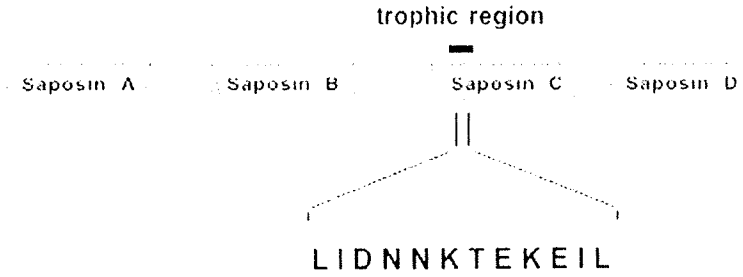

Fig. (3). Neurotrophic functional site localizes in the domain for saposin C.
Prosaposin and saposin C showed neurotrophic activity in promoting neurite outgrowth in NS20Y cells, while no activity was demonstrated with saposin A, B, and D. The neurotrophic activity was further investigated various synthetic peptides derived from saposin C. The active region was pinpointed to a 12-amino acid-sequence in the N-terminal sequence of saposin C [20].

representing an important determinant of cell fate.

The ganglioside binding nature of prosaposin leads us to propose that prosaposin-ganglioside complex may be a structural component of membrane signaling lipid domains as prosaposin-receptor-G protein complex [7, 64]. Lipid rafts are small and highly dynamic structures that have lateral assemblies of specific lipids and proteins including G protein-coupled receptors in cellular membranes [65, 66]. Those domains are enriched in certain lipids [sphingolipids including gangliosides, sphingomyelin and cholesterol], generally being insoluble in common detergents such as Triton-X100. A variety of specific proteins associated with signal transduction have also been detected within those domains [66, 67]. Our recent study suggests that exogenously added prosaposin might exert its versatile, trophic and anti-apoptotic activities by binding its target molecule within cell surface lipid rafts to induce ERK phosphorylation and SK activation.

It is notable that prosaposin effects both glial cells and neurons. Experiments *in vitro* demonstrated that prosaposin prevented cell death of Schwann cells and oligodendrocytes via the ERK and phosphoinositide 3-kinase [PI3K]/Akt pathways [22, 24]. It also increased the cellular level of a myelin lipid, sulfatide, in primary Schwann cells and CG4 oligodendrocytes through a $G_{0\alpha}$ protein-coupled receptor-mediated mechanism [22-24, 68]. It is well-known that IGF-I induces both proliferation and differentiation of Schwann cells [69], while the action of prosaposin on glial cells was not proliferative but mitogenic, unlike IGF-I.

The biological effect of prosaposin can be observed in diverse cell types. For example, an ERK-mediated biological effect of prosaposin was demonstrated in monocytic cells [63]. This suggests that prosaposin could be considered a new player in the regulation of the apoptotic signal transduction pathway in cells of non-neurological origin. In this regard, prosaposin may be an additionally required molecule involved in the apoptotic machinery homeostatically controlling cell differentiation and cell numbers. It may also have a role in organogenesis, the elimination of damaged cells, and carcinogenesis. A recent study demonstrated that saposin C can up-regulate urokinase plasmonogen activator, its receptor, and the immediate early gene c-Jun. In prostate stromal and cancer cells, these effects lead to the activation of p42/44 and SAPK/JNK MAPK pathways leading to increased cell proliferation, migration, and invasion [70]. This strongly suggests that saposin C may act as a potent growth factor associated with prostate carcinogenesis. In a separate study, prosaposin was shown to enhance multiple signal transduction pathways potentiating an anti-apoptotic effect in the prostate cancer cells [71]. This finding implicates prosaposin over-expression in prostate cancer and suggests that the prosaposin gene is androgen-regulated gene responsible for normal and neoplastic growth of the prostate [72-74]. Prosaposin is potentially one of molecules associated with the process of carcinogenesis in the prostate.

An *in vitro* effect of prosaposin on oxidative stress was recently reported [75]. The study using PC12 cells demonstrated that prosaposin inhibited hydrogen peroxide-induced apoptotic cell death by suppressing neurotoxic cascades. A brief treatment with hydrogen peroxide promoted caspase-3 activation in PC12 cells, which was inhibited by simultaneous treatment with prosaposin. In accordance with the previous findings, prosaposin was demonstrated to use ERK and PI3K/Akt kinase pathways to prevent cell death induced by oxidative stress. Importantly, PI3K and MEK inhibitors neutralized the anti-apoptotic effects of prosaposin, thus demonstrating that the dual activation mechanism is crucial for prosaposin-mediated survival of PC12 cells from oxidative stress. At the same time, it was evident that prosaposin inhibits caspase-3 activation in mediating its anti-apoptotic effect. H_2O_2-induced phosphorylation of the downstream signaling targets of Akt such as JNK and p38 was completely knocked down by prosaposin stimulation. Collectively these findings clearly indicate that prosaposin utilizes a variety of signaling pathways linked to transcription factor activation to exert its versatile biological actions.

5. MOLECULAR GENETICS OF PROSAPOSIN

Human prosaposin gene encompasses a genomic sequence over 19 kb with 15 exons ranging in size from 57 to 1200 base pairs [bp] [76]. The regions encoding saposins A, B, and D consist of three exons, while the saposin C region has two exons. Based on the regularity of the positions of introns, the prosaposin gene is theorized to have evolved through sequential duplication of an ancestral gene. The transcriptional regulation of the prosaposin gene locus is of interest in understanding the neuro-biological and neuro-developmental significance of prosaposin [see Sections 6 and 7]. A TATA-less promoter sequence was found in the 5'

region of the human prosaposin gene, in which three potential transcription initiation sites were located in an region upstream of the initiation codon [77]. Positive and negative regulatory elements were also detected upstream of the ATG initiation codon. AP-1, Oct-1 and two orphan nuclear receptor [RORalpha] binding sites reside in the promoter region of the human prosaposin gene. In the mouse prosaposin gene, Sp family members [Sp1, Sp3, and Sp4], RORalpha, and an unknown transcription factor were shown to bind to this promoter region [78]. Using transgenic mice with 5'-flanking deletions of the prosaposin gene, it was determined that prosaposin expression is nearly exclusively localized to the cerebrum, cerebellum and eyes of the transgenic mice. Deletion of RORE and the Sp1 elements significantly decreased prosaposin gene expression in the CNS, which suggests that the prosaposin gene expression in the CNS could be due to the RORE and Sp1 elements in the promoter region. Interestingly, the human promoter lacks a Sp1 cluster and has AP-1, Oct-1 and two RORalpha sites instead [77]. In a separate study with transgenic approaches, RORalpha and Sp1 sites in the 5'-flanking region were shown to mediate developmental regulation in the CNS, and the presence of additional regulatory elements outside the 5' region of the promoter element was implicated in the physiological control of the prosaposin locus [78].

