バイオサイエンス研究科　博士論文要旨

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題　目	Expression and function of neuropsin after injury to the central nervous system. (中枢神経損傷後におけるニューロプシンの発現と機能)		

要旨

Injury to the central nervous system (CNS) causes a variety of phenomena such as neuronal and glial cell degeneration, phagocytosis, degradation of the extracellular matrix, inflammatory responses, and the subsequent demyelination and remyelination. Proteases such as tissue plasmingogen activator (tPA), plasmin, and matrix metalloproteases (MMPs) play very important roles in this complex process after injury to the CNS. TPA and plasmin participate in the excitotoxin-induced neuronal degeneration. MMPs family is reportedly mediated both extracellular matrix degradation and inflammatory demyelination after injury to the CNS. Neuropsin is a trypsin-type serine protease that is specifically expressed in the limbic system of adult mouse brain. Knife cut injury to mouse spinal cord and intraperitoneal injection with kainate could newly induce neuropsin mRNA in peri-lesioned regions of the CNS, and the cells that expressed neuropsin mRNA were located mainly in axon fiber bundles. In the current study, I performed neuronal degeneration induced by intrahippocampal kainate injection and axonal degeneration induced by enucleation on adult mice to explore the expression and function of neuropsin after injury to the CNS.

After neuronal degeneration induced by intrahippocampal injection with kainate, neuropsin mRNA was detected by in situ hybridization. The expression of neuropsin mRNA of pyramidal cells in CA1 and CA3 of hippocampus decreased significantly, however neuropsin mRNA was newly expressed around the degenerated region, such as the corpus callosum, fimbria and molecular layer of the hippocampus. The newly expression of neuropsin mRNA began to appear at 1 day, peaking during 4 days to 8 days, disappeared at 14 days after intrahippocampal injection with kainate, and the expression of neuropsin mRNA correlates with the degenerated neurons.

Double in situ hybridization confirmed that most of the cells expressing neuropsin mRNA were PLP-positive mature oligodendrocytes. To further examine whether the expression of neuropsin mRNA in oligodendrocytes was induced by directly kainate toxicity or indirectly through neuronal degeneration, I examined neuropsin expression in the optic nerve after axonal degeneration induced by enucleation in adult mice. Neuropsin mRNA was also expressed in oligodendrocytes of the enucleated optic nerves, but not expressed in the sham-operated optic nerves. Those results showed that neuropsin mRNA was expressed following both neuronal and axonal degeneration.

After neuronal degeneration and axonal degeneration, neuropsin protein was detected by immunohistochemistry and immunoelectron microscopic analysis. Neuropsin immunoreactivity was observed in cell bodies and processes by immunohistochemistry. Under immunoelectron microscopic analysis, neuropsin immunoreactivity was observed in cytoplasm of oligodendrocyte and myelin.

Neuropsin is possibly involved in demyelination because the time course of neuropsin expression matches that for demyelination and neuropsin protein was observed in myelin after injury to the CNS. Myelin proteins are reportedly good substrate of proteases. To see if neuropsin degrades myelin protein, myelin protein was purified from mouse brains and incubated with recombinant neuropsin. I used gel-blue staining and western bolting analysis to examine the myelin protein degradation. I found that density of myelin basic protein (MBP) decreased by incubating with active-type recombinant neuropsin, and new small MBP fragments appeared. Those results showed neuropsin degrades myelin protein in vivo. To further examine the function of neuropsin in vivo after injury to the CNS, I used neuropsin gene knockout mice. I did histology microscopic analysis to compare optic nerves after enucleation between wildtype and knockout mice. Both light and electron microscopic analysis showed neuropsin knockout mice have more preserved myelin than that of wildtype mice. Those results showed neuropsin is involved in the demyelination after injury to the CNS.

Expression and function of neuropsin after injury to the central nervous system
(中枢神経損傷後におけるニューロプシンの発現と機能)

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Contents

Introduction

Injury to the central nervous system (CNS) causes a variety of phenomena such as neuronal and glial cell degeneration, degradation of the extracellular matrix, inflammatory responses, phagocytosis, and the subsequent demyelination and remyelination. These biochemical events occur not only in neural injury, but also in various common diseases, including Alzheimer's disease, brain ischaemia and epilepsy. The development of new therapeutic strategies to these phenomena depends on an understanding of pathology. Understanding of this pathological process is an area of intense medical interest. Proteases are essential factors in these pathological conditions. In the present study, I explored the expression and function of a serine protease, neuropsin, after experimental neural injury.

Proteases play very important roles in the complex process after injury to the CNS. Tissue plasminogen activator (tPA) and plasmin participate in the excitotoxin-induced neuronal degeneration, because mice knockout tPA or plasminogen are resistant to kainate-induced neuronal degeneration [Tsirka et al, 1995; Tsirka et al 1997]. TPA-mediated fibrinolysis protects against axonal degeneration and demyelination after neural injury [Akassouglou et al, 2000]. Matrix metalloproteases (MMPs) family are multifunctional proteases, mediated both extracellular matrix degradation and inflammatory demyelination after injury to the CNS [Romanic and Madri, 1994; Rosenberg 1995; Cuzner et al 1999].

Neuropsin is a trypsin-type serine protease whose cDNA was originally isolated

from adult mouse hippocampus. Northern and in situ hybridization analyses demonstrated that neuropsin mRNA is expressed specifically in the limbic system of the adult mouse brain. Neuropsin mRNA is expressed in the limbic areas such as the hippocampus, the amygdaloid complex, the septal areas, and has the highest concentration in pyramidal neurons of the hippocampal CA1-3 subfields [Chen et al, 1995]. Biochemical analysis of recombinant and native neuropsin suggested that it is an extracellular trypsin-type protease with a relatively narrow spectrum of substrates, and this protease was shown to hydrolyze extracellular matrix fibronectin at restricted sites [Shimizu et al, 1998]. Physiological studies indicated that neuropsin is associated with neural plasticity. The amounts of neuropsin mRNA and protein increased after plasticity events such as kindling and long-term potentiation [Chen et al, 1995; Okabe et al, 1996], and the blockade of neuropsin's function with a specific monoclonal antibody drastically induced the delay of kindling formation and reduced the amplitude of LTP. [Momota et al, 1998; Komai et al, 2000]. Studies of the ontogeny of neuropsin mRNA expression showed a widespread localization in the developmental stages and suggested that neuropsin is also involved in development [Suzuki, et al, 1995; Chen, et al, 1998]. By the study of neuropsin gene knockout mice, neuropsin is involved in hippocampal synapse formation. Mice defective in neuropsin showed severe hippocampal synaptic loss and significant functional loss [Hirata, et al.2001]. All the studies on neuropsin indicated that neuropsin is a multiple functional protein involved in neural plasticity, development, synapse formation.

The myelin sheath, which is formed as an extension of the oligodendrocytes in the

CNS, appears as tubes surrounding the axon and serves as insulation for the nerve fiber and thereby facilitates impulse conduction. One of the most common reactions of the nervous system after neural injury is the degradation of myelin. Vesicular degeneration of myelination, together with stripping of myelin by macrophages, was reported 7 days after spinal cord injury [Blight, et al 1985]. Degenerating myelin sheaths were observed at 8 days after wallerian degeneration in rat optic nerves [James et al, 1970]. Extensive demyelination begins between 4 and 7 days after ischemic injury [Olby and Blakemore, 1996]. Demyelination process after kainate-induced neuronal depletion in the CNS was reported to lead, within two weeks, to an almost completely disappearance of myelin sheaths [Dusart et al, 1992]. Proteases are involved in demyelination because it is a degradative process in which myelin proteins are broken down [Cuzner and Norton, 1996; Cuzner and Opdenakker, 1999]. Several proteases such as trypsin, plasmin and metalloproteinases have been shown to degrade myelin protein. Inuzuka et al reported that trypsin and plasmin caused extensive loss of myelin basic protein and some loss of other myelin proteins by incubating with human and rat myelin preparations [Inuzuka et al, 1984]. Cammer et al suggested that plasmin potentiates the degradation of myelin proteins for a mechanism of inflammatory demyelination [Cammer et al., 1986]. Chantry et al reported that the myelin-associated metalloproteinases could play roles in both myelinogenesis and in pathological demyelination [Chantry et al., 1989]. There are also suggestions that myelin has endogenous protease activity with the potential to degrade myelin proteins [Sato et al. 1982; Berlet et al., 1984; Chantry et al. 1989].

Neuropsin mRNA was expressed around the lesion region of the CNS after knife

cut injury to mouse spinal cord and intraperitoneal injection with kainate, and the cells which expressed neuropsin mRNA were located mainly in axon fiber bundles [Tomizawa et al. 1999]. Neuropsin, as a serine protease, was newly expressed in the early period after injury to the CNS, it is possible that neuropsin functions in the complex process after injury to the CNS, such as demyelination. An understanding of the mechanism of demyelination is important to any therapeutic approach to the CNS injury. However, it is still not clear how the demyelination occurred after neural injury. In the current study, I investigated the possible mechanism of demyelination. I produced neuronal degeneration induced by intrahippocampal kainate injection and axonal degeneration induced by enucleation on adult mice to analyze the expression and functions of neuropsin after injury to the CNS. My analysis revealed neuropsin is an important factor in the process of demyelination after neural injury.

