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Extracellular environment and extracellular serine proteases in the central nervous system

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Review

Extracellular Environment and Extracellular Serine Proteases in the Central Nervous System

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Abstract: Extracellular serine proteases play important roles in the central nervous system (CNS) by modifying extracellular environments. Prothrombin is expressed in the CNS and thrombin has been shown to have inhibitory function on neurite extension, however, its physiological function is not fully understood. Tissue plasminogen activators (tPA) has important function on neural plasticity in ocular dominance and long-term potentiation (LTP). tPA also has crucial function in the excitotoxic neuronal cell death through the activation of plasminogen. Beyond these proteases, several kallikrein family proteases including neuropsin and protease M are expressed in the CNS. Neuropsin is mainly expressed in the neurons of the limbic system and has important in the neural plasticity such as LTP and kindling. Following injury, neuropsin is induced in oligodendrocytes and may be related to demyelination. Protease M is constitutively expressed in oligodendrocytes and may have scavenging function. Other kallikrein family proteases are expressed in the CNS but their functions remained to be clarified.

Key words: serine protease, tissue plasminogen activator, kallikrein, neuropsin, protease M

INTRODUCTION

Cumulative evidence has shown that extracellular proteases play important roles by modifying extracellular moieties. Several well-known serine proteases such as thrombin and tissue plasminogen activator (tPA) has been known to function in the central nervous system (CNS) in the physiological and pathological conditions. Beyond these classical serine proteases, serine proteases most of which belong to kallikrein family have been identified in the CNS. Although many proteases including those described above had been known to be expressed in the CNS, their target substrates were not well understood. Recent progress of studies successfully shown the substrates of some of serine proteases and the mechanisms of how they function in some situations. I discuss current status of researches on several serine proteases in the nervous system.

Thrombin

Thrombin is a well-known coagulant factor and was known to have inhibitory effect against process formation of neuronal and glial cells *in vitro*[1]. The function of thrombin in the CNS has been attracted more attention by the finding that prothrombin mRNA is expressed by many neuronal cells including neurons and glial cells[2]. The discovery of thrombin's unique action on cells through tethered ligand receptor[3] prompted studies of protease-activated receptors (PARs) in the brain. PAR2 was the first PAR identified in the brain[4]. This study showed that ligand peptide specific for PAR-2 increased intracellular Ca^{++} concentration and decreased neuronal survival of cultured hippocampal cells. Subsequent studies revealed that all 4 types of PARs were expressed in the brain and that PAR expressions were upregulated after CNS injury and PAR may be involved in apoptotic cell death of neurons[5]. However, (pro)thrombin's physiological function *in vivo* is to be clarified.

tPA

tPA is the best-studied serine protease of the brain. The function of tPA can be divided into three: participation in cell migration and axon prolongation, contribution to the neural plasticity and to neuronal death. I will discuss the latter two points. The role of tPA has been studied on 2 forms of neural plasticity, ocular dominance plasticity and long-term potentiation (LTP). Visual cortex forms a discrete arrangement of cells that receive input from ipsilateral or contralateral retina during a certain period of early postnatal life. If a single eye is closed during this critical period, this arrangement changes and more cells receive input from the open eye. This is one of the examples of neural plasticity earliest identified. Single eye closure increased tPA expression in

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the visual cortex[6] and the application of inhibitor of tPA blocked the ocular dominance plasticity[7]. Furthermore, tPA-knockout mice did not show this plasticity[8]. LTP is another typical example of neural plasticity. When high frequency stimulation (tetanus stimulation) is applied to a certain brain region such as hippocampus, synapse transmission is facilitated for long time period. Therefore, this facilitation is called long-term potentiation. Most prominent induction is induced in the hippocampus and this region is the essential for memory formation, therefore, LTP is thought to be an important phenomenon as a cellular basis of memory. tPA-knockout mice showed limited continuation of the potentiation[9,10]. Moreover, those mice reduced learning activity in the behavioral experiments. Interestingly, tetanus stimulation to hippocampal slices of tPA-overexpressing transgenic mice induced larger induction of LTP. These mice learned more rapidly and retained memory better than wild type animals[11]. These studies clearly indicated significant role of tPA in neural plasticity. However, the target substrate of tPA in neural plasticity is not well recognized yet. Studies in this line have been pursued on the involvement of plasmin in LTP[12-14]. Inhibitors of plasmin reduced LTP induction and plasmin itself partially enhanced potentiation when applied to hippocampal slice cultures[12]. The same authors further suggested that plasmin may degrade laminin during induction of LTP[14]. Further studies are necessary to see whether the function of tPA is all though laminin degradation.

