition, rat trypsin IV (p23) was described as partially inhibitor resistant, presumably due to the presence of the negatively charged Asp^{198} [45]. A recent study revealed the roles of rat trypsin IV (p23) in PAR activation and PAR-2-dependent neurogenic inflammation [38]. Thus, it would not be surprising if new mesotrypsin orthologs were found in other species in the future.

P22

P22, a trypsin-like serine protease of 22 kDa, was purified from the incubation medium of rat brain slices by gelatine zymography [13]. The purified P22 preferentially cleaves serine protease substrates, such as Boc-Gln-Ala-Arg-MCA, Boc-Asp(OBzl)-Pro-Arg-MCA and Pyr-Gly-Arg-MCA. The enzymatic activity of P22 can be completely inhibited by serine protease inhibitors, diisopropyl fluorophosphate and benzamidine. Physiological studies demonstrate that P22 can efficiently digest the ECM proteins laminin and type IV collagen [13]. Laminin potentiates neuronal survival and neurite outgrowth [46]. Previously, it has been shown that microinjection of kainate into mouse hippocampus induces the digestion of laminin in the injected region and subsequently causes neuronal cell death [47]. Therefore, it was supposed that an excessive release of P22 produces the degradation of laminin and the change in the interaction between neurons and ECM, leading to neuronal cell death [13]. On the other side, type IV collagen is supposed to control the permeability of brain blood vessels [48]. Thus, P22 may be involved in the acute increase in the permeability of brain capillaries after the injury of the brain [13].

Besides degrading the ECM, P22 also signals to cells by activating PAR-2. Purified P22 has been shown to induce a pronounced and transient intracellular calcium mobilization in a human glioblastoma cell line, A172 cells, which express PAR-2, as shown by reverse transcription-PCR and the intracellular calcium response to the agonist peptide SLIGKV [13]. The pre-treatment of A172 cells with PAR-2 agonist peptide completely abolished the intracellular calcium response to P22, and *vice versa.* This indicates that P22 activates PAR-2 on A172 cells. Therefore, P22 could be a good candidate for neural PAR-2 activation. It was shown that PAR-2 is present in the rat hippocampus and associated with neuronal degeneration [49]. Enhanced PAR-2 expression in neurons in conjunction with neuroinflammation in the brain tissue from patients with HIV-1-associated dementia is thought to rescue neuronal cells [50]. In addition, it was also found that the deficiency of the *PAR-2* gene increases the acute ischemic cerebral injury. The injury process was associated with suppression of neuronal extracellular signal-related kinase activation and reactive astroglial activation [51]. Our recent data demonstrated that PAR-2 activation protects both primary astrocytes [40] and cortical neurons [41] from toxic insults *via* regulating release of the chemokine GRO/CINC-1. These data imply that PAR-2 might be involved in neuroprotection. On the other side, PAR-2 was also shown to modulate neuroinflammation in

experimental autoimmune encephalomyelitis and multiple sclerosis [52]. Thus, P22 might play a pivotal role in physiological and/or pathological processes in the brain *via* PAR-2.

More interestingly, Sawada et al. [13] showed that P22 appears to be enhanced after mechanical brain injury in a time-dependent manner. Three hours after the penetration insult, the proteolytic activity of P22 appears both on the ipsilateral and the contralateral sides of lesioned areas in the cerebral cortex and hippocampus. The expression of P22 is significant on the ipsilateral side of the lesion in the hippocampus 8 h after the injury, and remains for at least 24 h [13]. Taken together, P22 might be involved in cytotoxicity or neuroprotection events *via* either the digestion of the ECM or the stimulation of PAR-2.

Neurosin

Neurosin was originally identified from a primary breast tumor using differential display [14]. The same gene was also cloned from a human colon adenocarcinoma cell line and from brains of Alzheimer's patients [53, 54]. Neurosin is known as protease M, myelencephalon-specific protease (MSP, rat homologue) and zyme [14, 54, 55], and is now named human kallikrein 6 by the nomenclature for the kallikrein genes [56]. The gene encoding neurosin has been localized by fluorescence *in situ* hybridization analysis to human chromosome 19q13.3 [14, 54]. Distribution studies have demonstrated that neurosin is preferentially expressed in the adult brain [53, 54], suggesting that neurosin is an important trypsin-like serine protease in the brain.