Materials and Methods

Intrahippocampal kainate injection and enucleation

The procedure was approved by the Institutional Committee for Experimental Animals. DDY mice (eight- to thirteen-week old) were injected intraperitoneally with ethyl carbonate (1.2 g/kg of body weight) for anesthesia. They were placed in a stereotaxic apparatus and injected unilaterally with 1.5 nmol or 0.6 nmol kainate in 0.3μM phosphate buffered saline (PBS, 0.1 M, pH 7.4) and the same volume of PBS in the controlateral side. The coordinates of the injection were bregma -2.5 mm, medio-lateral 1.7 mm, and dorso-ventral 1.6 mm. Kainate was delivered over 30 seconds. After injection, the needle remained in place for another 2 min to prevent reflex of fluid. Mice were anesthetized and decapitated at different time points after injection. The brains were removed and frozen in dry ice and stored at -80°C until use. Enucleation of the left eye was gently performed on ddY, C57BL/6J, C57BL/6J neuropsin gene knockout mice (eight- to thirteen-week old) under anesthesia with ethyl carbonate. The animals were killed under anesthesia at designated times. The brains and optic nerves were removed, frozen in dry ice and stored at -80°C until use. For immunohistochemistry, some animals were perfused through the heart with 0.85% NaCl, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brains and optic nerves were removed, post-fixed in the same fixative overnight at 4°C, and then immersed in 30% sucrose, 0.1 M PB for 48 hours at 4°C and frozen at -80°C until use.

In situ hybridization

For neuropsin mRNA detection, in situ hybridization with ^{35}S-labeled and non-RI, digoxygenin (DIG)-labeled was performed using B41 plasmid covering 691-1131 of neuropsin cDNA. Frozen brain and optic nerve sections 20 μm thick were cut on a cryostat, and thaw-mounted onto glass slides coated with propyltriethoxysilane. The slides were fixed in 4% formaldehyde in 0.1M phosphate buffer for 20 min. After being washed with a 0.1 M phosphate buffer, sections were treated with 10 μg/ml protease K in 50 mM Tris / 5mM EDTA (PH 8.0) for 5 min at room temperature. Sections were postfixed in the same fixative and acetylated with acetic anhydride in 0.1M triethanolamine, then, rinsed with phosphate buffer, and dehydrated through an ascending alcohol series. The ^{35}S-labeled and non-RI, digoxygenin (DIG)-labeled probe for neuropsin mRNA (B41) was placed on the sections in hybridization buffer, which consisted of 50% deionized formamide, 0.3 M NaCl, 20mM Tris0HCl, 5mM EDTA, 10% dextran, 1×Denhardt's solution, 0.2% sarcosyl, 250 μg/ml yeast tRNA, 400 μg/ml salmon testis DNA, and 20mM dithiothreitol (PH8.0). After overnight incubation with probes at 55°C, sections were washed at 57°C (DIG) or 65°C (RI) in High Stringency solution (50% formamide, 2×SSC, and 10% 2-mercaptoethanol) for 30 min. After being washed with RNase buffer (0.5 M NaCl, 10mM Tris-HCl, and 1mM EDTA, PH 7.2) for 10 min three times, sections were then treated with 1 μg/ml RNase A in RNase buffer for 30 min at 37°C. Sections rinsed in RNase buffer for 10 min and high stringency solution for 30 min again.

Sections processed DIG-labeled probes were washed by buffer 1 (0.1 M Tris-HCl, 0.15 M NaCl) for 10 min and blocked by blocking buffer (1% blocking reagent in buffer

1), and then incubated with 1: 800 alkaline phosphatase-anti-digoxygenin antibody (Boeringer) in blocking buffer for overnight. After being washed in 0.1% Tween20, buffer1 for 15 min four times, sections were blocked with buffer 3 (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$) for 10 min and reacted with visualization solution, which consists of nitroblue tetrazolium (NBT) and chromogens, 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in buffer 3. The visualization usual took 3 days at room temperature. Sections processed by ^{35}S-labeled probe were dehydrated though an ascending alcohol series, and dried in air. The slides were dipped in Kodak NTB-2 emulsion (KODAK). The slides were developed after exposure for 3 weeks at 4°C. Once developed, the sections were quickly dehydrated and mounted in Entellan (Merck). They were counter-stained with thionin.

Double in situ hybridization was performed with the RNA probes for neuropsin mRNA (^{35}S-labeled) and PLP mRNA (digoxygenin-labeled). Except that the sections were hybridized with the two probes simultaneously, the protocol of hybridization was the same as the method of in situ hybridization with DIG-labeled probe. After hybridization, the sections were incubated with alkaline phosphatase-anti-digoxygenin antibody (Boeringer) overnight at 4°C and visualized with NBT and BCIP. After being air-dried, the sections were exposed to the emulsion NTB-2 (KODAK) for 3 weeks.

The number of neuropsin mRNA-positive cells was counted in a 0.35 mm^2 measuring frame for the forebrain sections (Fig.4F). The area for counting was selected as adjacent to the needle track of kainate injection and included deep layers of the cerebral cortex, the corpus callosum, the alveus and the stratum oriens of the CA1

subfield of the hippocampus.

Immunohistochemistry

Twenty-micrometer thick sections of the fixed brains and optic nerves were cut on a cryostat. The free floating sections were immersed in 0.1 M PBS containing 1% Triton X-100 and 0.3% H_2O_2 for 30 min at room temperature, and were blocked with 0.1 M PBS containing 5% bovine serum albumin (BSA) for 60 minutes at room temperature. The sections were incubated overnight at 4°C with or without anti-neuropsin 20μm/ml monoclonal antibody, B5. The antibody was extensively characterized in the previous studies [Inoue et al, 1998; Momota et al., 1998; Shimizu et al., 1998]. After being washed with 0.1 M PBS, the sections were incubated with biotinylated rabbit anti-rat IgG (Dako) diluted 1:1000 in 5% BSA in 0.1 M PBS overnight at 4°C. After being washed with 0.1 M PBS, the sections were incubated with streptoavidine-horse radish peroxidase (Vector) for 2 hours at room temperature. After being washed with 0.1 M PBS, the sections were treated with 0.5 mg/ml diaminobenzidine (DAB), 0.03% H_2O_2 and 0.4% ammonium nickel sulfate hexahydrate in 50 mM Tris-HCl (pH 7.4) for 2 minutes. For immunoelectron microscopy, the sections with a positive reaction were postfixed in 1% osmium tetroxide for 60 min, dehydrated and embedded in epoxy resin. Ultrathin sections 70nm thick were cut on an ultramicrotome and observed under a H7100 electron microscope (Hitachi).

Proteolysis of myelin protein by neuropsin

Myelin was purified from the brains of 8-week-old male mice according to the method of Norton and Poduslo (Norton and Poduslo, 1973). The myelin-enriched pellet was dispersed in 10mM Tris-HCl (pH 7.0) and sonicated. The protein concentration was determined with a BCA Protein Assay kit (Pierce, Rockford, IL) and adjusted to 1 μg/μl. The solubilized myelin was incubated with various doses of pro- or active-type recombinant neuropsin for 2 to 8 hours. The recombinant neuropsin has been characterized extensively (Shimizu et al., 1998). Twenty micrograms of the treated or non-treated myelin protein was loaded on 15% polyacrylamide gels for electrophoresis. After electrophoresis, GelCode Blue Stain Reagent (Pierce) to examine the changes of myelin proteins stained one of the two equally sample-loaded gels. The other gel was transferred to a nitrocellulose membrane and processed by western blotting for myelin basic protein. The membrane was blocked with 5% skim milk in 50mM Tris-Cl, pH 7.5, 150mM NaCl, and 0.1% Tween-20 (TBS-T) for 2 hours at room temperature, then incubated with anti-MBP antibody (DAKO) diluted 1:2000 in 5% skim milk in TBS-T at 4°C for 12 hours. After being washed with TBS-T, the blot was incubated with alkaline phosphatase-goat anti-rabbit IgG (BioRad) for 2 hours at room temperature. After a further wash, the blot was incubated with chemiluminescence substrate (Immun-Star, BioRad) and exposed to an X-ray film. The density of the MBP-positive bands of 14 kD to 21.5 kD was measured with a densitometer (ATTO, JAPAN) (n=3).

Histology microscopic analysis

Mice were perfused through the heart with 0.85% NaCl, followed by 2% PFA and

2.5% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The optic nerves were removed, and stored in the same fixative at 4°C until use. Optic nerves were postfixed in 1% osmium tetroxide for 60 min, dehydrated in an ascending series of ethanol and embedded in epoxy resin. Semi-thin sections (1000nm thick) and ultra-thin sections (100nm thick) were cut on an ultra-microtome. Semi-thin sections were stained with 1% heated (70°C) toluidine blue (4 min) and observed in light microscope. Ultra-thin sections were counter-stained with uranyl-acetate (15 min) and lead-citrate (10 min), and examined under a H7100 electron microscope (Hitachi).