An important finding on tPA's participation in neuronal cell death was reported by Strickland's laboratory[15]. Excitotoxin upregulated tPA in the hippocampus and tPA-knockout mice showed no hippocampal cell death by the injection of excitotoxins. Pyramidal neurons in the hippocampus are most vulnerable cells to excitotoxins and injection of excitotoxins such as kainic acid induce cell death selectively of pyramidal neurons. Plasminogen-knockout mice were also resistant to excitotoxins, indicating that the target of tPA in the hippocampus is plasminogen as in the blood coagulate-fibrinolysis system. However, fibrinogen-knockout mice did not show this resistance[16] but instead, extracellular matrix, laminin emerged as a convincing candidate for the substrate of plasmin[17]. Chen et al. found that laminin was degraded after application of excitotoxin in the wild-type animal and not in the tPA knockout mice. Moreover, when anti-laminin antibody was applied to the tPA-knockout mice with excitotoxin, hippocampal neurons degenerated. This finding strongly suggested that excitotoxin upregulates tPA and tPA activates plasminogen to plasmin. Then, activated plasmin degrades laminin directly or through activating other proteases such as matrix metalloproteases. The combination of hyper-excitement by excitotoxins and laminin degradation by activated proteses results in neuronal cell death (Fig. 1).

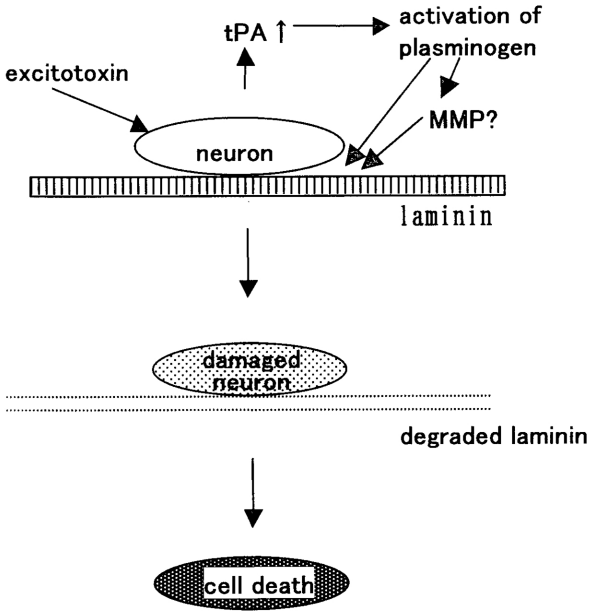

Fig. 1. Putative role of tPA in neuronal cell death caused by excitotoxins.

Kallikrein-related Proteases

Several kallikrein-related proteases are expressed in the CNS. Tissue (glandular) kallikrein is a protease that process kininogen and produce active kinins such as bradykinin. In human genome three highly conserved genes had been identified in the chromosomal locus, 19q13.3-q13.4 and three symbols were assigned, namely, KLK1 (tissue kallikrein), KLK2 and KLK3. The protease encoded by KLK3 is prostate-specific antigen (PSA) that is clinically used as a tumor marker for the prostate cancer. Although both KLK2 and KLK3 are highly expressed in the prostate, their substrates and functions are not fully understood. These three genes share several characteristics beyond sequence similarity: 1. The open reading frame is composed of 5 exons. 2. Mature protein begins in the 2nd of 5 exons. 3. His, Asp and Ser residues essential for serine protease catalysis are in 2nd, 3rd and 5th exon, respectively. 4. Coding size of exons are similar. 5. Exon-intron phases are all conserved. From 1994 many cDNAs of human, rat and mouse serine proteases similar to these kallikreins has been identified. They have 40-50% similarity to KLK1-KLK3. Along with the progress of human genome projects, the genes for these proteases have been found adjacent to kallikrein genes in the same locus tandemly without interruption by other genes. These genes also shared characteristics of kallikrein genes described above. In 2000, the nomenclature for these kallikrein family genes was revised and new codes, KLK1-KLK15 were assigned to the genes in the order from the direction of centromere (KLK3 and KLK15 are exceptions of this order due to the circumstance of identification (Fig. 2)[18]. The translated products of KLK1-KLK15 are named as hK1-hK15. The sequence similarities of amino acids among these proteases are summarized in Fig. 3. As Fig.