Neurosin is synthesized as the preproenzyme, secreted into the extracellular space as the inactive zymogen after the signal peptide removal, and activated after cleavage of the peptide bond between Lys^{21} and Leu^{22}. Interestingly, a low enzymatic activity of pro-neurosin is detected, but it is rapidly decreased due to autolysis between Arg^{80} and Glu^{81} [57]. Further experiments demonstrate that the recombinant mutant neurosin (R80Q) contains the active/mature neurosin (L^{22}VHGGPC-) and the partially truncated form of pro-neurosin (N^{20}KLVHGGP-). The latter is completely inactive, although it could be fully activated after incubation with lysyl endoprotease. In contrast, the purified enzymatically dead mutant of neurosin, in which the catalytic $serine^{197}$ is replaced by alanine, displays the complete pro-neurosin (A^{16}EEQNKLV-) [58]. Therefore, these data suggest that neurosin is able to autoactivate and autoinactivate itself. The mechanism of activation of neurosin involves a two-step process in which the peptide bond Q^{19}-N^{20} of the pro-neurosin is recognized and cleaved first, followed by the removal of the dipeptide $N^{20}K^{21}$ to yield an active/mature neurosin. The residue Arg^{80} can be self-targeted by mature neurosin, resulting in inactivation of the enzyme [58]. Recently, it was reported that the ability of autoactivation of neurosin is relatively limited when compared to the rate of autoinactivation or to the ability of enterokinase and plasmin to activate pro-neurosin [59].

Neurosin preferentially cleaves arginine-ending substrates Phe-Ser-Arg-AMC and Val-Pro-Arg-AMC, but has very low cleavage efficiency for the Val-Leu-Lys-AMC [57]. Neurosin does not hydrolyze chymotrypsin-, plasmin-, urokinase- or elastase-specific substrates [57, 58], suggesting that neurosin displays relatively restricted substrate specificity. Recently, it was shown that neurosin cleaves the Arg^{36}-Ser^{37} peptide bond at the N terminus of PAR-2 and activates the receptor [60]. However, neurosin fails to activate PAR-1 and PAR-4, although it can cleave the peptide bond after Arg at the activation domain of PAR-1 or PAR-4 shown by HPLC and mass spectrometry [60]. These data demonstrate that neurosin is a potential PAR-2 agonist in the brain. PAR-2 might mediate the physiological and pathological functions of neurosin in the brain. The consequence of the interaction between PAR-2 and neurosin in the brain needs to be investigated in future.

Previous studies have indicated that neurosin is involved in neurodegenerative diseases, like Alzheimer's disease, Parkinson's disease and multiple sclerosis. Neurosin was identified in brains of Alzheimer's disease, and locates in microglia and the perivascular region, as shown by immunohistochemistry [54]. In cells cotransfected with neurosin and APP cDNA, amyloid β (Aβ) is detected in the culture media [54]. Neurosin is able to cleave the N terminus of APP at three different sites [57]. Therefore, neurosin might contribute to the accumulation of Aβ in the brain and regulate the pathogenesis of Alzheimer's disease.

It has also been shown that neurosin degrades α-synuclein, an acidic synaptic protein involved in the pathogenesis of Parkinson's disease [61]. Immunohistochemistry indicates that neurosin locates at the core of Lewy inclusion bodies in brains of patients with Parkinson's disease. The subcellular localization studies further reveal that neurosin is colocalized with cytochrome c and resides at the mitochondria. Upon stress stimulation, neurosin is released from the mitochondria to the cytosol and thereby hydrolyzes α-synuclein [61]. Therefore, the degraded fragments of α-synuclein accumulate as insoluble particles in the brain and prevent α-synuclein polymerization, eventually contributing to the development of Parkinson's disease.