All the ultra-thin sections were obtained from the optic nerve within 1mm near the enucleated site. Fibers (axon and myelin) in optic nerves, based on their maximal diameters and pathologic condition, were categorized as small (<1.0 μm), medium (1.0-1.5 μm), large A or large B (> 1.5 μm). Fibers in large A have normal myelin sheaths. Fibers categorized into large B were either fused sheaths composed of more than one myelin sheathes, sheaths which were separated from axons, or sheaths in markedly pathological state. [Griffiths et al, 1983; Ludwin et al, 1992; Rosenberg et al, 1999]. Electron microscopic pictures were taken in 2K and 5K magnification under a H7100 electron microscope. Fibers were categorized and counted in morphometic grids (20 μm × 15 μm) using electron microscopic pictures.

Results

Neuropsin mRNA was expressed in oligodendrocytes after intrahippocampal injection with kainate.

Two days after intrahippocampal injection with 1.5 nmol kainate, pyramidal cells in CA1 and CA3 degenerated, where there was no neuronal degeneration occurred in the PBS-control side (Fig.1). The examination of time course of neuronal degeneration after intrahippocampal injection with kainate, just as reported previously [Andersson et al., 1991], pyramidal cells began to degenerate 6 hours, completely degenerated in 2 days and then disappeared and were replaced by glial cells [Fawcett and Asher, 1999] (Fig.2). Neuropsin mRNA was detected by in situ hybridization 4 days after intrahippocampal injection with 1.5 nmol kainate. The expression of neuropsin mRNA in pyramidal cells of CA1 and CA3 decreased significantly, however neuropsin mRNA was newly expressed in the kainate injected hippocampal areas, for example, corpus callosum, alveus, fimbria (Fig.3 A and C). Neuropsin mRNA was not expressed on the PBS-injected control side (Fig.3 B and D). We next examined the time course of neuropsin mRNA expression after intrahippocampal injection with kainate. Neuropsin mRNA began to appear 1 day after the kainate injection and disappeared at 14 days, the peak expression was from 4 to 8 days after the injection (Fig.4). To examine accurately which cells express neuropsin mRNA, we performed double in situ hybridization (RI for neuropsin and alkaline phosphate for PLP) on mice after intrahippocampal injection with kainate. Silver grains (neuropsin mRNA) were observed in the cells stained with

alkaline phosphatase (PLP mRNA) (Fig. 5). Most of the cells (77.9±1.4%, n=5) expressing neuropsin mRNA were positive for PLP mRNA, a marker of mature oligodendrocytes. This result of double in situ hybridization indicated neuropsin mRNA was induced in oligodendrocytes after intrahippocampal kainate injection. There was no marked difference in the number of PLP mRNA-expressing cells between kainate-injected and control side (data not shown).

Expression of neuropsin mRNA was associated with neuronal degeneration.

Either through neuronal degeneration or excitotoxity to oligodendrocytes, the expression of neuropsin mRNA could be induced in oligodendrocytes. To examine the pathway of neuropsin mRNA induction, I decreased the injected kainate dose from 1.5 nmol to 0.6 nmol. Pyramidal cells in CA3 were partly degenerated after intrahippocampal injection with 0.6nmol kainate. Neuropsin mRNA was found to express only in the area around the degenerated neurons (Fig.6). This result indicates expression of neuropsin mRNA was associated with degenerative neurons and was induced following neuronal degeneration.

Neuropsin mRNA was also expressed in oligodendrocytes after axonal degeneration.

To determine if axonal degeneration induces neuropsin expression, unilateral enucleation was performed on adult mice. By in situ hybridization, we found that cells in the optic nerve and optic chiasm from the operated side expressed neuropsin mRNA, and neuropsin mRNA began to be expressed as early as 12 hours after enucleation (Fig.

7A). The peak of the expression was at 4 days after the operation. There was no neuropsin mRNA expression in the contra-lateral optic nerve or sham operated mice. Double in situ hybridization revealed that most of the neuropsin mRNA-positive cells were also positive for PLP mRNA as was observed after kainate injection to the hippocampus (74.6±5.9%, n=5, data not shown). This result indicates that expression of neuropsin mRNA in oligodendrocytes was induced not only following neuronal degeneration, but also following axonal degeneration.

Neuropsin immunoreactivity was expressed in oligodendrocytes and myelins.

We examined the localization of neuropsin protein after neuronal and axonal degeneration using immunohistochemistry. After kainate injection, neuropsin immunoreactivity was shown in the corpus callosum and alveus of the kainate injected sides (Fig.8A). The expression pattern of neuropsin protein was similar to that of neuropsin mRNA. With closer observation, immunoreactive cell processes were also found (arrowheads) as well as cell bodies (arrows) (Fig. 8C and D). There was no immunostaining in the PBS contra-lateral side (Fig. 8B). No immunoreactivity was observed in sections incubated without primary antibody (data not shown). After enucleation, neuropsin immunoreactivity in the optic nerve of the enucleated side was also observed (Fig. 9A), whereas no immunoreactivity was observed in the optic nerve of contralateral side (Fig. 9B). The immunoreactivity was observed in cell bodies (diameter about 5 to 10 μm, arrows) and in the cell processes (arrowheads). (Fig.9C). The localization of immunoreactive cell bodies was very similar to that of the mRNA.

Immunoelectron microscopic analysis was performed after enucleation. Since a high concentration of detergent (1% Triton X-100) was necessary to obtain immunoreactivity, the fine structure was not well preserved. Nevertheless, dark immunoreactive products were observed in the cytoplasm and myelin (Fig. 9D). The neuropsin-immunoreactive cells had typical clumped chromatin characteristic of oligodendrocytes. This result was consistent with neuropsin mRNA being expressed in PLP mRNA-positive cells.

Recombinant neuropsin degraded myelin protein in vitro.

To examine if proteins in myelin are possible substrates for neuropsin, we incubated myelin protein with recombinant neuropsin. GelCode Blue staining revealed that recombinant neuropsin at 1/100 of myelin protein degraded proteins corresponding to MBP (Fig. 10A). Western blot analysis confirmed that MBP was degraded by recombinant neuropsin and MBP-immunoreactive small fragments appeared (arrows in Fig. 10B, lane 4-6). Fig. 10C shows the result of quantitative analysis of the degradation of MBP. About 34% of myelin basic protein were degraded over 8 hours incubation (Fig. 10.C). Eight-hour incubation without r-neuropsin resulted in a slight decrease in MBP probably due to endogenous proteolysis activity in the myelin (Fig. 10B, lane 2) (Sato et al., 1982).

Neuropsin knockout mice showed more preserved myelin than that of wild-type mice after enucleation.

To further examine the function of neuropsin in vivo after injury to the CNS, we used neuropsin gene knockout mice. We did both light and electron microscopic analysis to compare optic nerves after enucleation between wildtype and knockout mice. Semi-thin sections of optic nerves stained with toluidine blue showed that knockout mice had more preserved myelin in optic nerves than wildtype mice 8 days after enucleation under light microscopy (Fig. 11). Under electron microscopic analysis, we found knockout mice had more preserved myelin in optic nerves after enucleation than knockout mice (Fig. 12). Table.1 showed the quantitative analysis of the different fibers between wildtype and knockout mice. The result demonstrated that neuropsin gene knockout mice have more small fibers than of wild-type mice (P=0.0088), and the number of large pathologic fibers in knockout mice decreased significantly than that of wild-type mice (P=0.0398). These results indicate neuropsin gene knockout has protective effect in the demyelination after injury to the CNS.

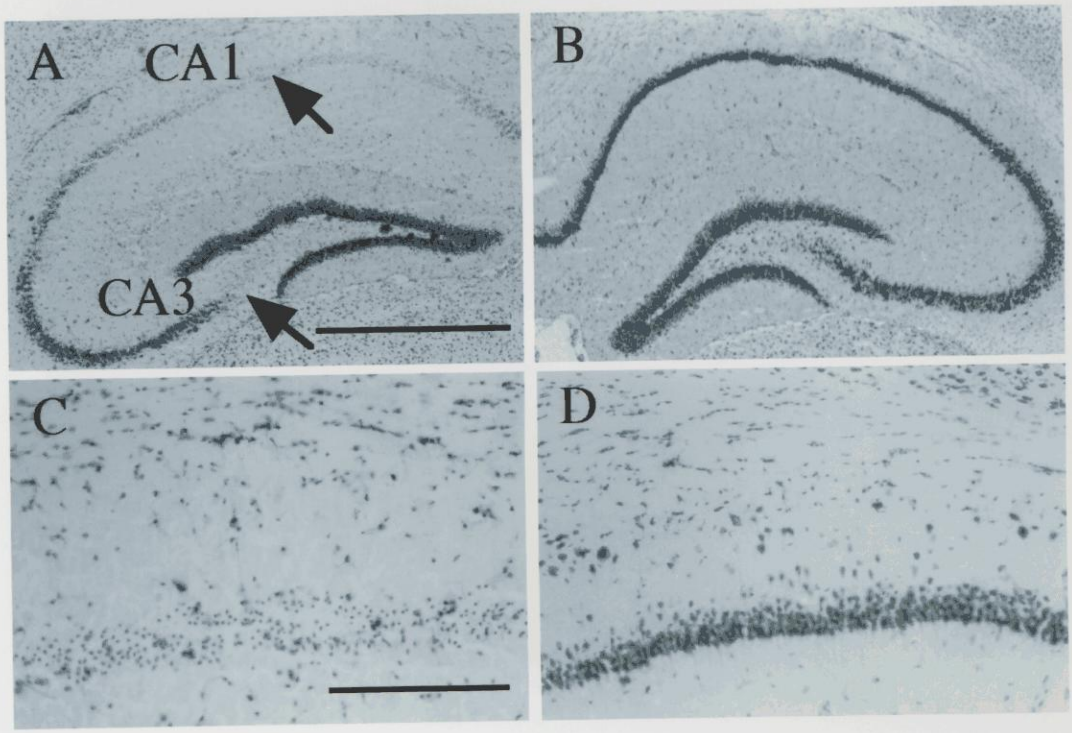

Fig.1 Neuronal degeneration was induced by intrahippocampal injection with kainate. After intrahippocampal injection with 1.5 nmol kainate, coronal brain sections were processed for Nissl staining. A and C, Ipsilateral side 2 days after kainate injection; B and D, Contralateral side 2 days after kainate injection. Higher magnification of CA1 region in A and B are shown in C and D. Neuronal degeneration (arrows) was only observed in the pyramidal cells of CA1 and CA3 of kainate injected side. Scale bars, 0.6 mm in A and B, 0.2 mm in C and D.