6. PROSAPOSIN REGULATION FOLLOWING NEURO-INJURY

The expression level of various cytokines and their receptors is altered in response to traumatic insult to the CNS and PNS [79]. The expression level of prosaposin transcript also changes following nerve injury. An early study with molecular approaches identified the prosaposin gene as one of the differentially expressed genes associated with sciatic nerve crush injury [80]. The study showed that the prosaposin mRNA level was increased 6-fold over controls after 7 days of sciatic nerve crush and returned to the control level by 28 days after the operation. In addition, subsequent studies employing rat models of global cerebral ischemia and peripheral nerve injury identified the prosaposin gene as one of differentially induced genes the following nerve damages [80, 81]. Those findings implicate a significant role for prosaposin in the nerve degeneration and regeneration processes following injury. Prosaposin regulation following nerve injury was further investigated using two rat models: middle artery occlusion and cortical stab wound injury [82]. In ischemic brains the level of prosaposin mRNA was elevated greater than 400% over controls within 5 days after ischemic insult and sustained over 3 weeks (Fig. **4**). Immunohistochemical staining and *in situ* hybridization demonstrated an enhanced signal distribution of prosaposin mRNA and injury-induced prosaposin protein around the lesion. The data suggest that the expression and processing of prosaposin mRNA may be crucially regulated not only for cerebral homeostasis but also during nerve regenerative and degenerative processes.

More importantly, the nerve injury-induced response in prosaposin gene expression was found to be accompanied by a qualitative change. The human prosaposin gene generated three alternatively spliced forms of the transcript [83]. The domain for saposin B is coded by four exons, Exons-6 to -9,

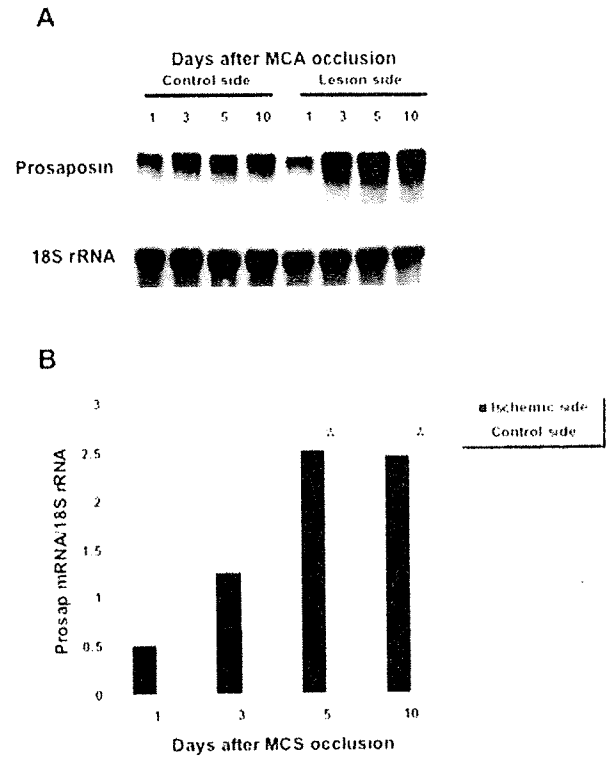

Fig. (4). Enhanced expression of prosaposin mRNA following cerebral ischemia.
Prosaposin mRNA expression following cerebral ischemia **[A]**. Focal cerebral ischemia was induced by middle cerebral artery occlusion. After appropriate periods of reperfusion, brains were removed and processed for Northern blot analysis [79]. The blot was prehybridized and then hybridized with a prosaposin probe, and then reprobed with a 1.5 kbp fragment of human 18 S rRNA cDNA for normalization. Expression of prosaposin mRNA was assessed by the densitometric ratio of prosaposin mRNA to 18S rRNA **[B]**. Difference from controls with $*p < 0.01$.

of which Exon-8, consisting of only 9 bp, is involved in the alternative splicing. This alternative splicing gives rise to prosaposin variants with two and three extra amino acid-insertions, Gln-Asp-Gln and Asp-Gln, and with no insertion within the saposin B domain. Our recent study using rat models with neuro-injury showed that this Exon-8 splicing was altered in response to nerve injury (Fig. **5**). In normal brain, two prosaposin mRNA species with and without the 9-base insertion were expressed at a ratio of 85:15; however, this equilibrium reverted to 5:95 following ischemic injury. The prosaposin mRNA variant with 6-base insertion was under detectable levels in those samples. Although the precise role of Exon-8 splicing remains unknown at this point, available evidence indicates that the alternative splicing in the prosaposin gene is tissue specific and conserved from fish to humans. The expression of Exon-8 is evident in brain and reproductive organs but only slightly in other major organs such as liver, kidney and spleen. Despite several potential implied roles for the splicing mechanism [55, 84, 85], a recent study with molecular biological approaches demonstrated that the prosaposin variant with Exon 8-derived se-

