Neuroscience Research (2001) 39(4):455-462.

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Xiao-Ping He [a], Sadao Shiosaka [a] and Shigetaka Yoshida [a,b]

[a] Division of Structural Cell Biology, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-0101 Japan

[b] Department of Anatomy and Neuroscience, Hyogo College of Medical School, 1-1 Mukogawacho, Nishinomiya, Hyogo 663-8501 Japan

Corresponding author:

Shigetaka Yoshida

Department of Anatomy and Neuroscience

Hyogo College of Medicine

1-1 Mukogawacho, Nishinomiya, Hyogo 663-8501

Japan

Phone +81 79845 6416

FAX +81 79845 6417

email syoshida@hyo-med.ac.jp

Present address: Department of Anatomy, Asahikawa Medical College

Midorigaoka-higashi 2-1-1-1 Asahikawa

Hokkaido 078-8510 Japan

19 text pages and 5 figures

Abstract

Proteases are involved in a variety of processes including demyelination after injury to the CNS. Neuropsin is a serine protease which is constitutively expressed in the neurons of the limbic system. In the present study, intrahippocampal kainate injection and enucleation were performed on adult mice. Neuropsin mRNA and protein expression was detected by in situ hybridization and immunohistochemistry. Double in situ hybridization confirmed that the mRNA expression was induced in oligodendrocytes. One day after kainate injection to the hippocampus, neuropsin mRNA was expressed, peaking 4 to 8 days postoperatively and disappearing at 14 days. Immunohistochemistry and immunoelectron microscopy revealed that neuropsin was expressed in the cell body of oligodendrocytes and myelin. To see if neuropsin degrades myelin protein, purified myelin was incubated with recombinant neuropsin. A decrease in the intensity of the bands of myelin basic protein was observed. These results indicate that neuropsin is involved in demyelination.

Keywords

Injury; demyelination; serine protease; myelin basic protein;

1. Introduction

Injury to the central nervous system causes neuronal and glial cell death, inflammatory responses and subsequent demyelination and remyelination. Extensive demyelination begins between 4 and 7 days after ischemic injury (Olby and Blakemore, 1996). An understanding of the mechanism of demyelination and remyelination is important to any therapeutic approach to the CNS insult. Proteases are involved in these processes because demyelination 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, 1984; Cammer et al., 1986; Chantry et al., 1989). There have been 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). Our previous experiments showed that stab wounds induced expression of neuropsin mRNA in the area surrounding the lesion in the CNS, and the cells which expressed the mRNA were located mainly in axon fiber bundles (Tomizawa et al. 1999). Neuropsin is a serine protease whose mRNA is constitutively expressed specifically in the neurons of the limbic system of the adult mouse brain (Chen et al., 1995; Yoshida and Shiosaka, 1999; Shiosaka and Yoshida, 2000). 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 (Okabe et al., 1996; Momota et al., 1998; Komai et al., 2000). It is possible that neuropsin plays some role in this complex process such as demyelination after injury to the CNS under pathological conditions. However, the function of this protease was not known. Therefore, the purpose of the present study was to investigate the expression of neuropsin after axonal degeneration, to clarify the localization of the protein and to examine possible substrates for neuropsin.

2. Materials and Methods

2.1 Intrahippocampal kainate injection and enucleation

The procedure was approved by the Institutional Committee for Experimental Animals. Eight- to thirteen-week old ddY mice 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 kainate and the same volume of 0.1 M phosphate buffered saline (PBS, pH 7.4) in the contralateral 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. After 12 hours to 14 days, the mice were anesthetized and decapitated. The brains were removed and frozen in dry ice and stored at -80^{o}C until use. Enucleation of the left eye was gently performed under anesthesia with ethyl

carbonate. The animals were killed under anesthesia at postoperative 12 hours to 7 days. 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, postfixed 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.