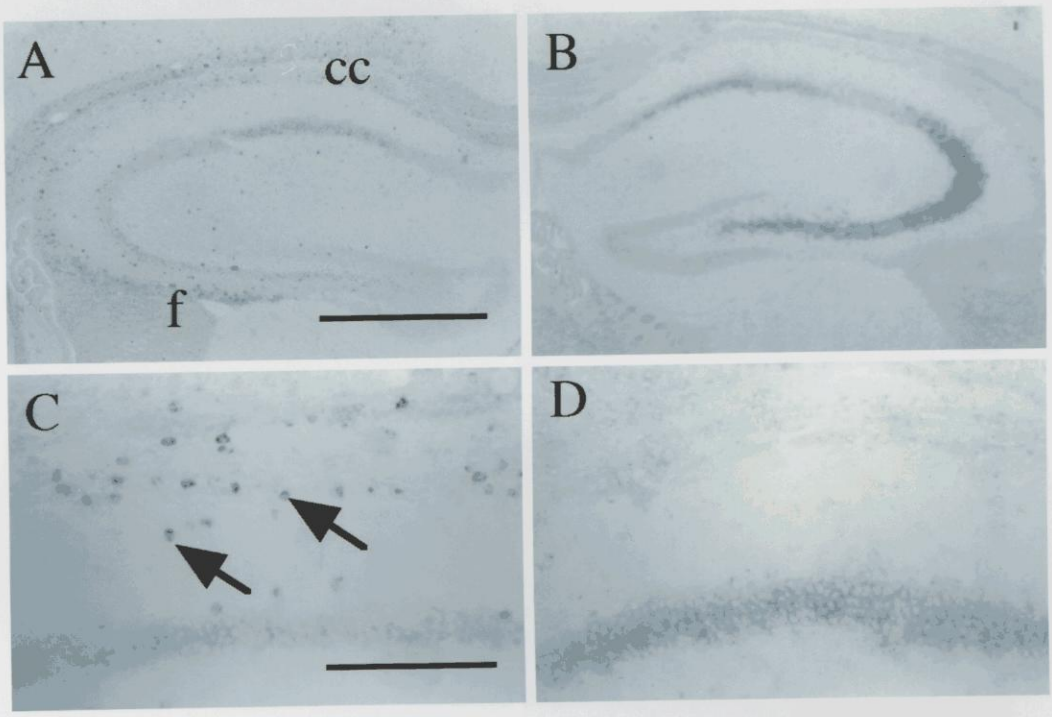

Fig.2 Expression of neuropsin mRNA was induced 4 days after intrahippocampal injection with kainate. After intrahippocampal injection with 1.5 nmol kainate, in situ hybridization was performed on coronal brain sections. A and C, Ipsilateral side 4 days after kainate injection. B and D, Contralateral side 4 days after kainate injection. Higher magnification of CA1 region in A and B are shown in C and D. The expression of neuropsin mRNA of pyramidal cells in CA1 and CA3 of hippocampus decreased significantly, however neuropsin mRNA was expressed around the degenerated region, such as the corpus callosum (cc), fimbria (f). Arrows marked the cells which induced neuropsin mRNA. Scale bars: 0.6 mm in A and B; 0.2 mm in C and D.

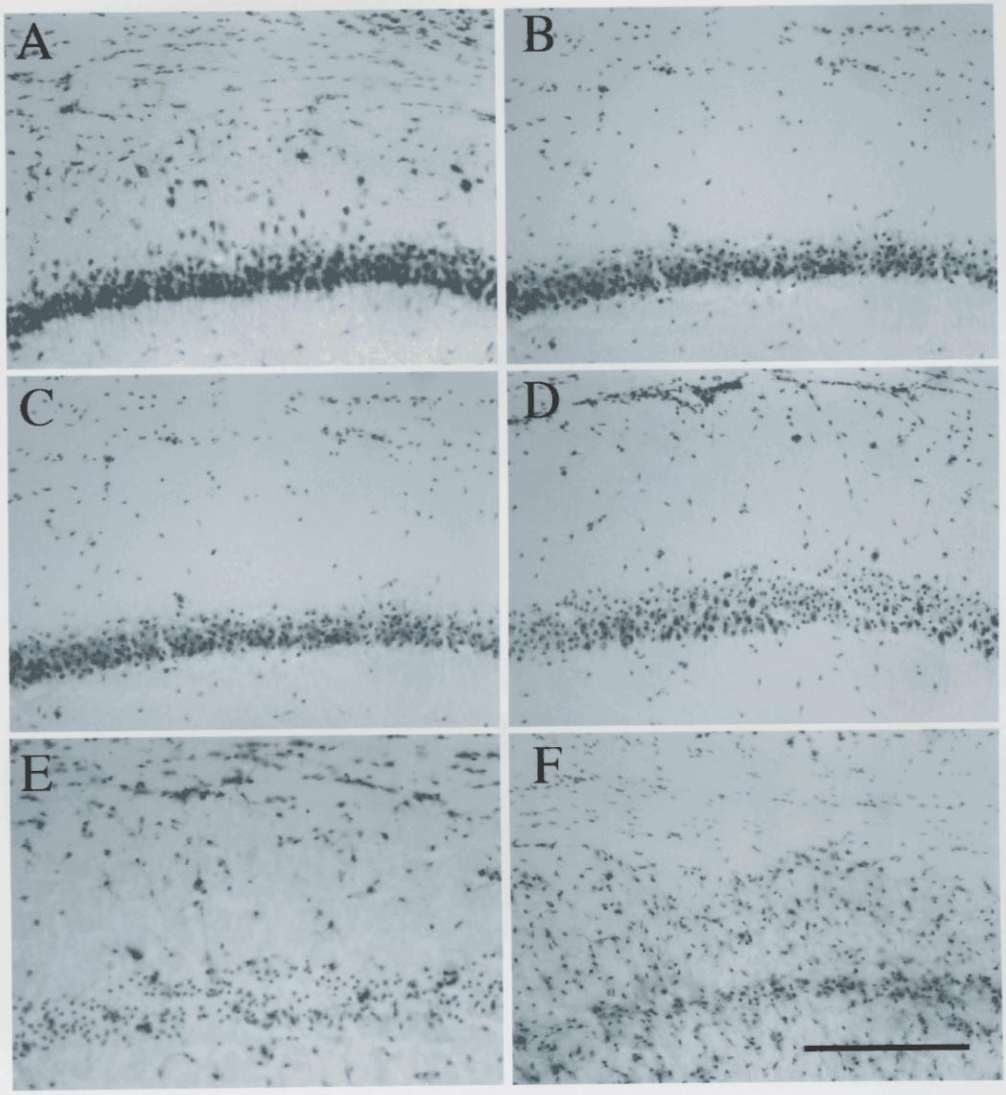

Fig.3 Time course of neuronal degeneration of CA1 after intrahippocampal injection with kainate. After intrahippocampal injection with 1.5 nmol kainate, coronal brain sections were processed for Nissl staining. A: PBS-contralateral side 2 days after kainate injection. Kainate-injected sides respectively 6 hours (B), 12 hours (C), 1 day (D), 2 days (E) and 14 days (F). Pyramidal cells began to degenerate at 6 hours, completely degenerated at 2 days, and gliosis appeared at 14 days. Scale bar: 0.2 mm.