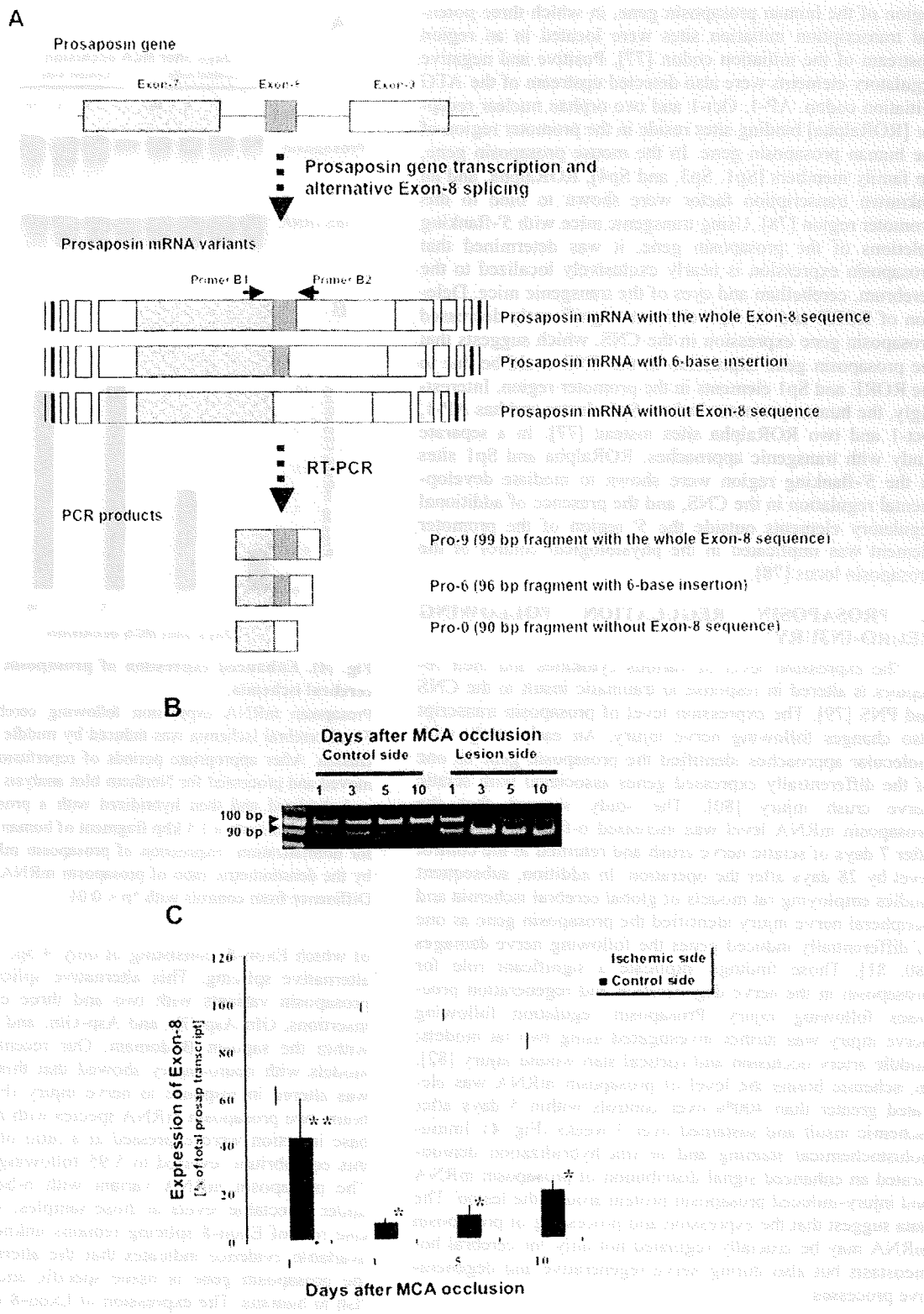

Fig. (5). Alternative Exon-8 splicing and nerve injury.

Alternative 9-base splicing and brain injury. **[A]** A primer set [B1 and B2] encompassing a sequence region within Exon-7 and Exon-8 was designed for RT-PCR to amplify prosaposin variant transcripts as fragments of 99 bp [Pro-9], 96 bp [Pro-6] and 90 bp [Pro-0]. The data shows that 9-base splicing of prosaposin mRNA was induced following brain injuries [79]. Change in Exon-8 alternative splicing was assessed as the densitometric ratio of Pro-9/Pro-0 in each sample **[B** and **C]**. Difference from controls with $*p < 0.01$.

quence is dispensable for normal mouse development and fertility [86]. Collective findings on prosaposin gene regulation implicate a physiological significance of the pre-prosaposin mRNA processing mechanism in maintaining the nervous system, although no conclusive evidence showing the precise role of alternatively spliced prosaposin variants has been provided. In terms of the neurobiological significance of alternative gene splicing, there is accumulating evidence suggesting a potential involvement of an alternative splicing mechanism in nerve development, regeneration, and degeneration processes [87]. The tau gene represents a typical example of a gene that is transcribed into two functionally different alternatively spliced mRNA species. The first is mainly found in developing nervous system and was selectively induced in response to sciatic nerve axotomy [88, 89]. The second variant was shown to be present in Alzheimer's disease brains at a high concentration [90]. This raises the possibility that the tau splicing mechanism may be one of the contributors to the neuropathology of Alzheimer's disease.

Prosaposin expression was also shown to be regulated at protein level following nerve injury. An *in situ* experiment using a rat model demonstrated that prosaposin was secreted into endoneurial fluids in response to sciatic nerve transection [91]. Prosaposin was not detected in homogenates of normal uninjured sciatic nerve, suggesting that it was secreted as consequence of nerve injury. Those findings clearly indicate that prosaposin expression is governed immediately in response to neuronal damage, implicating its crucial involvement in the injury-repair mechanism in the both PNS and CNS. The regulation of prosaposin following nerve injury may be associated in part with intrinsic cellular repair mechanisms in the nervous systems. In addition, the resulting autocrine or paracrine transported prosaposin may act as a myelinotrophic and injury-repair protein on injured nerves.

7. PROSAPOSIN AS POTENTIAL NEURO-THERAPEUTIC

i. Prosaposin-Derived Neurotrophic Peptide, Prosaptide

Since prosaposin is a large molecule, it is more realistic to use its peptide mimetics for therapeutic purpose, in particular, for CNS application. Peptide mimetics generally have significant advantages over the parent polypeptide due to a variety of reasons: economical production, greater chemical stability, enhanced pharmacological properties and reduced antigenicity. In order to explore the applicability of prosaposin in clinic, we have designed a variety of peptides emcompassing the neurotrophic region of prosaposin [prosaptide]. The first to be developed was a neurotrophic 14-mer prosaptide TX14[A] and ultimately we developed an 11-mer D-amino acid-peptide [all D; LLEETANNDLL] based on the sequence of TX14[A]. Both were tested *in vitro* and *in vivo*. This second generation prosaptide D5 was able to overcome the physical and the enzymatic barriers to crossing the blood-brain barrier and it remained intact in the brain [92]. By taking advantage of such properties, it can be expected that the use of retro-inverso peptide will be profitably applied to therapeutic strategies for treatment of patients with neuro-degenerative disorder, in particular, in the CNS. The following studies were conducted with not only prosaposin but also the peptide mimetics of prosaposin.

ii. Neuro-Efficacy of Prosaposin and Prosaptide

Cerebral Ischemia

The neuro-protective effect of prosaposin was first demonstrated by a study using a gerbil model of cerebral ischemia [93]. Ischemia was induced by an occlusion of common carotid arteries and prosaposin was infused into the lateral ventricles at a dose of 100 or 240 ng/day for 7 days. Behavioral and histochemical examinations indicated that post-ischemic learning disabilities and neural loss were significantly reduced by prosaposin infusion. Using the same model, an 18-mer rat prosaptide was also shown to have neuro-protective activities. It was capable of suppressing ischemia-induced learning disabilities, hippocampal CA1 neuronal loss, ischemia-induced space navigation disability, the cortical infarction, and thalamic degeneration [94]. The efficacy of the rat prosaptide in cerebral ischemia was further confirmed by a study using an occlusion model in stroke-prone spontaneously hypertensive rats [95]. Furthermore, a retro-inverso prosaposin peptide, D5, was tested for its effectiveness and pharmacological behaviour in models of cerebral ischemia. In an occlusion model in rats, systemic injection of D5 was shown to improve post-ischemic behavioral impairment and to reduce the sizes of infarct areas [96].

