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NEUROPSIN PROMOTES OLIGODENDROCYTE DEATH, DEMYELINATION AND AXONAL DEGENERATION AFTER SPINAL CORD INJURY

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Abstract—Previous studies indicated that the expression of neuropsin, a serine protease, is induced in mature oligodendrocytes after injury to the CNS. The pathophysiology of spinal cord injury (SCI) involves primary and secondary mechanisms, the latter contributing further to permanent losses of function. To explore the role of neuropsin after SCI, histochemical and behavioral analyses were performed in wild-type (WT) and neuropsin-deficient (neuropsin$^{-/-}$) mice using a crush injury model, a well-characterized and consistently reproducible model of SCI. *In situ* hybridization revealed that neuropsin mRNA expression was induced in the spinal cord white matter from WT mice after crush SCI, peaking at day 4. Neuropsin$^{-/-}$ mice showed attenuated demyelination, oligodendrocyte death, and axonal damage after SCI. Although axonal degeneration in the corticospinal tract was obvious caudal to the lesion site in both strains of mice after SCI, the number of surviving nerve fibers caudal to the lesion was significantly larger in neuropsin$^{-/-}$ mice than WT mice. Behavioral analysis revealed that the recovery at days 10–42 was significantly improved in neuropsin$^{-/-}$ mice compared with WT mice in spite of the severe initial hindlimb impairments due to SCI in both strains. These observations suggest that neuropsin is involved in the secondary phase of the pathogenesis of SCI mediated by demyelination, oligodendrocyte death, and axonal degeneration.

Key words: serine protease, secondary injury, *in situ* hybridization, immunofluorescence labeling, tract tracing, behavior.

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Abbreviations: ALP, alkaline phosphatase; ANOVA, analysis of variance; APC, adenomatous polyposis coli; BBB, Basso Beattie Bresnahan; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; BDA, biotinylated dextran amine; CST, corticospinal tract; DIG, digoxigenin; GAPDH, glyceraldehyde phosphate dehydrogenase; IR, immunoreactive; MBP, myelin basic protein; NBT, nitroblue tetrazolium; neuropsin$^{-/-}$, neuropsin deficient; PB, phosphate buffer; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PLP, proteolipid protein; PLSD, protected least significant difference; SCI, spinal cord injury; SSC, standard saline citrate; tPA, tissue plasminogen activator; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-fluorescein nick end labeling; WT, wild-type.

The pathophysiology of spinal cord injury (SCI) involves primary and secondary mechanisms. Following an initial impact after SCI, there is a delayed and progressive period of secondary damage that contributes to the loss of sensory and voluntary motor function (Lu et al., 2000; Park et al., 2004). This secondary phase of injury includes ischemia, inflammation and glutamate excitotoxicity, together with neuronal and glial cell death. These processes are mediated by changes in the constitution of a variety of extracellular matrix components (Sanes, 1983, 1989), growth factors (Mocchetti and Wrathall, 1995), and proteolytic cascades (Krystosek and Seeds, 1981; Monard, 1988; Seeds et al., 1990). Several groups have now demonstrated that oligodendrocyte death and subsequent demyelination are involved in the pathology after SCI (Lu et al., 2000; Blight, 2002; Dong et al., 2003; Park et al., 2004). Oligodendrocyte loss and demyelination result in inadequate nerve conduction and the appearance of neurological deficits, which are potential targets for improving function after SCI even in the absence of axonal regeneration.

Since oligodendrocytes have protease activity which might contribute to the pathology after SCI (Berlet et al., 1984), the identification and characterization of key enzymatic players may suggest new therapeutic targets to reduce further damage and promote remyelination. Kallikreins are serine proteases that comprise a recently identified large and closely related 15-member family in humans. Mice are reported to carry up to 37 kallikrein genes including genes homologous to newly identified human kallikreins. These kallikreins serve a variety of physiological functions including regulation of blood pressure, neuronal health, and the inflammatory response (Borgono and Diamandis, 2004; Borgono et al., 2004; Diamandis et al., 2004). Neuropsin is a kallikrein-like 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). *KLK8* was assigned as the gene for the human analog (Yoshida et al., 1998; Yousef et al., 2003). A biochemical analysis of recombinant and native neuropsin suggested that it is an extracellular trypsin-type protease with a relatively narrow spectrum of substrates (Shimizu et al., 1998). Our previous experiments showed that traumatic, excitotoxic and immunological injury induced expression of neuropsin mRNA in the area surrounding the lesion in the CNS (Tomizawa et al., 1999; Terayama et al., 2004, 2005a). Combined *in situ* hybridization and immunohistochemistry revealed that most of

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the cells expressing neuropsin mRNA were mature oligodendrocytes (Terayama et al., 2004).

Although upregulation of neuropsin expression after injury to the CNS has been reported, the functional roles of neuropsin are not fully characterized. We therefore examined changes in the expression of neuropsin in wild-type (WT) mice and assessed the pathophysiological response in neuropsin-deficient (neuropsin$^{-/-}$) mice. We used a crush injury model, a well-characterized and consistently reproducible model of SCI that allows evaluation of both cell biology and behavior (Inman et al., 2002; Faulkner et al., 2004) to examine the differences between WT and neuropsin$^{-/-}$ mice in pathological hallmarks and functional outcome after SCI.