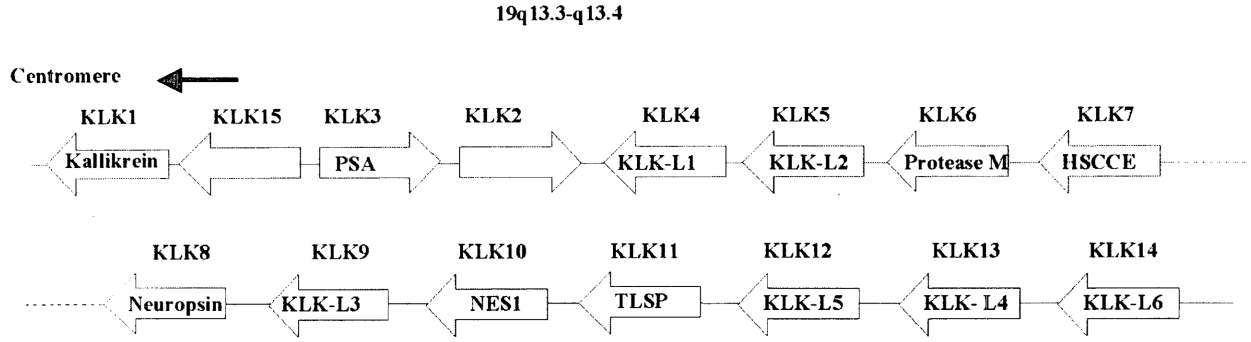

Fig. 2. Human kallikrein gene locus.
Arrows indicate the direction of transcription. Trivial names are shown in arrows.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
KLK1														
KLK2	**65**													
KLK3	**61**	**79**												
KLK4	39	41	41											
KLK5	39	44	42	52										
KLK6	39	46	43	41	46									
KLK7	41	43	41	46	48	41								
KLK8	45	56	36	42	48	47	46							
KLK9	41	42	41	37	49	44	41	50						
KLK10	35	39	36	36	40	40	43	45	39					
KLK11	44	45	41	42	51	46	44	50	57	40				
KLK12	38	39	38	41	44	46	45	47	42	45	45			
KLK13	40	45	45	43	50	52	45	50	47	42	52	45		
KLK14	44	45	44	45	50	47	45	49	48	42	49	43	51	
KLK15	39	42	42	39	44	46	44	47	49	42	52	44	46	48

Fig. 3. Amino acid homologies among human kallikrein family genes.
Numbers, 1-14 on the top line indicate KLK1-KLK14, respectively. The comparison was based on the sequences of mature forms.

3 shows, hK1-hK3 are>60% similar to each other, whereas, hK4-hK15 are 40-50% similar among them and to hK1-hK3. In this review, I will refer to proteases or protease genes 60% or more similar to KLK1 as kallikrein-type, those whose sequence similarities are less than 50% as kallikrein-related and both collectively as kallikrein family. From this sequence similarity, it is speculated that KLK1 (or one of KLK1-KLK3) and KLK4-KLK15 were produced in the early evolutional stage and later KLK2 and KLK3 were produced by gene duplication of KLK1.