Cortical Stab Wound

The neuro-protective effect of prosaposin on brain injury was demonstrated using a rat model of bilateral stab wound injury [97]. In this study, prosaposin was injected in each lesion at 120 ng/day for 3 days following a stab wound. After 3 weeks of operation, histological examination and Morris water maze task were employed to assess the efficacy of prosaposin. The histological study showed that the prosaposin-treated group retained a well-preserved morphology of the hippocampus and less glial reaction around the stab wound (Fig. **6**), thus suggesting that prosaposin could facilitate structural preservation of the damaged brain tissue. Reduction in cavity formation was also evident in the prosaposin-treated group. In the transfer test, the prosaposin-treated group showed improved crossings of the platform place.

Sciatic Nerve Injury

In an experiment using a guinea pig model of sciatic nerve transection, prosaposin treatment dramatically increased the number of regenerating nerve fibers, thus suggesting a potential use of prosaposin for the treatment of peripheral nerve injuries [98]. The study determined the degree of atrophy and chromatolysis of neurons to demonstrate that prosaposin could promote peripheral nerve regeneration through its action on alpha-motor neurons in the anterior horn and on small sensory neurons in the dorsal root ganglion. In independent studies, prosaposin-derived 14-mer peptide TX14[A] was shown to prevent structural and functional abnormalities associated with peripheral neuropathy in diabetic rats [99, 100]. The peptide treatment exhibited acute anti-hyperalgesic properties in various pain models in rats [101, 102]. Using a range of models in which allodynia is induced to the peripheral nerve with various degrees of damage, pre-treatment with the peptide was shown to alleviate tactile allodynia and to prevent onset of allodynia in the pa-

clitaxel and formalin injection models. These results demonstrate the anti-allodynic properties of prosaposin in diverse models of neuropathic pain and therapeutic potential of prosaposin for treatment of both degenerative neuropathy and neuropathic pain.

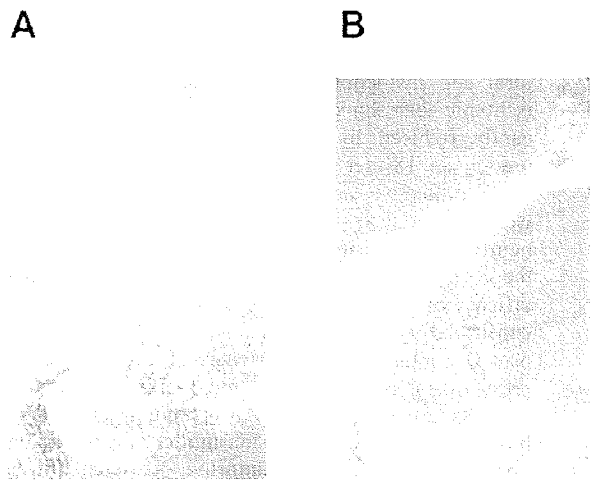

Fig. (6). Neuro-efficacy of prosaposin on cortical stab wound injury.

Sprague-Dawley rats were anesthetized and bilateral stab wounds were made in the motor area of the frontoparietal cortex. Under anesthesia in a stereotaxis apparatus, rats had been injected in each stab wound with 1 μl of prosaposin [120 ng/μl per day] for 3 days post-operation. 0.1 M phosphate-buffered saline [PBS] was used as control [93]. After 3 weeks post-operation, brain sections from rats treated with prosaposin **[A]** and PBS **[B]** were stained with Hematoxylin-Eosin stain. Note that the glial reaction around the stab wound, especially the corpus callosum was more suppressed than that in stab-wound rats treated with PBS.

In the Seltzer rat model of neuropathic pain, the D5 peptide reduced thermal hyperalgesia in the returning paw withdrawal latency to control levels [92]. The effect became evident within 3 hours after injection and was sustained over at least 48 hours. *In vitro* analysis demonstrated that prosaposin and D5 inhibits K1-stimulated synaptosomal $^{45}Ca^{2+}$ uptake similar to v-conotoxin MVIIC, which was abolished by pertussis toxin treatment prior to D5 treatment. Collectively, these findings indicate the involvement of a G-protein-associated voltage-dependent calcium channel mechanism in the effect of prosaposin on hyperalgesia in the Seltzer model.

Effect in Developing Rats

The myelinotrophic effect of D5 in developing rats has been investigated [103]. This study demonstrated that subcutaneous D5-injection to neonate rats increased sulfatide concentrations in the brains and sciatic nerves by 250 and 150% over controls, respectively. These results suggested that D5 treatment might stimulate the myelination process in developing rats, although no significant changes in the level of other myelin markers such as myelin-associated glycoprotein and P0 protein were demonstrated. The findings suggest that prosaposin and its peptide mimetic might be applicable agents to treat both neuroinjury after trauma and unmyelinated tracks in premature infants such those found in periventricular leukomalacia.

SUMMARY

With the increasing number of patients with neurodegenerative disorders, there is increasing demand for therapeutics that control disease symptoms with greater efficacy to improve the quality of life for patients. The neuropathological findings in prosaposin deficiency in man and in prosaposin knock-out mice clearly indicate the physiological significance of prosaposin in the nervous system. The regulation of prosaposin gene and prosaposin protein expression following nerve injury may be associated, in part, with intrinsic cellular repair mechanisms in the nervous system. Ample evidence through various studies conducted with neuro-injury models justify the rationale and validate the relevance of prosaposin as a therapeutic target. In addition, collectively these studies provide future perspective in exploring therapeutic strategies using prosaposin and prosaposin peptides for treatment of patients with neurodegenerative disorders.