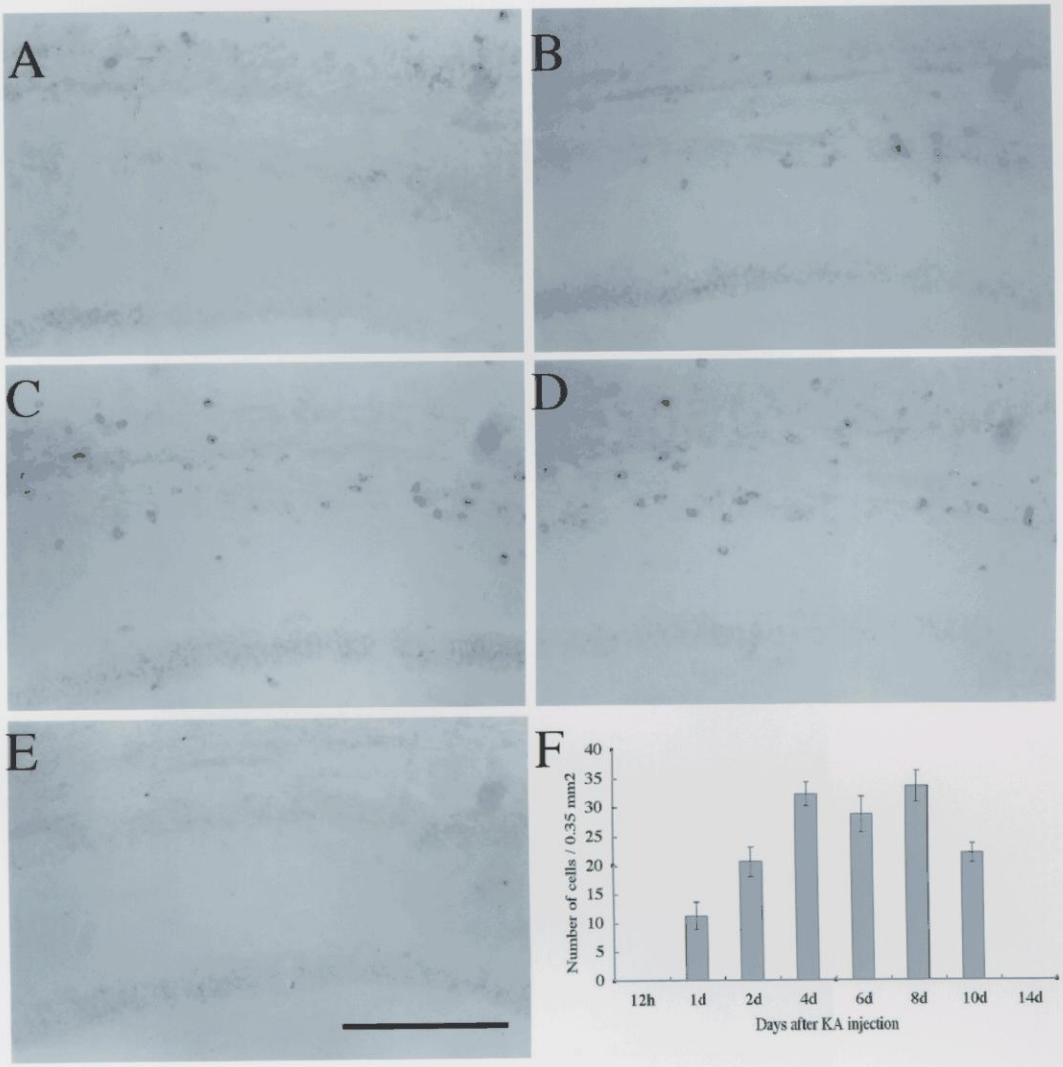

Fig.4 The time course of expression of neuropsin mRNA in CA1 area after intrahippocampal injection with kainate. After intrahippocampal injection with 1.5 nmol kainate, in situ hybridization was performed on coronal brain sections. Neuropsin mRNA expression at 1 day (A), 2 days (B), 4 days (C), 8 days (D) and 14 days (E) is shown. Expression of neuropsin began to appear at 1 day, peaked during 4 days to 8 days, disappeared at 14 days after kainate injection. Quantitative analysis of the number of cells expressing neuropsin mRNA was shown in (F). The cells in a 0.35 mm^2 measuring frame adjacent to the needle track of kainate injection were enumerated (±SD). Scale bars, 0.2 mm.

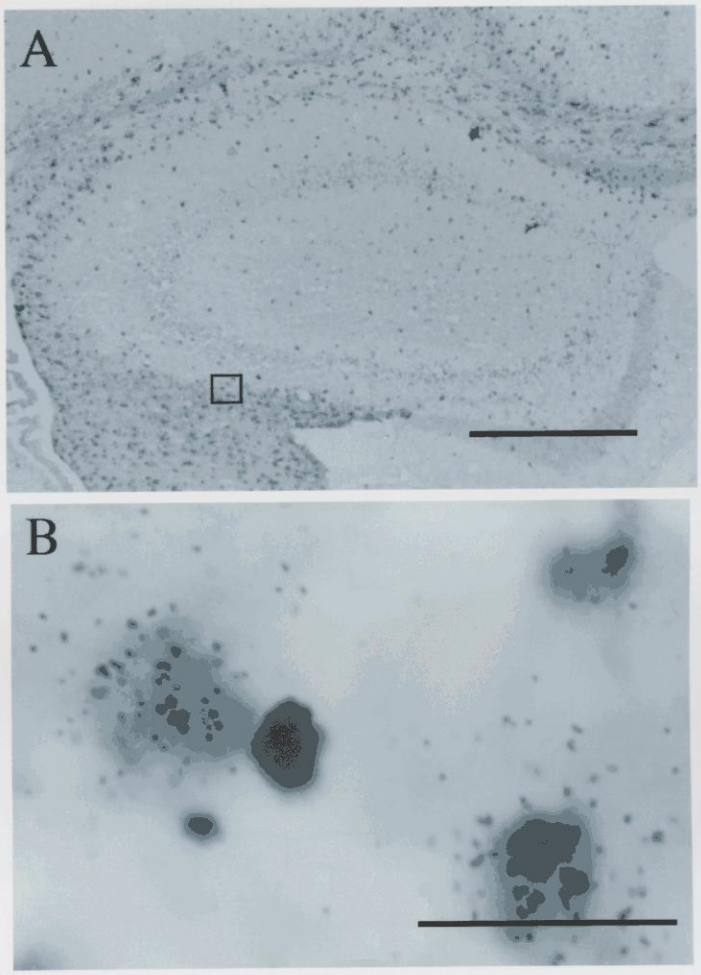

Fig.5 Neuropsin mRNA is expressed in mature oligodendrocytes. Double in situ hybridization (RI for neuropsin and alkaline phosphatase for PLP) was performed on the sections 4 days after kainate injection. The signal of silver grains (neuropsin mRNA) were observed in the cells which are stained with alkaline phosphatase (PLP mRNA). The cells which expressed neuropsin mRNA were positive for oligodendrocyte marker, PLP. Higher magnification of the boxed area in A is shown in B. Scale bars: 0.6 mm in A and 0.01mm in B.

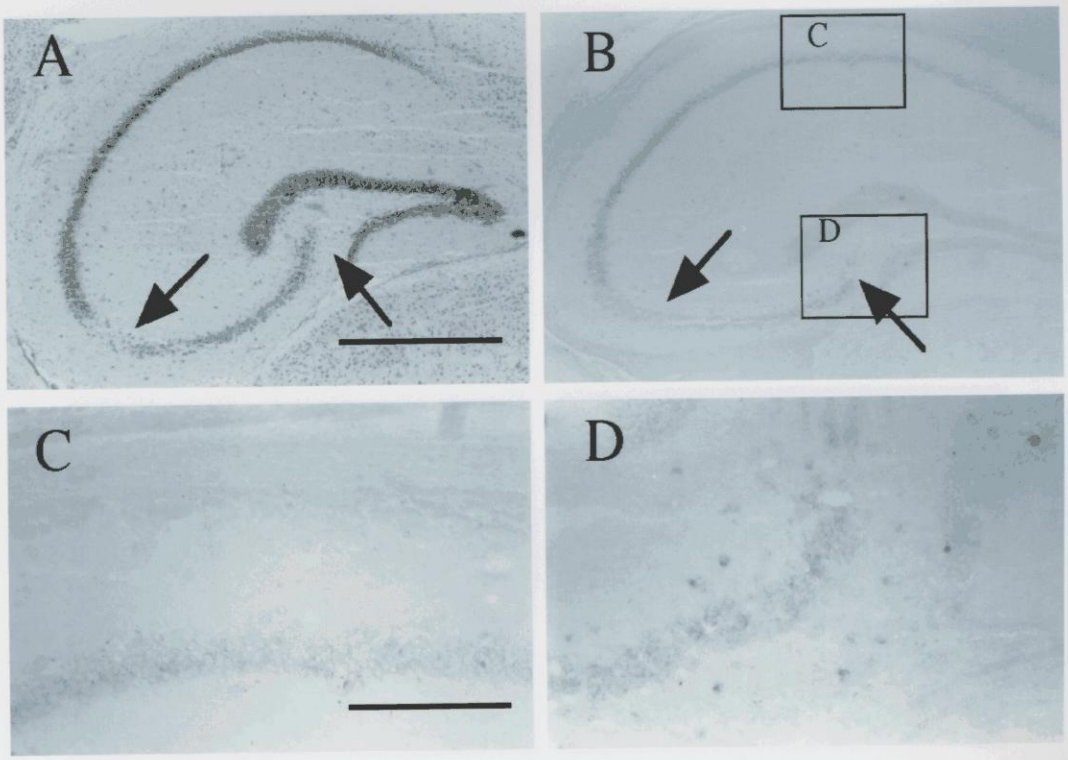

Fig.6, Expression of neuropsin mRNA was associated with degenerative neurons. Changes 1 day after kainate injection (0.6nmol) were shown by Nissl staining (A) and in situ hybridization for neuropsin mRNA (B). Higher magnification of boxed areas in (B) is shown in (C) and (D). Neuropsin mRNA was induced only in the areas which was close to degenerated neurons (arrows) (D), was not induced in the area where has not degenerated neurons (C). Scale bars: 0.6 mm in A, B and 0.2 mm in C, D.