Recently, it was found that neurosin is predominantly expressed in CNPase-positive oligodendrocytes, and plays an important role in multiple sclerosis. In mice with myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE), the expression of neurosin is up-regulated in the white and gray matter surrounding demyelinating lesions in the brain [62]. The cells expressing neurosin display immunoreactivity for CNPase and NG2, specific markers for oligodendrocytes and their progenitors, respectively. It was also shown that neurosin is expressed in perivascular and parenchymal inflammatory cells in brains of proteolipid protein peptide (PLP)-primed mice [63]. Inhibition of enzymatic activity of neurosin by anti-neurosin antibody delays the onset of disease and reduces clinical disease scores in mice with PLP-induced EAE. Similar inhibitory effects by anti-neurosin antibody were observed in mice with MOG-induced EAE. Moreover, reduced clinical deficits by anti-neurosin antibody are associated with reduced meningeal inflammation and white matter parenchymal pathology at days 12 or 21 post-PLP priming in mice [63]. These studies suggest that neurosin is involved in the pathogenesis of multiple sclerosis. In contrast, Bando et al. [64] suggest that neurosin is important for the maintenance of myelination after brain insults. Under that condition, neurosin is down-regulated in glutathione-*S*-transferase-π-positive oligodendrocytes during demyelination in mice fed with cuprizone, but neurosin is increased during remyelination when mice are returned to the normal diet [64].

Abnormal expression of neurosin in the brain has also been observed in animal models of other neurodegenerative disorders. Spinal cord injury induces up-regulation of neurosin mRNA in the white and gray matter surrounding the lesion [65]. The expression of neurosin is increased mainly in oligodendrocytes in the corpus callosum and around the ischemic area in rats subjected to transient middle cerebral artery occlusion [66]. In addition, a higher level of neurosin mRNA and protein expression around the cryogenic area is detected at 2–7 days after cryogenic injury. Double immunohistochemical staining reveals that neurosin is expressed mainly in oligodendrocytes around the lesion [67]. Taken together, it seems important that neurosin regulates PAR-2 physiology and is involved in neurodegenerative disorders in the brain.

Neuropsin

Neuropsin is a trypsin-like serine protease in the brain, although it is also present in other organs, like skin and ovary, known as kallikrein 8 [68, 69]. Initially, it was found that neuropsin was mainly expressed in the limbic structures of mouse brain and was localized at highest concentrations in pyramidal neurons of the hippocampal CA1–3 subfield [15]. Subsequently, Mitsui et al. [70] isolated cDNA clones encoding two novel forms of human neuropsin, designated as type 1 and type 2 neuropsin, which are generated by alternative splicing in human brain. Type 1 neuropsin is identical in length to mouse neuropsin, whereas type 2 neuropsin is a species-specific splice variant, containing an insert of 45 amino acids between the leader peptide and the proenzyme peptide of type 1 neuropsin. The essential three amino acids in the active site triad, His, Asp, and Ser, and the single putative *N*-glycosylation site are conserved in human and mouse neuropsin. The distribution of neuropsin mRNA in human brain appears to be different from that in mouse. Assessment of the adult mouse brain by *in situ* hybridization revealed that neuropsin mRNA is concentrated in hippocampal pyramidal neurons and weak signals are observed in neocortex [15, 71]. In contrast, human type 1 neuropsin mRNA is predominantly present in pancreas; whereas type 2 neuropsin is preferentially expressed in broad areas of the adult brain including hippocampus [70]. Although human fetal brain expresses both type 1 and type 2 neuropsin in comparable amounts, the localization of neuropsin in human fetal brain is still unclear [70]. Secretion of both type 1 and type 2 neuropsin from insect cells can be detected at the protein level in conditioned media, suggesting that type 2 neuropsin is a protein present in the human brain [70].

Neuropsin is secreted into the extracellular space in the soluble fraction as a nonactive zymogen and activated by processing at the Lys^{32}-Ile^{33} peptide bond after its release [72]. The activation of neuropsin is regulated by neural activity in a *N*-methyl-D-aspartate (NMDA) receptor-dependent manner [73]. Containing the complete triad (His-Asp-Ser) of the serine protease domain, both active neuropsins from brain and the recombinant enzymes have proteolytic activity. They preferentially cleave Arg-X and to a lesser extent Lys-X bonds in the synthetic chromogenic substrates. The highest specific activity was found with Boc-Val-Pro-Arg-4-methylcoumaryl-7-amide [72], which is also a substrate for thrombin. Therefore, it would be interesting to see whether neuropsin is able to activate or inactivate PARs in the brain, and whether neuropsin *via* regulating PAR activation plays a role in LTP, neurite outgrowth, and synaptic plasticity.