ABBREVIATION

AP-1	=	Activator protein-1
bp	=	Base pair
CD	=	Circular dichroism
ChAT	=	Choline acetyltransferase
CNS	=	Central nervous system
ERK	=	Extracellular signal-regulated kinase
IGF-1	=	Insulin-like growth factor-1
LDL	=	Low-density lipoprotein
M6P	=	Mannose-6-phosphate
Oct	=	Octamer transcription factor
PBMC	=	Peripheral blood mononuclear cell
PE	=	Phosphatidylethanolamine
pI	=	Isoelectric point
PI3K	=	Phosphoinositide 3-kinase
PNS	=	Peripheral nervous system
ROR	=	Orphan nuclear receptor
RORE	=	ROR response element
S1P	=	Sphingosine-1-phosphate
SDS-PAGE	=	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
SK	=	Sphingosine kinase
Sp	=	Sp family member

REFERENCES

[1] Kishimoto, Y.; Hiraiwa, M.; O'Brien, J.S. Saposins: structure, function, distribution and molecular genetics. *J. Lipid Res.*, **1992**, *33*, 1255-1267.

[2] Kolter, T.; Sandhoff, K. Principles of lysosomal membrane digestion: stimulation of sphingolipid degradation by sphingolipid acti-

vator proteins and anionic lysosomal lipids. *Annu. Rev. Cell Dev. Biol.*, **2005**, *21*, 81-103.

[3] Hiraiwa, M.; Martin, B.M.; Kishimoto, Y.; Conner, G.E.; Tsuji, S.; O'Brien, J.S. Lysosomal proteolysis of prosaposin, precursor of saposins [sphingolipid activator proteins]:its mechanism and inhibition by gangliosides. *Arch. Biochem. Biophys.*, **1997**, *341*, 17-24.

[4] Hineno, T.; Sano, A.; Kondoh, K.; Ueno, S.; Kakimoto, Y.; Yoshida, K. Secretion of sphingolipid hydrolase activator precursor, prosaposin. *Biochem. Biophys. Res. Commun.*, **1991**, *176*, 668-674.

[5] Hiraiwa, M.; O'Brien, J.S.; Kishimoto, Y.; Galdzicka, M.; Fluharty, A.L.; Ginns, E.I.; Martin, B.M. Isolation, characterization, and proteolysis of human prosaposin, the precursor of saposins [Sphingolipid Activator Proteins]. *Arch. Biochem. Biophys.*, **1993**, *304*, 110-116.

[6] Fu, Q.; Carson, G.F.; Hiraiwa, M.; Grafe, M.; Kishimoto, Y.; O'Brien, J.S. Occurrence of prosaposin as a neuronal surface membrane component. *J. Mol. Neurosci.*, **1994**, *5*, 59-67.

[7] Misasi, R.; Sorice, M.; Garofalo, T.; Griggi, T.; Campana, W.M.; Giammatteo, M.; Pavan, A.; Hiraiwa, M.; Pontieri, G.M.; O'Brien, J.S. Colocalization and complex formation between prosaposin and monosialoganglioside GM3 in neural cells. *J. Neurochem.*, **1998**, *71*, 2313-2321.

[8] Sano, A.; Hineno, T.; Mizuno, T.; Kondoh, K.; Ueno, S.; Kakimoto, Y.; Inui, K. Sphingolipid hydrolase activator proteins and their precursor. *Biochem. Biophys. Res. Commun.*, **1989**, *165*, 1191-1197.

[9] Sprecher-Levy, H,; Orr-Urtreger, A.; Lona, P.; Horowitz, M. Murine prosaposin: expression in the reproductive system of a gene implicated in human genetic disease. *Cell. Mol. Biol.*, **1993**, *39*, 287-299.

[10] Sun, Y.; Witte, D.P.; Grabowski, G.A. Developmental and tissue-specific expression of prosaposin mRNA in murine tissues. *Am. J. Pathol.*, **1994**, *145*, 1390-1398.

[11] Kreda, S.M.; Fujita, N.; Suzuki, K. Expression of sphingolipid activator protein gene in brain and systemic organs of developing mice. *Dev. Neurosci.*, **1994**, *16*, 90-99.

[12] Harzer, K.; Paton, B.C.; Poulos, A.; Kustermann-Kuhn, B.; Roggendorf, W.; Grisar, T.; Popp, M. Sphingolipid activator deficiency in a 16 week old atipical Gaucher disease patient and its fetal sibling: biochemical signs of combined sphingolipidoses. *Eur. J. Pediatr.*, **1989**, *149*, 31-39.

[13] Bradovà, V.; Smid, F.; Ulrich-Bott, B.; Roggendorf, W.; Paton, B.C.; Harzer, K. Prosaposin deficiency: further characterization of the sphingolipid activator protein-deficient sibs. *Hum. Genet.*, **1993**, *92*, 143-152.

[14] Sikora, J.; Harzer, K.; Elleder, M. Neurolysosomal pathology in human prosaposin deficiency suggests essential neurotrophic function of prosaposin. *Acta Neuropathol.*, **2007**, *113*, 163-175.

[15] Elleder, M.; Jerábková, M.; Befekadu, A.; Hrebícek, M.; Berná, L.; Ledvinová, J.; Hulková, H.; Rosewich, H.; Schymik, N.; Paton, B.C.; Harzer, K. Prosaposin deficiency -- a rarely diagnosed, rapidly progressing, neonatal neurovisceral lipid storage disease. Report of a further patient. *Neuropediatrics*, **2005**, *36*, 171-180.

[16] Hulková, H.; Cervenková, M.; Ledvinová, J.; Tocháckova, M.; Hrebícek, M.; Poupetová, H.; Befekadu, A.; Berná, L.; Paton, B.C.; Harzer, K.; Böör, A.; Smíd, F.; Elleder, M. A novel mutation in the coding region of the prosaposin gene leads to a complete deficiency of prosaposin and saposins, and is associated with a complex sphingolipidosis dominated by lactosylceramide accumulation. *Hum. Mol. Genet.*, **2001**, *10*, 927-940.

[17] Schnabel, D.;, Schroderl, M.; Furst, W.; Klein, A.; Hurwitz, R.; Zenk, T.; Weberg, J.; Harzer, K.; Paton, B.C.; Poulos, A.; Suzuki, K.; Sandhoff, K. Simultaneous deficiency of sphingolipid activator proteins 1 and 2 is caused by a mutation in the initiation codon of their common gene. *J. Biol. Chem.*, **1992**, *267*, 3312-3315.

[18] Fujita, N.; Suzuki, K.; Vanier, M.T.; Popko, B.; Maeda, N.; Klein, A.; Henseler, M.; Sandhoff, K.; Nakayasu, H.; Suzuki, K. Targeted disruption of the mouse sphingolipid activator protein gene: a complex phenotype, including severe leukodystrophy and wide-spread storage of multiple sphingolipids. *Hum. Mol. Genet.*, **1996**, *5*, 711-725.