2.2 In situ hybridization

Frozen sections 20 μm thick were cut on a cryostat. In situ hybridization was performed with the probes for neuropsin and proteolipid protein (PLP) mRNA (Tomizawa et al., 1999). The method used for in situ hybridization was described in a previous paper (Yoshida et al., 1994). Double in situ hybridization was performed with the RNA probes for neuropsin (^{35}S-labeled) and PLP mRNA (digoxygenin-labeled). The sections were hybridized with the two probes simultaneously, washed stringently, incubated with alkaline phosphatase-anti-digoxygenin antibody (Boeringer) overnight at 4°C and visualized with chromogens, 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT). After being air-dried, the sections were exposed to the emulsion NTB-2 (KODAK), for 3 weeks. Once developed, the sections were quickly dehydrated and mounted in Entellan (Merck).

The number of neuropsin mRNA-positive cells was counted

in a 0.35 mm^2 measuring frame for the forebrain sections. 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.

2.3 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 monoclonal antibody, B5, at 20 ng/ml. The antibody was extensively characterized in 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 a wash 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 70 nm thick were cut on an ultramicrotome and observed under a H7100 electron microscope (Hitachi).

2.4 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 10 mM 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, one of the two equally sample-loaded gels was stained by GelCode Blue Stain Reagent (Pierce) to examine the changes of myelin proteins and the other gel was transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk in 50 mM Tris-Cl, pH 7.5, 150 mM NaCl, and 0.1% Tween-20 (TBS-T) for 2 hours at room temperature, then incubated with anti-myelin basic protein (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)

3. Results

3.1. Neuropsin mRNA is expressed after intrahippocampal kainate injection.

Four days after intrahippocampal injection of 1.5 nmol kainate, pyramidal cells in CA1 and CA3 degenerated, and neuropsin mRNA disappeared from the pyramidal neurons. Neuropsin mRNA was detected in regions where it was not expressed on the PBS-injected control side (Fig. 1A and B). As was reported previously, pyramidal cells began to degenerate 12 hours after the kainate injection (Andersson et al., 1991), completely degenerated in 2 days and then disappeared and were replaced by glial cells (Fawcett and Asher, 1999). To examine accurately which cells express neuropsin mRNA, we performed double in situ hybridization (RI for neuropsin and alkaline phosphate for PLP). Silver grains (neuropsin mRNA) were observed in the cells stained with alkaline phosphatase (PLP mRNA) (Fig. 1C). Most of the cells (77.9±1.4%, n=5) expressing neuropsin mRNA were positive for PLP mRNA, a marker of mature oligodendrocytes. There was no marked difference in the number of PLP mRNA-expressing cells between kainate-injected and control sides (data not shown). We next examined the time course of neuropsin mRNA expression after kainate injection. Neuropsin mRNA began to appear 1 day after the kainate injection and disappeared at 14 days (Fig. 1D and H). The peak expression was from 4 to 8 days after the injection

(Fig. 1F and G).

3.2. Neuropsin protein is expressed in the cell body and cell processes.

We examined the localization of neuropsin protein after injury using immunohistochemistry. Fig. 2 shows the expression of neuropsin around the CA1 region of the hippocampus 4 days after intrahippocampal kainate injection. The immunoreactive cells were located in the alveus and the external capsule, as well as being scattered in other regions of hippocampus and neocortex. The expression pattern was similar to that of the mRNA. On closer observation, immunoreactive cell processes were also found (Fig. 2C and D, arrowheads) as well as cell bodies (arrow). Uninjured tissue did not show immunostaining (Fig. 2B)

3.3. Expression of neuropsin mRNA and protein was induced following enucleation.

To determine if axonal degeneration induces neuropsin expression, unilateral enucleation was performed. By in situ hybridization, we found that the cells in the optic nerve of the operated side and the optic chiasm began to express neuropsin mRNA as early as 12 hours after enucleation (Fig. 3A). The peak of the expression was at 4 days after the operation. There was no neuropsin mRNA expression in the contralateral 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).

Immunohistochemistry revealed neuropsin-like immunoreactivity in the optic nerve on the side ipsilateral to the site of enucleation (Fig. 4A), whereas no immunoreactivity was observed in the optic nerve of the contralateral side or the unoperated mice (Fig. 4B). No immunoreactivity was observed in sections incubated without primary antibody (data not shown). The immunoreactivity was within cell bodies about 5 to 10 μm in diameter (arrows in Fig. 4C) and in the cell processes (arrowheads). The localization of immunoreactive cell bodies was very similar to that of the mRNA. Immunoelectron microscopy was performed. 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. 4D). The neuropsin-immunoreactive cells had typical clumped chromatin characteristic of oligodendrocytes. This result is consistent with neuropsin mRNA being expressed in PLP mRNA-positive cells.