Multiple evidence has suggested that neuropsin plays an important role in synaptic plasticity. Neuropsin rearranges the ECM by degrading ECM proteins,

fibronectin and the cell adhesion molecule L1, whereby it regulates plasticity changes. Fibronectin, which is a major ECM protein expressed in the brain and plays critical roles in cell adhesion and migration, can be effectively cleaved by neuropsin [72]. It was shown that CHO α5B2 cells expressing integrin α5β1 bind less effectively to fibronectin that was treated with neuropsin than to intact fibronectin in adhesion assays. However, the fibronectin-induced migration of CHO α5B2 cells is not affected by neuropsin treatment [74]. The cleavage pattern of fibronectin by neuropsin is similar to that by plasmin but is distinct from those by thrombin, trypsin and tPA [75, 76]. Therefore, neuropsin regulates the ECM environment by changing the interaction between cells and fibronectin. This results in morphological changes in the synapse and synaptic plasticity.

L1 is known as a major adhesion molecule of growth cones, involved in elongation, fasciculation and pathfinding [77, 78]. It has been shown that L1 is a Schaffer collateral presynaptic component in the hippocampal CA1 subfield [73]. Neuropsin can rapidly and completely degrade L1 *in vitro*, suggesting that L1 is a most suitable substrate for neuropsin. Interestingly, neuropsin-induced cleavage of L1 is enhanced by NMDA stimulation in hippocampus, which can be completely inhibited by pre-treatment with NMDA receptor antagonist [73]. Furthermore, the neutralization of neuropsin by anti-neuropsin antibody blocks NMDA-induced L1 degradation and impairs the hippocampal Schaffer collateral early-phase LTP (E-LTP) [73, 79]. Therefore, neuropsin-dependent L1 processing might be involved in synaptic plasticity.

Nakamura et al. [80] further showed that active neuropsin plays an important role in synaptogenesis and maturation of Schaffer-collateral L1cam-immunoreactive synaptic boutons. The maturation of synaptic structures is inhibited by neuropsin deficiency, which causes an accumulation of immunoreactive L1 orphan and synaptic boutons. Therefore, neuropsin and L1 regulate the structural changes of the hippocampal synapse, which are important for neural plasticity, including learning and memory.

Data from neuropsin-deficient mice showed that the deficiency of neuropsin results in morphological changes of the pyramidal neuron soma, presumably *via* modification of ECM components [81]. Strikingly, the synaptic loss is significant in the stratum radiatum, the major terminal field of the Schaffer-collateral pathway, but not in the stratum oriens. Behavioural studies showed that neuropsin-knockout mice are significantly impaired in the Morris water maze and Y-mazes and fail to exhibit early LTP induced by a single tetanus administration [82], suggesting the involvement of neuropsin in learning. Interestingly, low concentrations of recombinant neuropsin induce LTP *in vivo*, whereas neuropsin at high concentrations elicits long-term depression *in vivo*. The neuropsin-induced phosphorylation at different sites on the GluR1 subunit of the AMPA receptor regulates two opposite types of synaptic plasticity [82]. Therefore, neuropsin-induced synaptic rearrangement is a major mechanism for synaptic plasticity, particularly in early LTP.

Conversely, neuropsin-deficient mice generated by the Edinburgh group display normal hippocampal LTP and exhibit no deficits in spatial navigation, as shown in the water maze [83]. However, neuropsin mutant mice possess an increased susceptibility for hyperexcitability (polyspiking) in response to repetitive afferent stimulation. Moreover, seizure activity after kainic acid administration is markedly increased in mutant mice, which is accompanied by elevated c-fos gene expression throughout the brain [83]. Therefore, in those mutant mice, neuropsin has little effect on synaptic remodeling but plays a role in limiting neuronal hyperexcitability induced by epileptogenic insults. These data are consistent with previous findings [84]. It was shown that neuropsin is significantly increased in the hippocampus and cerebral cortices in kindled mice. Blockade of neuropsin by its antibody inhibits the progression of kindling, suggesting the potential therapeutic role of neuropsin in epilepsy.