EXPERIMENTAL PROCEDURES

Mice

Neuropsin$^{-/-}$ mice were derived from heterozygous (neuropsin$^{+/-}$) mice after backcrossing with C57BL/6 mice for at least 10 generations. Complete loss of neuropsin mRNA was confirmed with a cDNA probe covering exons 2–4 including the histidine residue essential for proteolytic activity (Hirata et al., 2001; Kirihara et al., 2003). Adult (6- to 8-week-old) mice were used in all experiments (Table 1). Mice were bred in-house (Division of Laboratory Animal Resources at Asahikawa Medical College, Asahikawa, Japan), controlled for temperature (20 °C), and maintained with a daily light period of 12 h. Paralyzed mice were given easy access to food and hand watered at least twice daily. All procedures described herein were approved by the Animal Care Committee of Asahikawa Medical College, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23), revised 1996. Every attempt was made to minimize the number of animals used and to reduce their suffering at all stages of the study.

SCI

Mice were anesthetized with an i.p. injection of pentobarbital sodium (Nembutal, 40–50 mg/kg). Under a dissecting microscope, a laminectomy at the 10th thoracic (T10) level was performed to injure the spinal cord at first lumbar (L1) level with number 5 Dumont forceps ground down to a tip width of 0.5 mm. The forceps we used in this report were modified with a spacer so that at maximal closure a 0.6 mm space remained to make incomplete lesion (Faulkner et al., 2004). The spinal cord was compressed with the forceps laterally from both sides for 10 s (Fig. 1A, B). After the wound was sutured, animals were allowed to survive for 1–42 days.

Tissue preparation

At days 0, 4 and 14 post-injury, animals (n=5 per strain at each time point; Table 1) were re-anesthetized and perfused transcardially with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The spinal cord including the lesion was removed, postfixed overnight in the same fixative, and then immersed in 30% sucrose in 0.1 M PB for 1–2 days. The spinal cord was then frozen in powdered dry ice, embedded in Tissue-Tek (Miles, Elkhart, IN, USA) optimal cutting temperature compound, and stored at −80 °C prior to use. Frozen 14 μm transverse sections of the spinal cord were cut on a cryostat and mounted onto silane-coated slides. Sections were processed for detection of multiple markers to find the differences between WT and neuropsin$^{-/-}$ mice after SCI.

In situ hybridization

In situ hybridization was performed using a digoxigenin (DIG, Roche Molecular Biochemicals, Mannheim, Germany) -labeled cRNA probe for neuropsin and proteolipid protein (PLP) mRNA. The method used for *in situ* hybridization was described in previous papers (Yoshida et al., 1994; Chen et al., 1998; Terayama et al., 2004). In brief, slide-mounted sections were postfixed in 4% formaldehyde in 0.1 M PB, and treated with 10 μg/ml of proteinase K, followed by 0.25% acetic anhydrate in 0.1 M triethanolamine (pH 8.0) for 10 min at RT. The sections were dehydrated and defatted in graded alcohol solutions and chloroform, respectively and air-dried before application of the hybridization buffer at 55 °C for 1 h. Hybridization was performed overnight at 55 °C in a humidified chamber with hybridization buffer containing 10 ng/100 μl of the DIG-labeled cRNA probe (Terayama et al., 2004). The hybridized sections were washed in 4× standard saline citrate (SSC) buffer (pH 7.4) for 20 min and then in 50% formamide/4× SSC buffer at 60 °C for 30 min. After being treated with RNase A, sections were incubated with alkaline phosphatase (ALP)–anti-DIG antibody (Roche Molecular Biochemicals) at a dilution of 1:500. Sections were developed for 48 h in the dark with 10% polyvinyl alcohol containing 3.75 μl/ml of 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Roche Molecular Biochemicals) and 5 μl/ml of nitroblue tetrazolium (NBT, Roche Molecular Biochemicals). The specificity of the hybridization signal was verified by comparing adjacent sections hybridized with antisense and sense probes of comparable specific activity. In a given area (250 μm×180 μm), the number of PLP-positive cells in the white matter of five randomly selected sections within 400–600 μm rostral and caudal to the epicenter was counted at each survival time point. PLP-positive cells were defined as cells with dark blue staining in the cell body, regardless of staining intensity, but cells with very weak equivocal staining were considered negative. The data from three individual experiments were represented as the

Table 1. Numbers of animals used for each experimental procedure

Experimental procedure	Control	Spinal cord injury				
		Day 1	Day 4	Day 7	Day 14	Days 0–42
Transverse sections[a]	5/5		5/5		5/5	
Lesion size analysis					5/5	
Electron microscopy					3/3	
Anterograde tract tracing with BDA	5/5				5/5	
Real-time quantitative PCR[b]	3	3	3	3	3	
Western blot analysis	3/3		3/3			
Behavioral analysis						8/8

Values indicate numbers of WT/neuropsin$^{-/-}$ mice unless noted.

[a] Transverse sections were used for *in situ* hybridization, immunofluorescence labeling and TUNEL.

[b] WT mice only.