3.4. Neuropsin degraded myelin protein.

To see if proteins in the myelin were possible substrates for neuropsin, we incubated myelin protein with neuropsin. GelCode Blue staining revealed that recombinant neuropsin at 1/100 of myelin protein degraded proteins corresponding to MBP (Fig. 5A). Western blot analysis confirmed that MBP was degraded

by recombinant neuropsin and MBP-immunoreactive small fragments appeared (arrows in Fig. 5B, lane 4-6). Fig. 5C shows the result of quantitative analysis of the degradation of MBP. About 33% of myelin protein was degraded over 8 hours incubation. Eight-hour incubation without r-neuropsin resulted in a slight decrease in MBP probably due to endogenous proteolysis activity in the myelin (Fig. 5B, lane 2) (Sato et al., 1982).

4. Discussion

Injury to the CNS accompanies demyelination. The mechanism underlying the initial stage of demyelination is still unknown (Cuzner et al., 1996), partly because endogenous demyelination-specific molecules such as proteases within oligodendrocytes have not been identified. There are several extracellular proteases expressed in oligodendrocytes, namely, urokinase plasminogen activator (Dent et al., 1993), matrix metalloproteinase-9 (Uhm et al., 1998) and protease M/neurosin/MSP (Scarisbrick et al, 1997; Yamanaka et al., 1999; Scarisbrick et al., 2000). In contrast, neuropsin is constitutively expressed exclusively in the neurons in the limbic systems (Fig. 1B; Chen et al., 1995). The present and previous studies have clearly demonstrated that neuropsin mRNA and protein in oligodendrocytes were expressed following CNS injury, though there is no constitutive expression in oligodendrocytes (Tomizawa et al., 1999). In the present study, 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. 1C).

Intrahippocampal injection of kainate induced the expression of neuropsin mRNA in oligodendrocytes within 1 day. The expression peaked at 4-8 days and disappeared at 14 days. This time course matches that for the onset of demyelination observed after kainate injection (Dusart et al., 1992). Furthermore, by immunohistochemical staining and immunoelectron microscopic analysis, neuropsin was localized to oligodendrocytes and myelin, although the ultrastructural preservation was sacrificed by the high concentration of detergent, which is sometimes necessary when detecting antigens in myelin (Weruaga et al., 1998). The in vitro biochemical assay demonstrated that neuropsin degraded MBP. This activity would indicate that neuropsin functions in demyelination through proteolysis of myelin proteins. Myelin protein is degraded by proteases (Marks et al., 1976; Whitaker and Seyer, 1979; Berlet et al, 1984; Inuzuka et al, 1984; Cammer et al., 1986) and demyelination is completed when myelin and/or axons therein are phagocytosed by macrophage or microglia (Cuzner and Norton, 1996). We hypothesize that neuropsin functions at the onset of demyelination after injury and phagocytes clean up myelin which is partially broken down by internal proteases such as neuropsin. It will be important to see whether neuropsin is induced under demyelinating conditions such as experimental allergic

encephalopathy or multiple sclerosis in humans.

There were two possible explanations for how injury induced neuropsin expression, a direct effect of kainate on oligodendrocytes or an indirect effect through degenerating neurons and/or axons. The experiments of enucleation showed that neuropsin was induced in the optic nerve after Wallerian degeneration in a similar time course to that seen after intrahippocampal kainate injection. This result indicates that some signal from degenerating axons or loss of axonal contact induced neuropsin expression.

In conclusion, neuropsin is expressed in oligodendrocytes after injury. From the time course of its induction and its ability to degrade myelin proteins, it is likely that neuropsin functions in the process of demyelination. Further study is needed to elucidate the role of neurosin in demyelinating diseases such as multiple sclerosis and experimental allergic encephalopathy.