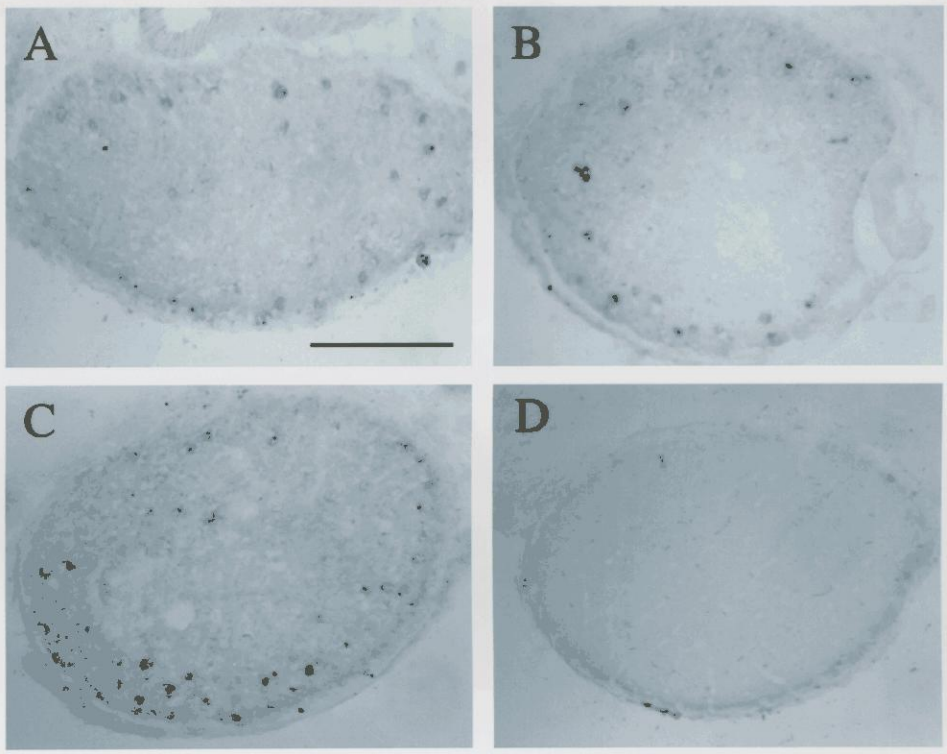

Fig. 7 Neuropsin mRNA was expressed in optic nerves after axonal degeneration. After enucleation, neuropsin mRNA was detected in optic nerves at different time points by in situ hybridization. Neuropsin mRNA was expressed at 12 hours (A), 2 days (B) and 4 days (C) after enucleation. Neuropsin mRNA was not expressed at 2 days after sham enucleation (D). Scale bar: 0.2 mm.

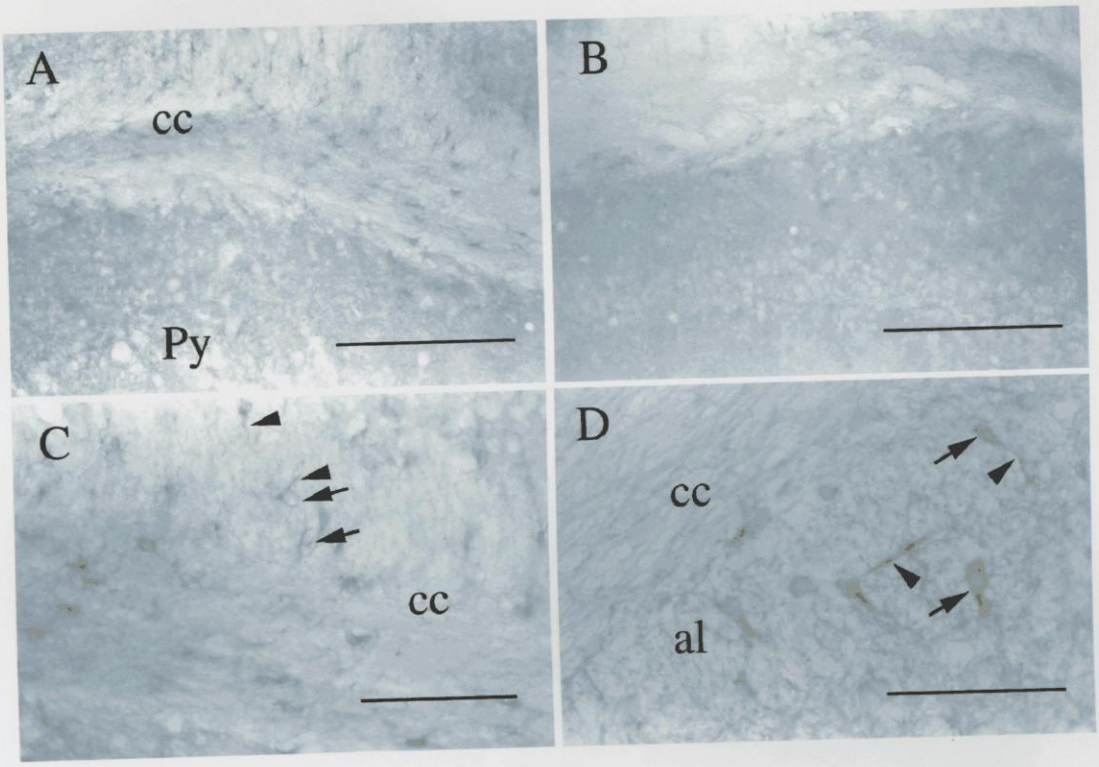

Fig.8 Neuropsin immunoreactivity after intrahippocampal injection with kainate. After intrahippocampal injection with 1.5 nmol kainate, immunohistochemistry was performed on mouse coronal sections using monoclonal anti-neuropsin, B5. A, Neuropsin immunoreactivity was observed in the CA1 subfield of the hippocampus on the kainate injected side. B, On the contralateral side, no immunoreactivity was observed. C and D, Neuropsin immunoreactive cells (arrows) and processes (arrowheads) were observed in the corpus callosum (C) and alveus (D) of CA1 subfield of the kainate-injected side. Abbreviations: corpus callosum (cc), alveus (al), pyramidal cells (Py). Scale bars, 0.2 mm in A and B, 0.1 mm in C, 0.04mm in D.

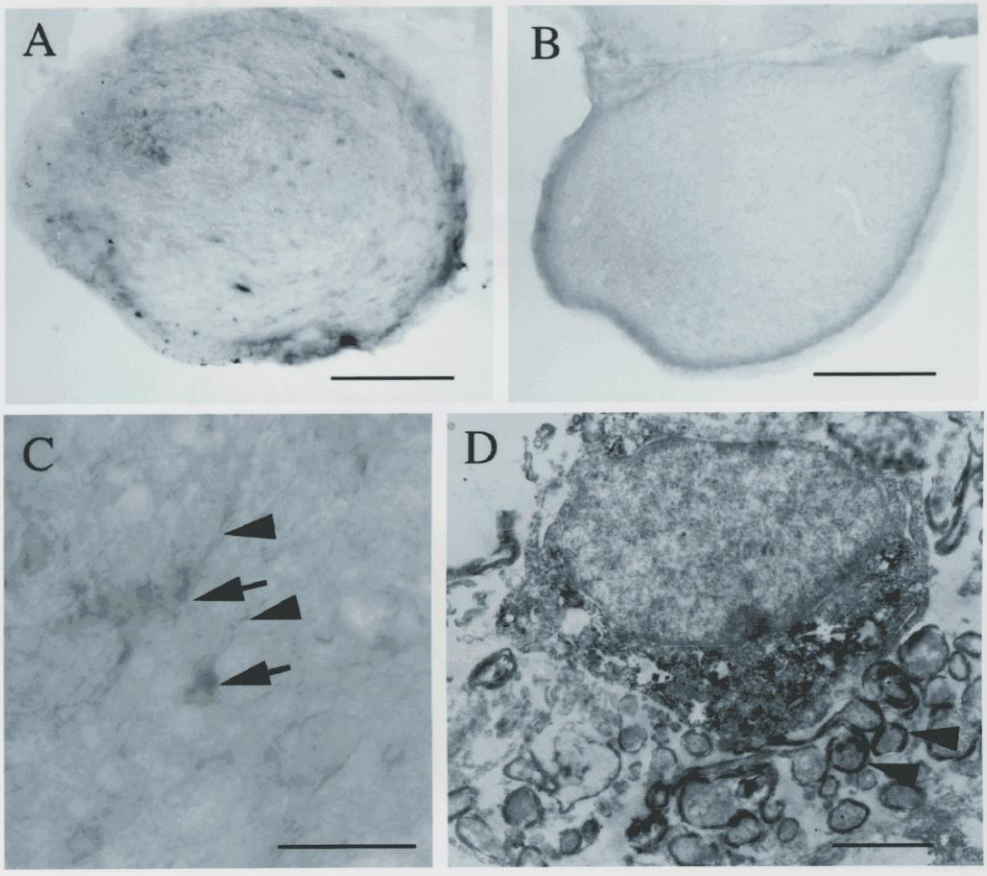

Fig.9 Neuropsin immunoreactivity in the optic nerve after enucleation. Immunohistochemistry was performed on mouse sections using monoclonal anti-neuropsin, B5. A, Neuropsin immunoreactivity was observed in ipsilateral optic nerve 4 days after enucleation. B, Contralateral optic nerve did not show immunoreactivity. Observation at higher magnification (C) revealed immunoreactivity in the cell body (arrows) and processes (arrowheads). The neuropsin immunoreactivity in the cytoplasm of oligodendrocytes and myelin (arrowheads) was detected by immunoelectron microscopy (D). Scale bars: 0.2 mm in A and B, 25 μm in C and 2 μm in D.