[19] Oya, Y.; Nakayasu, H.; Fujita, N.; Suzuki, K.; Suzuki, K. Pathological study of mice with total deficiency of sphingolipid activator proteins [SAP knockout mice]. *Acta Neuropathol.*, **1998**, *96*, 29-40.

[20] O'Brien, J.S.; Carson, G.S.; Seo, H.C.; Hiraiwa, M.; Kishimoto, Y. Identification of prosaposin as a neurotrophic factor. *Proc. Natl. Acad. Sci. USA*, **1994**, *91*, 9593-9596.

[21] O'Brien, J.S.; Carson, G.S.; Seo, H.C.; Hiraiwa, M.; Weiler, S.; Tomich, J.M.; Barranger, J.A.; Kahn, M.; Azuma, N.; Kishimoto, Y. Identification of the neurotrophic factor sequence of prosaposin. *FASEB J.*, **1995**, *9*, 681-685.

[22] Hiraiwa, M.; Taylor, E.M.; Campana, W.M.; Darin, S.J.; O'Brien, J.S. Cell death prevention, mitogen-activated protein kinase stimulation, and increased sulfatide concentrations in Schwann cells and oligodendrocytes by prosaposin and prosaptides. *Proc. Natl. Acad. Sci. USA*, **1997**, *94*, 4778-4781.

[23] Campana, W.M.; Hiraiwa, M.; O'Brien, J.S. Prosaptide activates the MAPK pathway by a G-protein-dependent mechanism essential for enhanced sulfatide synthesis by Schwann cells. *FASEB J.*, **1998**, *12*, 307-314.

[24] Campana, W.M.; Darin, S.J.; O'Brien, J.S. Phosphatidylinositol 3-kinase and Akt protein kinase mediate IGF-I- and prosaptide-induced survival in Schwann cells. *J. Neurosci. Res.*, **1999**, *57*, 332-341.

[25] Mehl, E.; Jatzkewitz, H. Eine *cerebrosidsulfatase* aus schweineniere. *Hoppe-Seyler's Z. Physiol. Chem.*, **1964**, *339*, 260-276.

[26] O'Brien, J.S.; Kretz, K.A.; Dewji, N.N.; Wenger, D.A.; Esch, F.; Fluharty, A.L. Coding of two sphingolipid activator proteins [SAP-1 and SAP-2] by same genetic locus. *Science*, **1988**, *241*, 1098-1101.

[27] Inui, K.; Kao, F.T.; Fujibayashi, S.; Jones, C.; Morse, H.G.; Law, M.L.; Wenger, D.A. The gene coding for a sphingolipid activator SAP-2, is on chromosome 10. *Hum. Genet.*, **1985**, *69*, 197-200.

[28] Fujibayashi, S.; Kao, F.T.; Jones, C.; Morse, H.; Law, M.; Wenger, D.A. Assignment of the gene for humansphingolipid activator protein-2 [SAP-2] to chromosome 10. *Am. J. Hum. Genet.*, **1985**, *37*, 741-748.

[29] Kao, F.T.; Law, M.L.; Hartz, J.; Jones, C.; Zhang, X.L.; Dewji, N.N.; O'Brien, J.S.; Wenger, D.A. Regional localization of the gene coding for sphingolipid activator protein [SAF1] on human chromosome 10. *Somat. Cell Mol. Genet.*, **1988**, *13*, 685 -688.

[30] Zhang, X.; Rafi, M.A.; DeGala, G.; Wenger, D.A. Insertion in the mRNA of the methacromatic leukodystrophy patient with sphingolipid activator-1 deficiency. *Proc. Natl. Acad. Sci. USA.*, **1990**, *87*, 1426-1430.

[31] Kretz, K.A.; Carson, G.S.; Morimoto, S.; Kishimoto, Y.; Fluharty, A.L.; O'Brien, J.S. Characterization of a mutation in a family with saposin B deficiency: a glycosylation site defect. *Proc. Natl. Acad. Sci. USA*, **1990**, *87*, 2541-2544.

[32] Zhang, X.; Rafi, M.A.; DeGala, G.; Wenger, D.A. The mechanism for a 33-nucleotide insertion in mRNA causing sphingolipid activator protein [SAP-1] deficient methacromatic leukodystrophy. *Hum. Genet.*, **1991**, *87*, 211-215.

[33] Schnabel, D.; Schroder, M.; Sandhoff, K. Mutation in the sphingolipid activator protein 2 in a patient with a variant of Gaucher disease. *FEBS Lett.*, **1991**, *284*, 57-59.

[34] Matsuda, J.; Vanier, M.T.; Saito, Y.; Tohyama, J.; Suzuki, K.; Suzuki, K. A mutation in the saposin A domain of the sphingolipid activator protein [prosaposin] gene results in a late-onset, chronic form of globoid cell leukodystrophy in the mouse. *Hum. Mol. Genet.*, **2001**, *10*, 1191-1199.

[35] Rafi, M.A.; Zhang, X.-L.; DeGala, G.; Wenger, D.A. Detection of a point mutation in sphingolipid activator protein-1 mRNA in patients with a variant from of metachromatic leukodystrophy. *Biochem. Biophys. Res. Commun.*, **1990**, *166*, 1017-1023.

[36] Spiegel, R.; Bach, G.; Sury, V.; Mengistu, G.; Meidan, B.; Shalev, S.; Shneor, Y.; Mandel, H.; Zeigler, M. A mutation in the saposin A coding region of the prosaposin gene in an infant presenting as Krabbe disease: first report of saposin A deficiency in humans. *Mol. Genet. Metab.*, **2005**, *84*, 160-166.

[37] Dewji, N.N.; Wenger, D.A.; O'Brien, J.S. Nucleotide sequence of cloned cDNA for human sphingolipid activator protein 1 precursor. *Proc. Natl. Acad. Sci. USA*, **1987**, *84*, 8652-8656.

[38] Nakano, T.; Sandhoff, K.; Stümper, J.; Christomanou, H.; Suzuki, K.Structure of full-length cDNA coding for sulfatide activator, a Co-beta-glucosidase and two other homologous proteins: two alternate forms of the sulfatide activator. *J. Biochem.*, **1989**, *105*, 152-154.

[39] Morimoto, S.; Martin, B.M.; Kishimoto, Y.; O'Brien, J.S. Saposin D: a sphingomyelinase activator. *Biochem. Biophys. Res. Commun.*, **1988**, *156*, 403-410.

[40] Fürst, W.; Machleidt, W.; Sandhoff, K. The precursor of sulfatide

activator protein is processed to three different proteins. *Biol. Chem. Hoppe-Seyler*, **1988**, *369*, 317-328.