Difference of Kallikrein Family Genes between Human and Rodents

There is a big difference in the kallikrein-type genes between human and rodents. Although only three kallikrein-type genes exists in the human genome, there are 27 kallikrein-type genes in the mouse genome (10 of them are pseudogenes) and 13 genes in the rat genome. They are highly homologous, generally>70%, each other. In 1991, new nomenclature was adopted and assigned mKlk1-mKlk27. Interestingly, it is difficult to identify mouse homologue of KLK1-KLK3 from sequence similarity only. Namely, no single mouse gene has significantly higher homology to for example, KLK1 than other mouse kallikrein-type genes. It is obvious, however, that mKlk1 is a functional homologue of human KLK1. Above these genes, mouse genome (presumably, rat too) also has kallikrein-related genes. In the mouse genome, these kallikrein-related genes exists close to kallikrein-type genes as in the human genome[19]. The mouse kallikrein-related genes have 40-50% similarities each other and to kallikrein-type genes as in the case of the human genes. In contrast to the kallikrein-type genes, there are one-to-one correspondence of kallikrein-related genes between human and mouse genomes. Currently there is a problem in regard to the nomenclature of kallikrein family genes. For example, the gene coding for neuropsin is assigned KLK8 in human, however, mKlk8 has been assigned to the unrelated gene belonging to kallikrein-type genes. This discord has to be corrected.

Kallikrein Family Genes in the CNS

Among kallikrein family genes, cells in the CNS reportedly express KLK6, KLK8, KLK9, KLK10, KLK11, KLK12 and KLK14 in the human, rat or mouse.

Physiological Function of KLK 8/Neuropsin

KLK8/neuropsin has been most extensively studied among kallikrein family genes expressed in the CNS. Neuropsin was originally identified from the mouse hippocampus and was expressed in neurons in several limbic structures including hippocampus CA1 and CA3, where LTP is most effectively induced[20]. Neuropsin exerted trypsin-type enzyme activity, namely cleaved after Lys or Arg residues but showed much narrower substrate specificity than trypsin. At least certain combination of three amino acid residues were necessary to be a substrate of neuropsin. Since neuropsin is an extracellular serine protease, several extracellular proteins were tested as a potential substrate for neuropsin. Neuropsin cleaved L1 and fibronectin most efficiently, but N-CAM or laminin

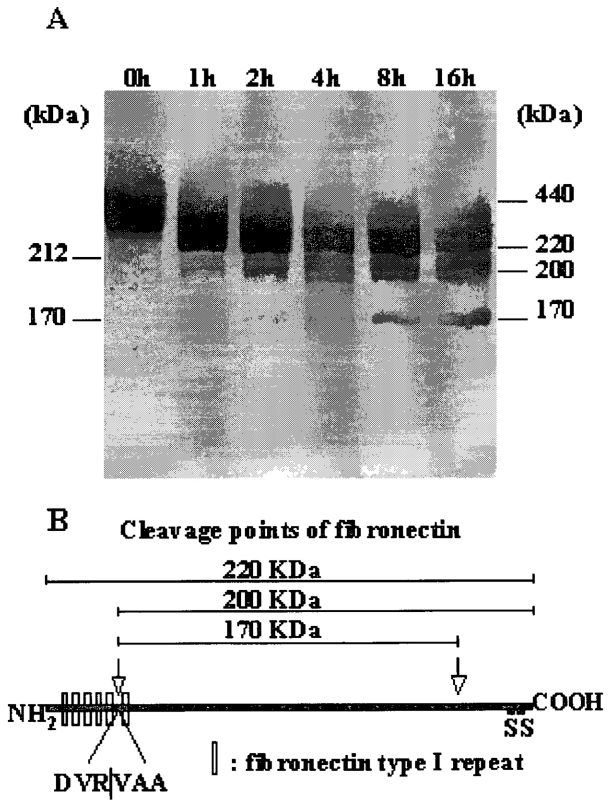

Fig. 4. Cleavage of fibronectin by neuropsin.
A Fibronectin was incubated with neuropsin for the time shown above the lanes. B Putative cleavage sites.

was not cleaved at all[21] (Fig. 4).