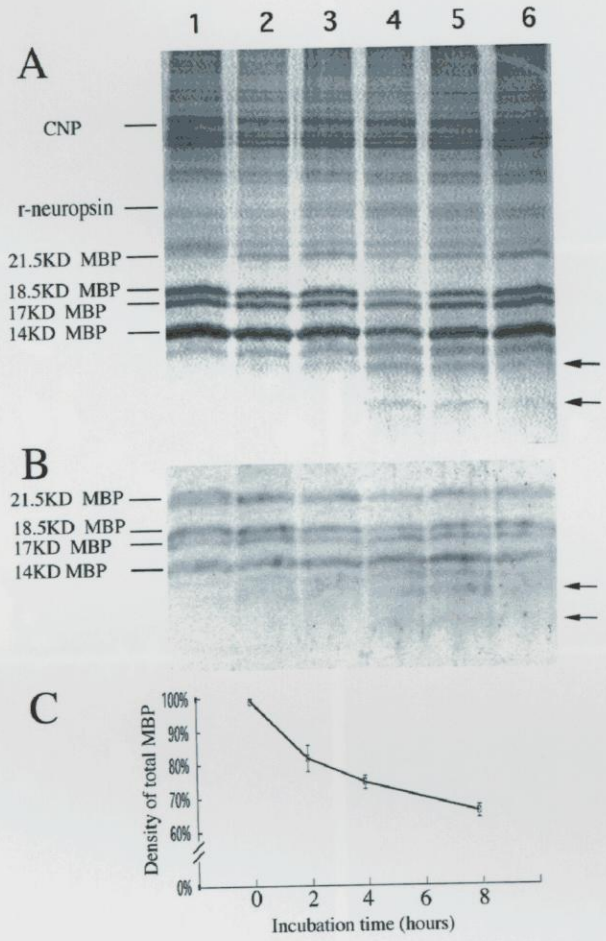

Fig.10 Neuropsin degrades myelin protein. Lane 1, freshly prepared myelin protein; lane 2, myelin protein incubated at 37°C for 8 hours in 10 mM Tris-HCl (pH 7.0); lane 3, myelin proteins incubated at 37°C with 1:100 pro-neuropsin (inactive form) for 8 hours; lane 4, 5 and 6, myelin proteins incubated at 37°C with 1:100 active neuropsin for 8, 4 and 2 hours, respectively. One gel was stained with GelCode Blue Stain (A) and the other for western blotting with anti-MBP antibody (B). CNPase (CNP), recombinant neuropsin (r-neuropsin) and 4 isoforms of MBP are shown. MBP was degraded by active r-NP and new fragments were observed (arrows). C, Change of density of the MBP-positive bands during incubation with r-neuropsin.

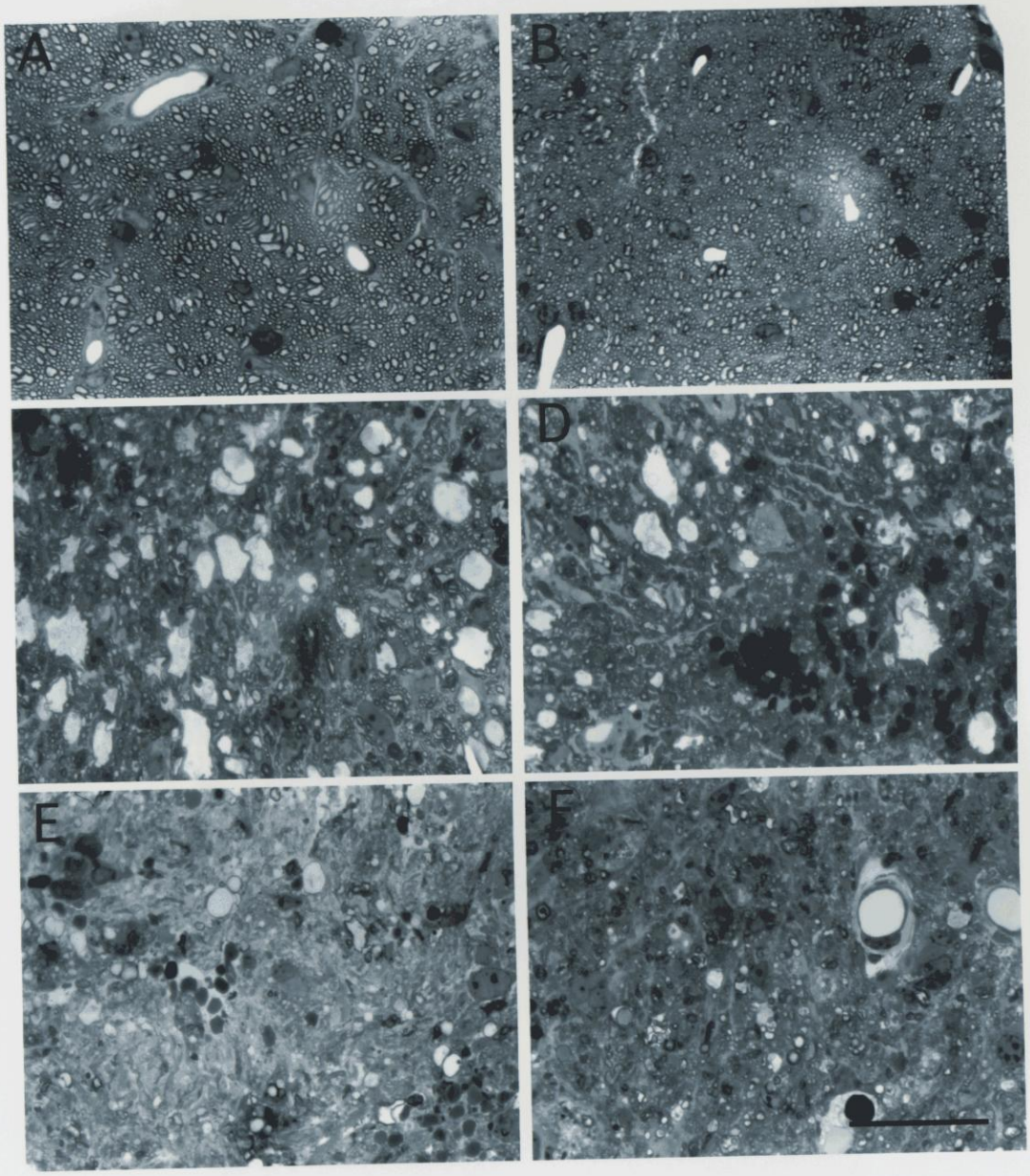

Fig.11 Neuropsin gene-knockout mice showed more preserved myelin than wild-type mice in optic nerves after enucleation. Semi-thin sections of optic nerves were stained with heated toluidine blue. Optic nerves on control side from wildtype (A) and knockout mouse (B). Optic nerves on enucleated side 4 days after enucleationof from wildtype (C) and knockout mouse (D). Optic nerves on enucleated side 8 days after enucleationof from wildtype (E) and knockout mouse (F). Neuropsin gene-knockout mice showed more preserved myelin 8 days after enucleation than wild-type mice. Scale bar: 0.04 mm.