Neuropsin has also been implicated to have a role in neuronal development, in addition to synaptic plasticity. Recombinant neuropsin induces morphological changes of hippocampal neurons, and enhances neurite outgrowth from soma and neuronal aggregation with neurite fascicles [85].

Several studies suggest that neuropsin exerts a pathological role in neurodegenerative diseases. Previously, it was shown that neuropsin mRNA expression is induced in the cell body of oligodendrocytes and in the myelin after brain injury [65, 86]. The expression of neuropsin mRNA peaks at 4–8 days and disappears at 14 days. Recently, it was found that neuropsin mRNA is up-regulated in CNPase-positive oligodendrocytes in the spinal cord white matter of mice with EAE. Neuropsin-knockout mice exhibit an altered EAE progression characterized by delayed onset and progression of clinical symptoms as compared to wild-type mice. Deficiency of neuropsin attenuates demyelination and oligodendrocyte death during the course of EAE [87]. These data suggest that neuropsin is involved in the pathogenesis of EAE regulated by demyelination and oligodendroglial cell death. Similarly, neuropsin was recently shown to regulate demyelination, oligodendrocyte death, and axonal degeneration, thereby contributing to the secondary phase of the pathogenesis of spinal cord injury [88].

Neurotrypsin

Neurotrypsin (PRSS12) was initially identified from a murine brain cDNA library using degenerated primers from the conserved catalytic sites of serine proteases [16, 89]. Human neurotrypsin was discovered later from a fetal brain cDNA library with primers based on the murine neurotrypsin [90]. Both murine and human neurotrypsin share a similar protein structure consisting of a kringle domain, followed by three or four scavenger receptor cysteine-rich repeats and a serine protease domain at the C terminus [16, 90]. The genomic sequence of the human NEUROTRYPSIN gene maps to chromosome 4q28 and has 13 exons.

It has been shown by Northern blot and *in situ* hybridization that neurotrypsin is strongly distributed in the brain, lung and kidney [16, 89]. During development, neurotrypsin mRNA expression is detectable in the 9-day embryo, and a moderate level of expression is maintained in the CNS in the 13–20-day embryo [91]. After birth, the expression of neurotrypsin mRNA in the mouse CNS peaks at postnatal day 10 (P10), decreases by P16 and continues with weak levels in the 7-month-old mouse brain. This developmental expression pattern suggests that neurotrypsin might play important roles in neural development [92]. At P10, neurotrypsin mRNA is localized in the limbic system, especially in the hippocampal CA1, cortical layers II/III, V and VIb in the cerebrum, and efferent neurons in the brainstem including the oculomotor nucleus, trochlear nucleus, mesencephalic and motor nuclei of the trigeminal nerve, abducens nucleus and facial nucleus [91]. *In situ* hybridization in the adult mouse brain revealed most prominent expression of neurotrypsin mRNA in neurons of the cerebral cortex, subicular complex, Ammon's horn, dentate gyrus, and lateral amygdala [16]. These structures are highly involved in learning and memory. By immune electron microscopy, the expression of neurotrypsin in the fetal human brain is confined to the presynaptic membrane and the presynaptic active zone of both asymmetrical (excitatory) and symmetrical (inhibitory) synapses [92]. Recent data on the intracellular localization further demonstrate that both mouse and human neurotrypsin locate in the cell body and to a much lesser extent along the dendrites and axons [93].

Biochemical studies demonstrated that the proteoglycan agrin is a unique substrate of neurotrypsin [94]. Neurotrypsin cleaves both Arg^{995}-Ala^{996} peptide bond at the α-cleavage site and Lys^{1754}-Ser^{1755} peptide bond at the β-cleavage site of agrin. The conserved Glu at the P2 position is important for recognition by the S2 subsite of neurotrypsin, which is essential for efficient and specific cleavage. Substitution of Glu at the β-cleavage site by Gln, Asp and Leu reduced the catalytic efficiency to ~20%. *In vitro* assays demonstrate that the enzymatic activity of neurotrypsin depends on calcium and pH value. The temporal expression pattern indicates that neurotrypsin-dependent cleavage of agrin is prominent during late stages of synaptogenesis, but decreases in the adult brain. It has been shown that neurotrypsin-solubilized agrin binds to the $\alpha 3$ subunit of the Na^+/K^+-ATPase and inhibits Na^+/K^+ pump functions in cortical neurons, resulting in membrane depolarization and increased action potential frequency of neurons [95]. Therefore, neurotrypsin plays an important role in reorganization of synapses.