[41] Morimoto, S.; Martin, B.M.; Yamamoto, Y.; Kretz, K.A.; O'Brien, J.S.; Kishimoto, Y. Saposin A: a second cerebrosidase activator protein. *Proc. Natl. Acad. Sci. USA*, **1989**, *86*, 3389-3393.

[42] Azuma, N.; Seo, H.C.; Lie, O.; Fu, Q.; Gould, R.M.; Hiraiwa, M.; Burt, D.W.; Paton, I.R.; Morrice, D.R.; O'Brien, J.S.; Kishimoto, Y. Cloning, expression and map assignment of chicken prosaposin. *Biochem. J.*, **1998**, *330*, 321-327.

[43] Kondoh, K.; Hineno, T.; Sano, A.; Kakimoto, Y. Isolation and characterization of prosaposin from human milk. *Biochem. Biophys. Res. Commun.*, **1991**, *181*, 286-292.

[44] Ito, K.; Takahashi, N.; Takahashi, A.; Shimada, I.; Arata, Y.; O'Brien, J.S.; Kishimoto, Y. Structural study of the oligosaccharide moieties of sphingolipid activator proteins, saposins A, C and D obtained from the spleen of a Gaucher patient. *Eur. J. Biochem.*, **1993**, *215*, 171-179.

[45] Hiraiwa, M.; Soeda, S.; Martin, B.M.; Fluharty, A.L.; Hirabayashi, Y.; O'Brien, J.S.; Kishimoto, Y. The effect of carbohydrate removal on stability and activity of saposin B. *Arch. Biochem. Biophys.*, **1993**, *303*, 326-331.

[46] Munford, R.S.; Sheppard, P.O.; O'Hara, P.J. Saposin-like proteins [SAPLIP] carry out diverse functions on a common backbone structure. *J. Lipid Res.*, **1995**, *36*, 1653-1663.

[47] Ahn, V.E.; Leyko, P.; Alattia, J.R.; Chen, L.; Privé, G.G. Crystal structures of saposins A and C. *Protein Sci.*, **2006**, *15*, 1849-1857.

[48] Ahn, V.E.; Faull, K.F.; Whitelegge, J.P.; Fluharty, A.L.; Privé, G.G. Crystal structure of saposin B reveals a dimeric shell for lipid binding. *Proc. Natl. Acad. Sci. USA*, **2003**, *100*, 38-43.

[49] Rossmann, M.; Schultz-Heienbrok, R.; Behlke, J.; Remmel, N.; Alings, C.; Sandhoff, K.; Saenger, W.; Maier, T. Crystal structures of human saposins C andD: implications for lipid recognition and membrane interactions. *Structure*, **2008**, *16*, 809-817.

[50] Hiraiwa, M.; Soeda, S.; Kishimoto, Y.; O'Brien, J.S. Binding and transfport of gangliosides by prosaposin. *Proc. Natl. Acad. Sci. USA*, **1991**, *89*, 11254-11258.

[51] Sylvester, S.R.; Morales, C.; Oko, R.; Griswold, M.D. Sulfated glycoprotein-1 [saposin precursor] in the reproductive tract of the male rat. *Biol. Reprod.*, **1989**, *41*, 941-948.

[52] Morales, C.R.; El-Alfy, M.; Zhao, Q.; Igdoura, S.A. Expression and tissue distribution of rat sulphated glycoprotein-1 [prosaposin] *J. Histochem. Cytochem.*, **1996**, *44*, 327-337.

[53] Fujibayashi, S.; Wenger, D.A. Biosynthesis of the sulfatide/GMl activator protein [SAP-1] in control and mutant cultured skin fibroblasts. *Biochim. Biophys. Acta*, **1986**, *875*, 554-562.

[54] Rijnboutt, S.; Aert, H.M.F.G.; Geuze, H.J.; Tager, J.M.; Strous, G.J. Mannose 6-phosphate-independent membrane: association of cathepsin D, glucocerebrosidase, and the sphingolipid activating protein in HepGZ cells. *J. Biol. Chem.*, **1991**, *266*, 4862-4868.

[55] Henseler, M.; Klein, A.; Glombitza, G.J.; Suzuki, K.; Sandhoff, K. Expression of the three alternative forms of the sphingolipid activator protein precursor in baby hamster kidney cells and functional assays in a cell culture system. *J. Biol. Chem.*, **1996**, *271*, 8416-8423.

[56] Vielhaber, G.; Hurwitz, R.; Sandhoff, K. Biosynthesis, processing and targeting of sphingolipid activator protein [SAP] precursor in cultured human fibroblasts. mannose 6 phosphate receptor-indipendent endocytosis of SAP precursor. *J. Biol. Chem.*, **1996**, *271*, 32438-32446.

[57] Hiesberger, T.; Huttler, S.; Rohlmann, A.; Schneider, W.; Sandhoff, K.; Herz, J. Cellular uptake of saposin [SAP] precursor and lysosomal delivery by the low density lipoprotein receptor-related protein [LRP]. *EMBO J.*, **1998**, *17*, 4617-4625.

[58] Vogel, A.; Schwarzmann, G.; Sandhoff, K. Glycosphingolipid specificity of the human sulfatide activator protein. *Eur. J. Biochem.*, **1991**, *200*, 591-597.

[59] Tsuboi, K.; Hiraiwa, M.; O'Brien, J.S. Prosaposin prevents programmed cell death of rat cerebellar granule neurons in culture. *Dev. Brain Res.*, **1998**, *110*, 249-255.

[60] Qi, X.; Kondoh, K.; Krusling, D.; Kelso, G.J.; Leonova, T.; Grabowski, G.A. Conformational and amino acid residue requirements for the saposin C neuritogenic effect. *Biochemistry*, **1999**, *38*, 6284-6291.

[61] Campana, W.M.; Hiraiwa, M.; Addison, K.C.; O'Brien, J.S. Induction of MAPK phosphorylation by prosaposin and prosaptide in PC12 cells". *Biochem. Biophys. Res. Commun.*, **1996**, *229*, 706-712.

[62] Misasi, R.; Sorice, M.; Di Marzio, L.; Campana, W.M.; Molinari, S.; Cifone, M.G.; Pavan, A.; Pontieri, G.M.; O'Brien, J.S. Prosaposin treatment induces PC12 entry in the S phase of the cell cycle and prevents apoptosis: activation of ERK's and sphingosine kinase. *FASEB J.*, **2001**, *15*, 467-474.