This cleavage activity of neuropsin may change extracellular cell adhesion structure and cause restructuring of synapses, which is important for neural plasticity such as LTP. Therefore, we produced neuropsin-knockout mice (neuropsin-KO) and examined tissue structures of the hippocampus of these mice. We found that neuropsin-KO had significantly smaller number of synapses than wild-type mice. On the contrary, neuropsin-KO had more axon terminal-like structure containing synapse vesicles but not forming synapses with postsynaptic structures[22]. This result suggests that neuropsin is involved in synapse formation by modifying the extracellular environment. To examine the role of neuropsin on neural plasticity, neuropsin was applied during LTP induction on hippocampal slices. With low concentration (1–5 nM) of neuropsin, LTP was facilitated. This facilitation was observed only when neuropsin was applied during tetanus stimulation. When applied after tetanus stimulation, neuropsin did not facilitate LTP. When intrinsic neuropsin was eliminated with anti-neuropsin monoclonal antibody, LTP was suppressed (Fig. 5)[23]. These results indicate that neuropsin is important in hippocampal neural plasticity. Another evidence supporting the function of neuropsin in neural plasticity was shown in the kindling paradigm. When a part of the brain, for example, the amygdala, is stimulated every day, abnormal circuits are formed in the hippocampus and the daily stimulation finally cause generalized seizure. This phenomenon is called kindling and is another example of hippocampal plasticity. Mice were daily stimulated in

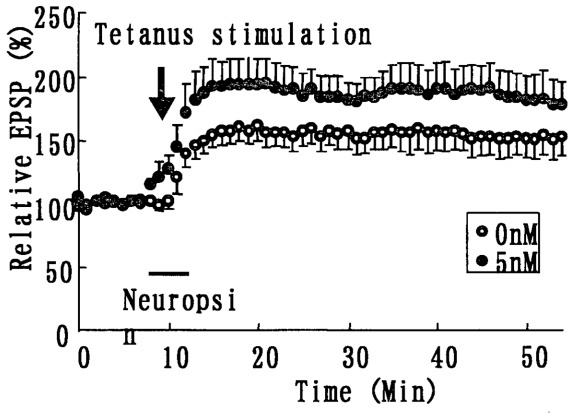

Fig. 5. The effect of neuropsin on long-term potentiation (LTP).
Tetanus stimulation was applied at the time shown by the arrow to hippocampal slices and anti-neuropsin antibody or control IgG was given for the period shown by the bar.

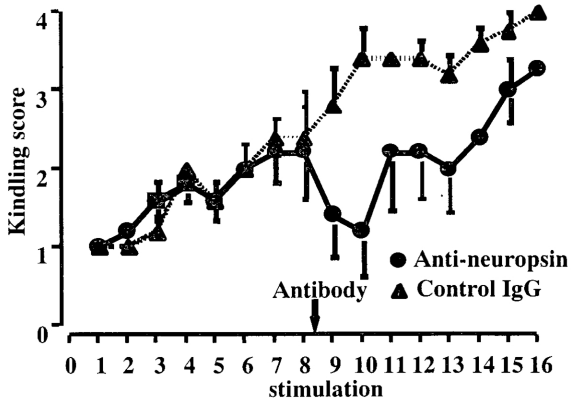

Fig. 6. The effect of neuropsin on kindling.
Below seizure-inducing stimulation was given to the amygdala once a day and at 8th day anti-neuropsin antibody (circle) or control IgG (triangle) was applied to the cerebrospinal space. Neuropsin significantly retarded kindling formation.

the amygdala in such a way that produce general seizure in 14 days. At the middle of kindling formation, anti-neuropsin antibody or control IgG was injected in the cerebrospinal space. This bolus injection of the antibody significantly retarded kindling formation, presumably by preventing abnormal circuits in the hippocampus (Fig. 6)[24].

Neuropsin in Pathological Conditions

Neuropsin has a quite different aspect in the pathological conditions. When the CNS of mice was injured, oligodendrocytes express neuropsin which is a neuron-specific protease in the physiological condition. This induction of neuropsin was observed mainly in the fiber tracts where most oligodendrocytes reside[25,26] (Fig. 7). Oligodendrocytes are glial cells which produce myelin sheaths around axons. So neuropsin might have functions regarding myelin turnover or myelin degradation. We severed optic nerves of neuropsin-knockout and wild type mice and compared ultrastructures 1 mm distal to