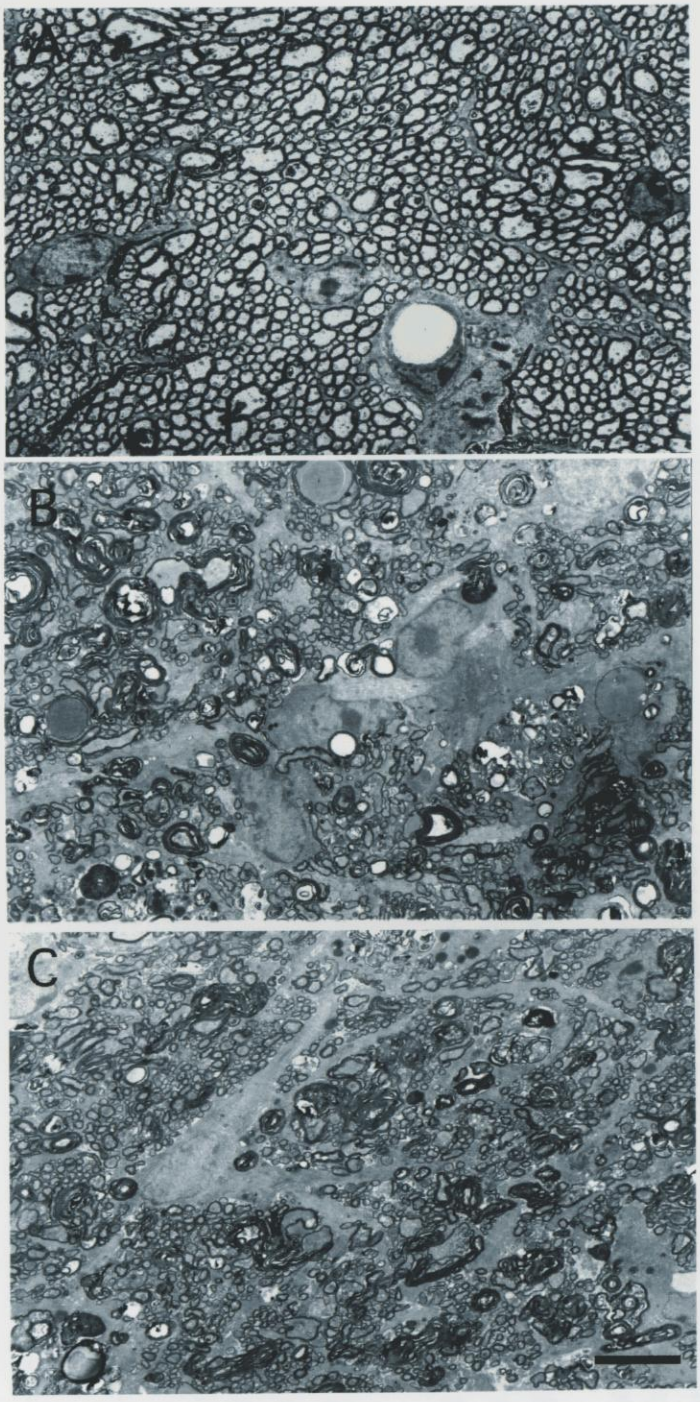

Fig.12 Electron microscopic analysise in optic nerves of wildtype and knockout mice 8 days after enucleation. A, optic nerve on control side. B, optic nerve on enucleated side after enucleation from wildtype mouse. C, optic nerve on enucleated after enucleation from knockout mouse. Neuropsin gene-knockout mice showed more preserved myelin in optic nerves 8 days after enucleation than wild-type mice under electron microscopic analysis. Scale bar: 5 μm.

Table.1: Counts of fibres in optic nerves between neuropsin wildtype and knockout mice.

Fibres	Normal (n= 3)	ENP8d(+/+) (n=3)	ENP8d(-/-) (n= 4)
total	140±10	96±22	126±13
Large.A (>1.5 μm)	14±3	5±1	5±1
Large.B (>1.5 μm)	1±1	10±2*	8±1*
Medium (1.0~15 μm)	33±6	17±6	17±5
Small (<1.0 μm)	92±6	64±14**	97±8**

Large.A (> 1.5 μm) : Fibre > 1.5 μm. Fibre has normal myelin sheath.

Large.B (> 1.5 μm) : Fibre > 1.5 μm. 1. Several fibres are fused into one fibre. 2. Myelin is separated from axon. 3. Myelin is in a markedly pathological state.

Value represent the mean ±SE number of fibres of 300 μm2 area of optic nerves from 10 measuring frames per treatment mouse.

* Value is significantly different between neuropsin wildtype and knockout mice.(P<0.05)

** Value is significantly different between neuropsin wildtype and knockout mice.(P <0.01)

Discussion

The results of the present study clearly demonstrate that neuropsin mRNA and protein were expressed after injury to the CNS and the expression was within two weeks after injury, which indicates that neuropsin might be involved in the acute response of the CNS following injury. There is a complex process in the acute response after injury to the CNS. One of the most common reactions of the nervous system after injury is demyelination. Demyelination process after kainate-induced neuronal depletion in the CNS was reported to lead, within two weeks, to an almost completely demyelination [Dusart et al, 1992]. The mechanism of demyelination is still unknown [Cuzner et al., 1996], partly because endogenous demyelination-specific molecules such as proteases within oligodendrocytes have not been identified. In my present studies: (1) Neuropsin mRNA and protein were expressed in oligodendrocytes following injury to the CNS, though there was no constitutive expression in oligodendrocytes. (2) Recombinant neuropsin degraded myelin basic protein of mouse brain in vitro. (3) Neuropsin gene knockout mice had more preserved myelin in optic nerves than that of wildtype mice after axonal degeneration. These results indicate that neuropsin is an important factor in the demyelination after injury to the CNS.

The expression of neuropsin mRNA after intrahippocampal injection with kainate began to appear at 1 day. The expression peaked at 4-8 days and disappeared at 14 days (Fig.4). This time course matches that for the demyelination observed after kainate injection (Dusart et al., 1992). Furthermore, double in situ hybridization for neuropsin

and PLP mRNA confirmed that most of the cells expressing neuropsin mRNA were positive for PLP. This result indicates that the mature myelinating oligodendrocytes expressed neuropsin mRNA after injury to the CNS (Fig.5). By immunohistochemical staining and immunoelectron microscopic analysis, neuropsin was found localized on oligodendrocytes and myelins (Fig.9), it further suggested neuropsin might be involved in the response of myelin after injury to the CNS.

Many studies showed that the proteins in myelin are good substrate of proteases [Marks et al., 1976; Whitaker and Seyer, 1979; Berlet et al, 1984; Inuzuka et al, 1984; Cammer et al., 1986]. Myelin protein is degraded by proteases and demyelination is completed when myelin and/or axons therein are phagocytosed by macrophage or microglia [Cuzner and Norton, 1996; James et al, 1970]. Myelin sheaths in the CNS are characteristic of laminated structures, these sheaths are composed of major dense and intraperiod lines [Peter et al., 1991]. Myelin basic protein is a main component of major dense lines and its function is to make myelin lamellae compact by fusing the cytoplasmic surfaces of oligodendrocyte plasma membrane. My in vitro biochemical assay demonstrated that recombinant active neuropsin degraded MBP and new small MBP immunoreactive fragments appeared (Fig. 10). This activity indicated that neuropsin can degrade myelin in vitro.

To examine if neuropsin is involved in demyelination after injury to the CNS in vivo, we further compared the myelin in optic nerves after enucleation between wildtype and knockout mice. Histology microscopic analysis showed knockout mice had more preserved myelin in optic nerves after enucleation than that of wildtype mice

(Fig. 11, 12) (Table.1). The in vivo analysis indicated that neuropsin is involved in demyelination after injury to the CNS.

In addition, the best substrate of neuropsin in vivo is the cell adhesion molecule, L1 (Ninomiya, in preparation). In developing sciatic nerve, L1 immunoreactivity was reportedly expressed both in axon and Schwann cell process, whereas the axolemma was always L1-negative at the nodes of Ranver; L1 was deduced involved in fasciculation, initial axon-Schwann cell interaction, and onset of myelination [Rudolf et al, 1986]. In adult rat pyramidal tract, L1 immunoreactivity was reportedly negative in compact myelin, but, interestingly, L1-IR was found localized in between the inner oligodendrocytic mesaxon and compact myelin. L1 was suggested to be important in adult rat pyramidal tract, not only with respect to the adhesion between unmyelinated pyramidal tract axons but also during the stabilization of the axon-oligodendrocyte interaction [Joosten, 1991]. These documents suggest that neuropsin might function in the demyelination process after injury to the CNS, both with breaking myelin lamellae structure by degrading myelin basic protein, and with breaking interaction between axon and myelin by degrading L1.

There were two possible pathways for intrahippocampal injection with kainate induces neuropsin expression in oligodendrocytes. (1) After intrahippocampal injection with kainate, at first, neurons occur degeneration, then following axonal degeneration, finally the oligodendrocytes which contacted with degenerated axons express neuropsin. (2) Neuropsin was expressed in oligodendrocytes directly by the kainate excitotoxity to kainate-receptor in oligodendrocytes. We changed the dose of injected kainate from 1.5

nmol to 0.6 nmol to induce neuronal degeneration in partly pyramidal cells. This experiment showed that the expression of neuropsin mRNA was associated with neuronal degeneration (Fig.6). We further performed enucleation on mice to produce axonal degeneration in optic nerves. The experiments of enucleation showed that neuropsin was expressed in the optic nerve after axonal degeneration in a similar time course to that seen after intrahippocampal kainate injection. These results demonstrated that neuropsin was not only expressed following neuronal degeneration, but also following axonal degeneration. This indicates that some signals from degenerating neurons or degenerating axons or loss of axonal contact induced neuropsin expression.

In conclusion, neuropsin was expressed in after injury to the CNS and was associated with degenerative neurons or degenerative axons. From both in vitro and in vivo experiments, my studies demonstrate that neuropsin is an important factor which participate in the process of demyelination after injury to the CNS. Further study is needed to elucidate the role of neuropsin in demyelinating diseases such as multiple sclerosis and experimental allergic encephalopathy. Because neuropsin is only induced in the CNS but not in PNS after neural injury, the different responses to neural injury in CNS and PNS showed that the possible roles of neuropsin in regeneration after neural injury is also an interesting study field.

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