Clinical studies demonstrate that a mutant neurotrypsin gene is associated with autosomal recessive nonsyndromic mental retardation in children [96]. In patients, a 4-bp deletion, located in exon 7 of the neurotrypsin gene, has been detected. The 4-bp deletion results in a shortened protein lacking the catalytic domain of the serine protease. These results indicate that neurotrypsin-mediated proteolysis is required for physiological functions of the synapse in the brain. Therefore, neurotrypsin is the first serine protease identified as a cause of a nonsyndromic autosomal recessive form of mental retardation. Further experiments on *Drosophila* demonstrate that Tequila, the *Drosophila* neurotrypsin orthologue, regulates long-term memory (LTM) formation [97]. Inactivation of Tequila causes a decrease in LTM in *Drosophila.* On the other side, after LTM conditioning, the expression of Tequila is time-dependently upregulated in the mushroom bodies, which suggests that Tequila is physiologically involved in neural plasticity. Moreover, it was found that specific inhibition of Tequila expression in adult mushroom bodies by RNA interference causes a reversible LTM defect [97]. Therefore, these results suggest that neurotrypsin is essential for information processing and functional plasticity.

Recent data demonstrate that after facial nerve axotomy neurotrypsin mRNA expression is decreased at day 14 in facial nerve nuclei at the anterior margin of the parotid gland in mice, but is significantly recovered by day 21 [98]. The time course of neurotrypsin expression is consistent with the recovery of the facial motor neuron function, suggesting that neurotrypsin might be involved in neuroregeneration.

Serine protease inhibitors in the brain

Secretory serine proteases play important roles in neural development, plasticity, degeneration and re-

generation in the nervous system [99]. In the brain, the effects of serine proteases are modulated by serine protease inhibitors (serpins). The mechanism of inhibition of proteolytic activity by serpins involves a profound change in molecular conformation. The serpins act by binding to the active site of their target proteases. After binding, the protease cleaves the reactive center loop of the serpin, releasing it and initiating a conformational transition in which the bound protease moves from the top to the bottom of the serpin molecule. At the same time the reactive center loop inserts into the β-sheet A of the serpin, and the protease is irrevocably inactivated by molecular rearrangement [100].

Protease nexin-1 (PN-1), the first identified neural serpin, is a potent inhibitor for thrombin in the brain [101]. PN-1 is localized around blood vessels and expressed in glia and neurons [102–104]. It can modulate the mitogenic effects of thrombin and promote stellation of astrocytes [105]. Interestingly, PN-1 is co-expressed with PAR-1 in spinal motoneurons [106]. After facial nerve transection, the expression of PN-1 is decreased, which is associated with that of PAR-1 in facial motoneurons. In addition, PN-1 also targets tPA and uPA in the brain [101, 107].

Neuroserpin, the second neural serpin, is secreted from the growth cones of neurons and strongly inhibits the enzyme tPA and to a lesser extent uPA and plasmin, but has no action against thrombin [108, 109]. In terms of both structure and function, neuroserpin is most closely related to PN-1 and plasminogen activator inhibitor type 1 (PAI-1). Neuroserpin provides a natural counter balance to excessive tPA in both health and disease. In the adult brain, neuroserpin and tPA are co-expressed in the hippocampus, hypothalamus, cerebellum, amygdala and sympathetic nerves, suggesting that neuroserpin might modulate tPA-induced learning and memory processes [110–112]. Recent studies demonstrated that mutant neuroserpin polymerizes and forms intracellular aggregates, and thereby contributes to familial encephalopathy with neuroserpin inclusion bodies, an autosomal dominant neurodegenerative disorder [113, 114].