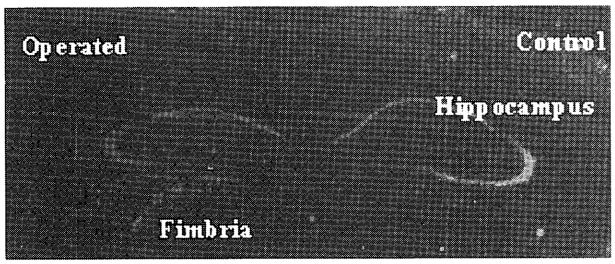

Fig. 7. The expression of neuropsin mRNA3 days after knife-cut to the fimbria that have input and output fibers of the hippocampus.
The lesion was anterior to the section. Neuropsin mRNA was induced in the operated side, whereas the expression in the neurons did not alter.

the stump. Preliminarily, neuropsin knockout mice showed significantly less degenerated myelins than wild type animals after optic nerve injury. This result suggests neuropsin may function in myelin degradation after CNS injury. However, it remains to be solved whether neuropsin directly degrades myelin proteins or neuropsin activates other factors then indirectly participates in myelin degradation.

There is also evidence suggesting that neuropsin is related to human disorders. mRNA expressions of a variety of kallikrein genes were studied in Alzheimer brain and age-matched control brains. Among kallikrein genes studied, only neuropsin gene (KLK8) expression was significantly higher in Alzheimer hippocampi than in control ones[27]. Currently there is no data available on whether the expression is the consequence of the disease or neuropsin is involved in the pathogenesis.

KLK6

KLK6 is a gene for protease named protease M, neurosin and zyme in humans, MSP in rats and BSSP and mBSP in mice[28-33]. In either species, the expression is most abundant in the brain and oligodendrocytes express the mRNA and protein at least in rats and mice[34]. Enzymatic assays have been done with rat and mouse homologues. This enzyme cleaved extracellular matrices, gelatin, laminin and fibronectin. As to the enzymatic activity for myelin proteins, myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG) were cleaved by this protease[35]. Although it is not of course direct comparison, this protease seems to have a broader substrate spectrum than neuropsin. The expression of this protease in the CNS pathology has been studied. After excitotoxin kainic acid administration and in experimental allergic encephalopathy (EAE, an animal model of multiple sclerosis), the expression of the protease was significantly increased. Interestingly, the expression under pathological condition was not confined into oligodendrocytes but was also found in immune cells such as T cells and macrophages[36]. Therefore, KLK6 may be involved in scavenging process of the injured myelin and other debris in the lesioned areas.

Other kallikrein family members in the CNS

KLK10 was reported to be expressed in Purkinje cells in the human cerebellum and some of neurons and astrocytes in the cerebral cortex[37]. mRNA of KLK11 was amplified from human cerebellum and hippocampal pyramidal neurons were observed to express the mRNA[38-40]. In the mouse, KLK11 mRNA was abundant in embryonic period rather than in the adult by northern blot analysis[39]. The expressions of KLK9, KLK12 and KLK14 were observed only by RT-PCR and the detail is to be studied.

Possible Substrates for Kallikrein Family Proteases

What are substrates for these proteases? Since each protease are supposed to be secreted, extracellular proteins are good candidates of proteolytic targets. It is naturally assumed that extracellular matrices and cell adhesion molecules be substrates for these proteases as in the case of KLK8/neuropsin and KLK6/protease M/neurosin/zyme. Another possibility is that these proteases are activating factors for other biologically active substances as in the case of KLK1/tissue kallikrein. KLK15 codes a protease expressed in the prostate and is identified as an activating factor for KLK3/PSA[41]. So it is probable that some of kallikrein family protease in the CNS are activating factor like tPA.

Inhibitors

Inhibitors play crucial roles in regulating activities of proteases. Neuroserpin was identified in 1996 as a potent inhibitor for tPA and this gene attracted attention when it was found to be the responsible gene of a certain type of familial dementia. Point mutation of the gene cause instability of neuroserpin which aggregates in the brain[42]. For KLK8/neuropsin two inhibitors has been identified[43]. The balance between proteases and inhibitors are important in the regulation of extracellular environments.

CONCLUSIONS

tPA, KLK8/neuropsin and KLK6 seem to have quite different function in physiological conditions and pathological conditions. It has to be clarified whether these protease cleave different substrates under different circumstances. It is also important to examine dynamics of extracellular proteins to understand neural functions in the CNS.

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