Acknowledgments

This work was supported by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan and a Sasagawa Memorial Grant from the Japan Foundation.

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Figure legends

Fig. 1 Induction of neuropsin mRNA in the hippocampus after intrahippocampal kainate injection. After unilateral kainate injection, in situ hybridization was performed on frontal sections with a probe for neuropsin. A, Hippocampal region 4 days after kainate injection. B, Hippocampal region of the side contralateral to A which was injected with PBS for control. C, Double in situ hybridization using probes for neuropsin (silver grains) and PLP (alkaline phosphatase). D-H, Time course of the expression of neuropsin mRNA after kainate injection into the hippocampus. Neuropsin mRNA expression at 1 day (D), 2 days (E), 4 days (F), 10 days (G) and 14 days (H) is shown. I, Quantitative analysis of the number of cells expressing neuropsin mRNA after kainate injection. The cells in a 0.35 mm^2 measuring frame adjacent to the needle track of kainate injection were enumerated (±SD).

Abbreviations: al, alveus; cc, corpus callosum; f, fimbria; Py, pyramidal cell layer of the hippocampus. Scale bars, 0.6 mm in A and B, 20 μm in C and 0.2 mm in D-H.

Fig. 2 Neuropsin (protein) localization after kainate injection. A, Neuropsin immunoreactivity 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 alveus and the stratum oriens of the CA1 subfield.

Abbreviations: see Fig. 1.
Scale bars, 0.2 mm in A and B, 0.1 mm in C and 40 μm in D.

Fig. 3 Neuropsin mRNA was induced by axonal degeneration of the optic nerve. Neuropsin mRNA was detected in the optic nerves after enucleation 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. 4 Neuropsin-like immunoreactivity in the optic nerve after enucleation. Neuropsin-like immunoreactivity in ipsilateral optic nerve 4 days after enucleation (A). Contralateral optic nerve did not show immunoreactivity (C). Observation at higher magnification revealed immunoreactivity in the cell body (arrows) and processes (arrowheads). The neuropsin immunoreactivity in the cytoplasm of oligodendrocytes and myelin (arrowheads) detected by immunoelectron microscopy (D). Scale bars, 0.2 mm in A and B, 25 μm in C and 2 μm in D.

Fig. 5 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.