PAI-1 is an inhibitor of uPA and tPA, and it may also inhibit thrombin production by inhibiting the cleavage of prothrombin to thrombin by activated factor X [115]. Unlike PN-1 or neuroserpin, PAI-1 is hardly detected in the nervous system [108], but it is strongly expressed in astrocytes after ischemia [116, 117]. In addition, PAI-1 can be induced in the brain after blood-brain barrier breakdown [118]. Recently, it was shown that activation of PAR-1 induces the secretion of PAI-1 from human brain microvascular pericytes [119], indicating a dynamic balance between serine proteases and serpins.

Colligin, also called heat shock protein 47, is a collagen-binding serpin. It inhibits thrombin through restructuring the ECM. Colligin localizes in the lumen of the endoplasmic reticulum and is induced in microglia and astrocytes after cerebral ischemia, subarachnoid hemorrhage and an intracerebral infusion of thrombin [120].

Serine proteinase inhibitor-3 (SPI3) and murinoglobulin I (MUG I) were identified as specific inhibitors of neuropsin in adult mouse brain [121]. SPI3 is co-expressed with neuropsin in pyramidal neurons of the hippocampal CA1–CA3 subfields. SPI3 is an intracellular protein, whereas active neurosin locates at the extracellular space. Thus, how SPI3 exerts its inhibitory effects on neurosin in the brain still awaits further study. Immunohistochemistry shows that MUG I is localized in the hippocampal neurons, and binds extracellularly to active neurosin in neurons of the CA1–CA3 subfields in the brain. Nevertheless, both SPI3 and MUG I serve to inactivate neuropsin and control the level of neuropsin in the adult brain [121].

Phosphatidylethanolamine-binding protein (PEBP) has been proposed as a novel type of serine protease inhibitor since it has no sequence homology with the serpins. PEBP exerts inhibitory activity against thrombin, chymotrypsin and neurosin *in vitro*, but it is not able to inhibit trypsin, tPA and elastase [122]. PEBP is widely expressed in multiple tissues, including brain, heart, testis, liver, kidney, epididymis [123]. Previously, PEBP was described as a Raf-1 kinase inhibitor protein by binding to Raf-1 [124]. Thrombin binds to PAR-1, resulting in Raf-1 phosphorylation [125]. Therefore, PEBP might modulate the cellular response to PARs.

Accumulating evidence has demonstrated that serpins play a neuroprotective role in the brain [113, 114, 117, 126]. The brain-related serine proteases targeted by certain serpins are listed in Table 2. The endogenous serpins of trypsin-like serine proteases, especially mesotrypsin/trypsin IV, in the brain are still largely unknown. The discovery of the endogenous serpins would significantly improve our understanding of the biological functions of serine proteases in the brain.

Future perspectives

Extrapancreatic trypsin and trypsin-like serine proteases have been identified in the brain. The expression of brain trypsins and their functions are summarized in Table 1. They appear to be involved in the neural development, plasticity and neuroregeneration of the CNS as well as in pathological processes of neurodegenerative diseases, *via* their proteolytic ac-

Table 2. Serine protease inhibitors (serpins) in the brain.

Serpins	Targeted serine proteases
Protease nexin-1	Thrombin, tPA, uPA [101, 107]
Neuroserpin	tPA, uPA, plasmin [108, 109]
Plasminogen activator inhibitor-1	uPA, tPA, thrombin (poor) [115]
Colligin	Thrombin [120]
Serine proteinase inhibitor 3	Neuropsin [121]
Murinoglobulin I	Neuropsin [121]
Phosphatidylethanolamine-binding protein	Thrombin, chymotrypsin, neurosin [122]

tivities. PARs are activated by certain serine proteases, like thrombin and trypsin and also mesotrypsin/trypsin IV, P22, and neurosin. Numerous studies demonstrate that PARs exert neuroprotective and neurodegenerative roles in the brain, depending on the amplitude and duration of agonist stimulation. A recent study reveals a novel role of PAR-1 in learning and memory [127]. Therefore, it is of great interest to investigate the activation of PARs by trypsin and trypsin-like serine proteases and their physiological significance in the brain. Serpins play an important role to keep in check the enzymatic activity of serine proteases. Understanding the interaction among brain trypsins, serpins, and PARs will provide invaluable insights into normal brain functions as well as the treatment of neural disorders.

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