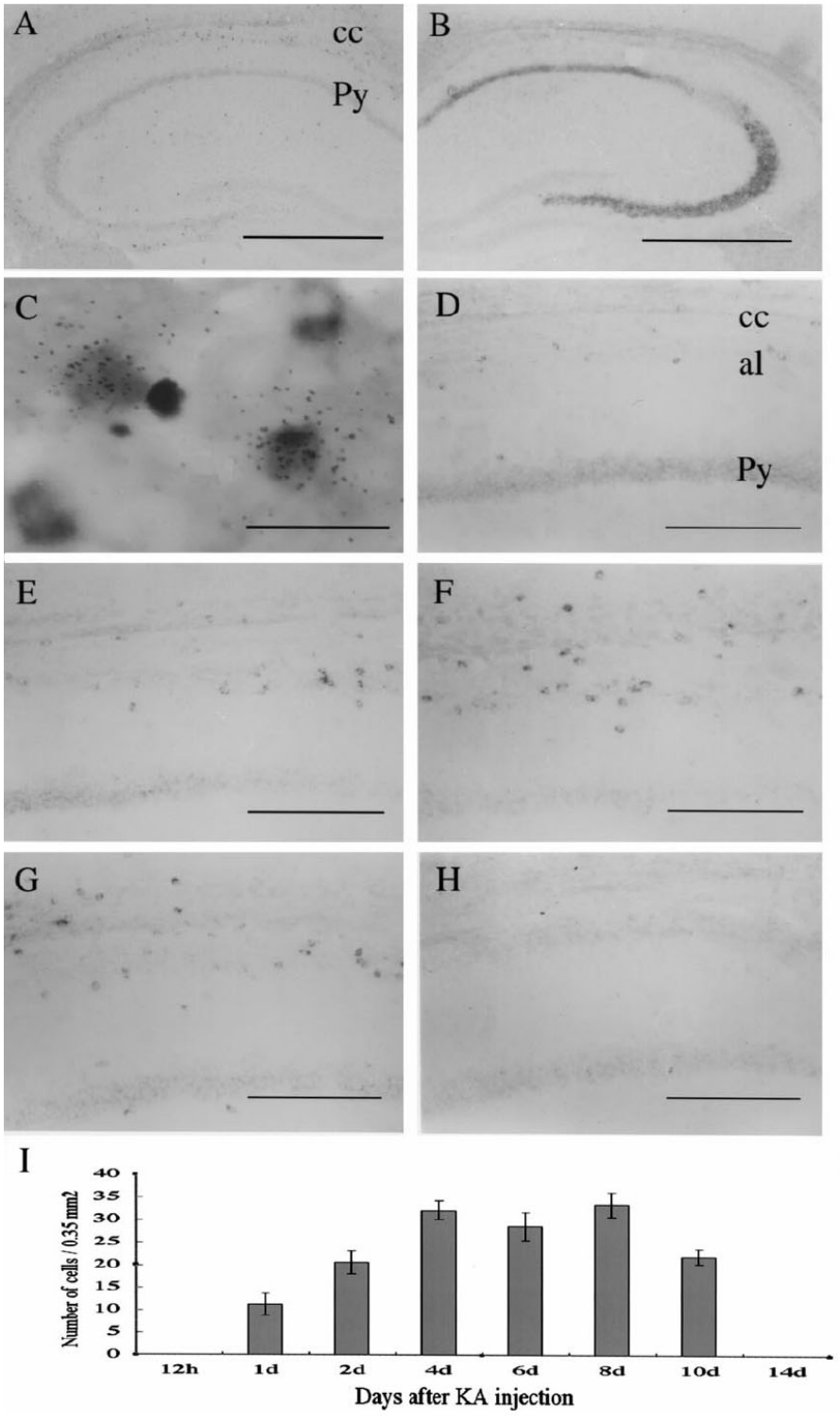
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Fig. 1

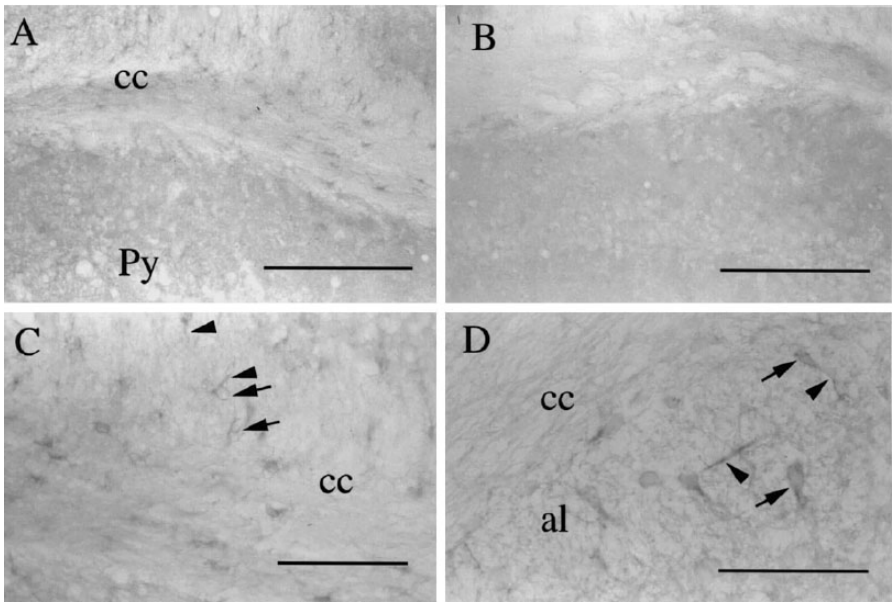


Fig. 2

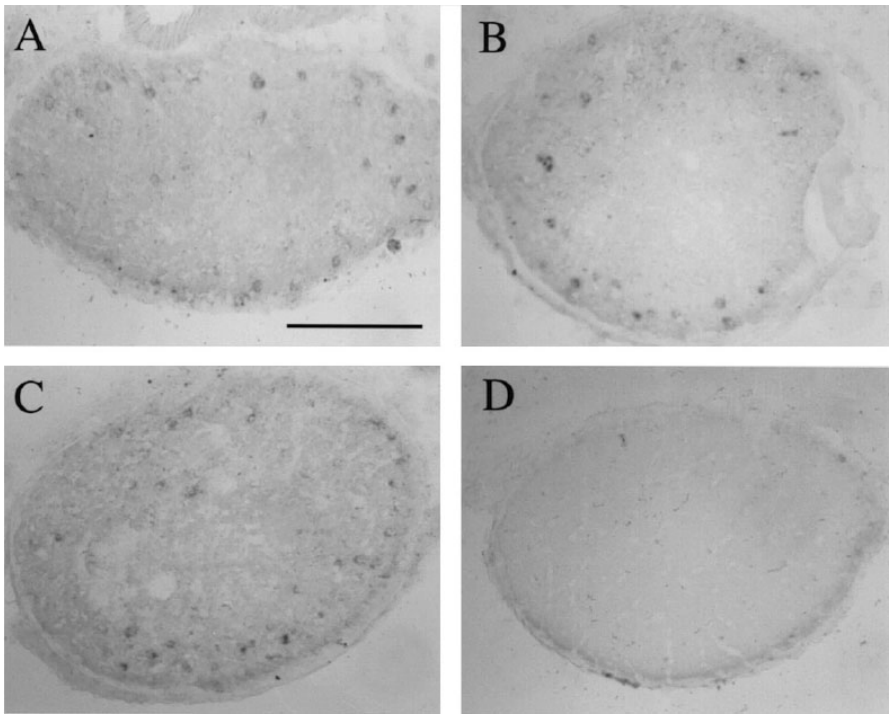


Fig. 3

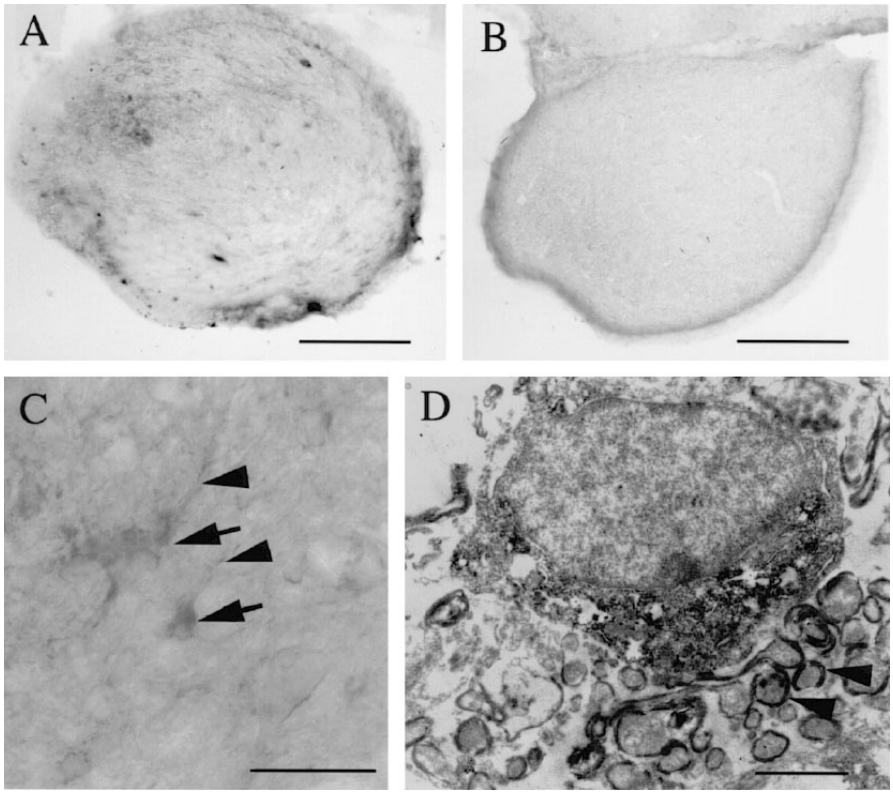
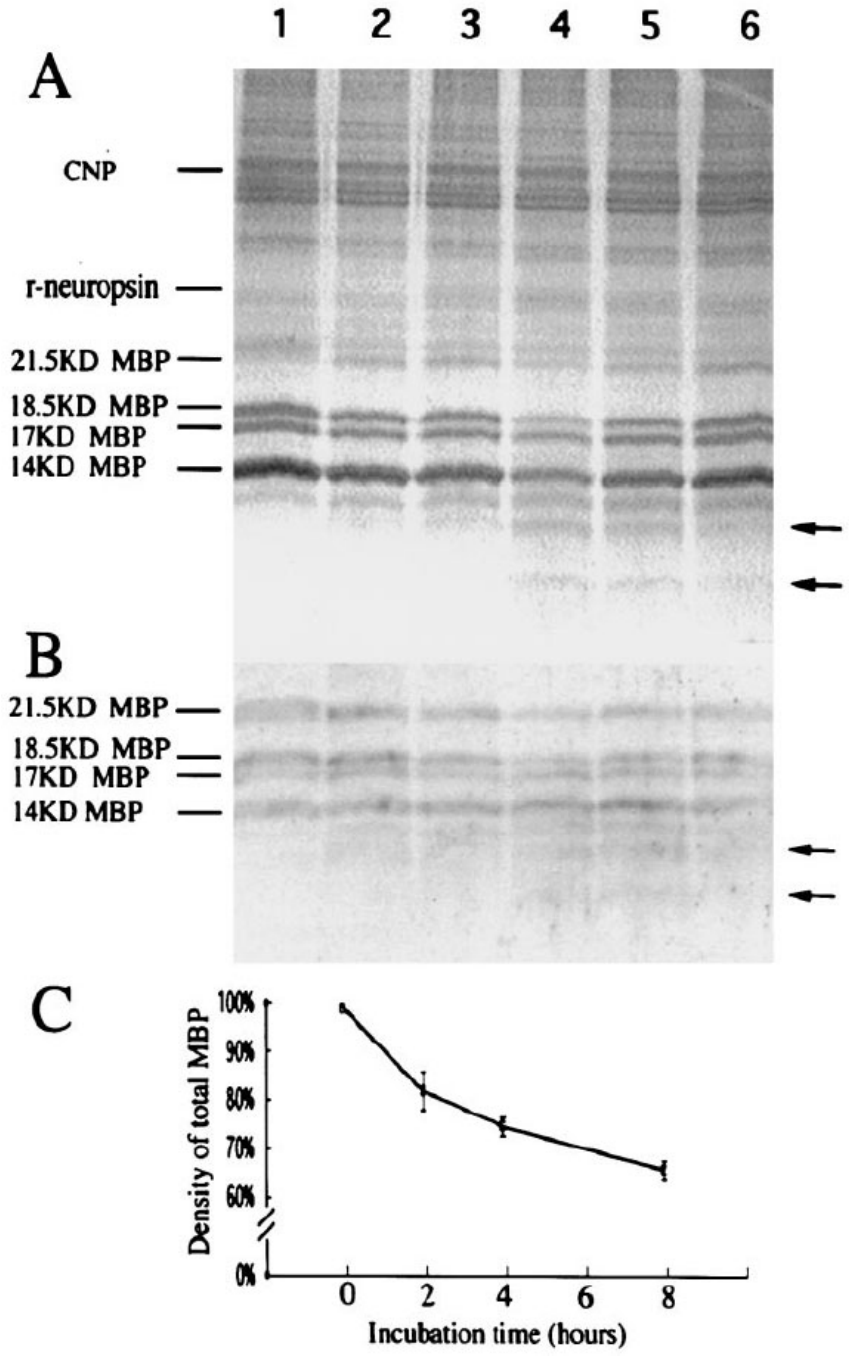


Fig. 4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17KD MBP
14KD MBP
C
Density of total MBP
100%
90%
80%
70%
60%
0%
0
2
4
6
8
Incubation time (hours)

Fig. 5