[63] Misasi, R.; Garofalo, T.; Di Marzio, L.; Mattei, V.; Gizzi, C.; Hiraiwa, M.; Pavan, A.; Cifone, M.G.; Sorice, M. Prosaposin: a new player in the cell death prevention of U937 monocytic cells. *Exp. Cell Res.*, **2004**, *298*, 38-47.

[64] Misasi, R.; Hiraiwa, M.; Sorice, M.; Garofalo, T.; Pavan, A.; Pontieri, G.M.; O'Brien, J.S. Functional role of ganglioside-prosaposin interaction in neuronal cells. *Recent Res. Devel. Neurochem.*, **2000**, *3*, 301-316.

[65] Ledesma, M.; Simons, K.; Dotti, C.G. Neuronal polarity: essential role of protein-lipid complexes is axonal sorting. *Proc. Natl. Acad. Sci. USA*, **1998**, *95*, 3966-3971.

[66] Simons, K.; Toomre, D. Lipid rafts and signal transduction, *Nat. Rev. Mol. Cell. Biol.*, **2000**, *1*, 31-41.

[67] Parolini, I.; Sargiacomo, M.; Lisanti, M.P.; Peschle, C. Signal transduction and glycophosphatidylinositol-linked proteins [lyn, lck, CD4, CD45, G-proteins, and CD55] selectively localize in Triton-insoluble plasma membrane domains of human leukemic cell lines and normal granulocytes. *Blood*, **1996**, *87*, 3783-3794.

[68] Hiraiwa, M.; Campana, W.M.; Martin, B.M.; O'Brien, J.S. Prosaposin receptor: evidence for a G-protein-associated receptor. *Biochem. Biophys. Res. Commun.*, **1997**, *240*, 415-418.

[69] Stewart, H.J.; Bradke, F.; Tabernero, A.; Morrell, D.; Jessen, K.R.; Mirsky, R. Regulation of rat Schwan cell P0 expression and DNA synthesis by insulin-like growth factors *in vitro*. *Eur. J. Neurosci.*, **1996**, *8*, 553-564.

[70] Koochekpour, S.; Sartor, O.; Hiraiwa, M.; Lee, T.J.; Rayford, W.; Remmel, M.; Sandhoff, K.; Minokadeh, A.; Patten, D.Y. Saposin C stimulates growth and invasion, activates p42/44 and SAPK/JNK signaling pathways of MAPK and upregulates uPA/uPAR. Expression in prostate cancer and stromal cells. *Asian J. Androl.*, **2005**, *7*, 147-158.

[71] Koochekpour, S.; Zhuang, Y.J.; Beroukhim, R.; Hsieh, C.L.; Hofer, M.D.; Zhau, H.E.; Hiraiwa, M.; Patten, D.Y.; Ware, J.L.; Luftig, R.B.; Sandhoff, K.; Sawyers, C.L.; Pienta, K.J.; Rubin, M.A.; Vessella, R.L.; Sellers, W.R.; Sartor, O. Amplification and overexpression of prosaposin in prostate cancer. *Genes Chromosomes Cancer*, **2005**, *44*, 351-364.

[72] Koochekpour, S.; Lee, T.J.; Wang, R.; Sun, Y.; Delorme, N.; Hiraiwa, M.; Grabowski, G.A.; Culig, Z.; Minokadeh, A. Prosaposin is a novel androgen-regulated gene in prostate cancer cell line LNCaP. *J. Cell Biochem.*, **2007**, *101*, 631-641.

[73] Koochekpour, S.; Lee, L.J.; Wang, R.; Sun, Y.; Delorme, N.; Hiraiwa, M.; Grabowski, G.A.; Culig, Z.; Minokadeh, A. Prosaposin is a novel androgen-regulated gene in prostate cancer cell line LNCaP. *J. Cell Biochem.*, **2007**, *101*, 631-641.

[74] Heemers, H.V.; Verhoeven, G.; Swinnen, J.V. Androgen activation of the sterol regulatory element-binding protein pathway: Current insights. *Mol. Endocrinol.*, **2006**, *10*, 2265-2277.

[75] Ochiai, T.; Takenaka, Y.; Kuramoto, Y.; Sakata, A.; Kimura, M.; Shimeno, H.; Hiraiwa, M.; Misasi, R.; Soeda, S. Molecular mechanism for neuroprotective effect of prosaposin against oxidative stress: its regulation of dimeric transcription factor formation. *Biochim. Biophys. Acta*, **2008**, *1780*, 1441-1447.

[76] Holtschmidt, H.; Sandhoff, K.; Furst, W.; Kwon, H.Y.; Schnabel, D.; Suzuki, K. The organization of the gene for the human cerebroside sulfate activator protein. *FEBS Lett.*, **1991**, *280*, 267-270.

[77] Rorman, E.G.; Scheinker, V.; Grabowski, G.A. Structure and evolution of the human prosaposin chromosomal gene. *Genomics*, **1992**, *13*, 312-318.

[78] Jin, P.; Sun, Y.; Grabowski, G.A. Role of Sp proteins and RORalpha in transcription regulation of murine prosaposin. *J. Biol. Chem.*, **1998**, *273*, 13208-13216.

[79] Lindvall, O.; Kokaia, Z.; Bengzon, J.; Elme'r, E.; Kokaia, M. Neurotrophins and brain insults. *Trends Neurosci.*, **1994**, *17*, 490-496.

[80] Gillen, C.; Gleichmann, M.; Spreyer, P.; Müller, H.W. Differential expressed gene after peripheral nerve injury. *J. Neurosci. Res.*, **1995**, *42*, 159-171.

[81] Yokota, N.; Uchijima, M.; Nishizawa, S.; Namba, H.; Koide, Y. Identification of differential expressed genes in rat hippocampus after transient global cerebral ischemia using subtractive cDNA clon-

ing based on polymerase chain reaction. *Stroke*, **2001**, *32*, 168-174.
[82] Hiraiwa, M.; Liu, J.; Lu, A.G.; Wang, C.Y.; Misasi, R.; Yamauchi, T.; Hozumi, I.; Imuzuka, T.; O'Brien, J.S. Regulation of gene expression in response to brain injury: enhanced expression and alternative splicing of rat prosaposin [SGP-1] mRNA in injured brain. *J. Nuerotrauma*, **2003**, *20*, 755-765.
[83] Holtschmidt, H.; Sandhoff, K.; Kwon, H.Y.; Harzer, K.; Nakano, T.; Suzuki, K. Sulfatide activator protein. Alternative splicing that generates three mRNAs and a newly found mutation responsible for a clinical disease. *J. Biol. Chem.*, **1991**, *266*, 7556-7560.
[84] Lamontagne, S.; Potier, M. Modulation of human saposin B sphingolipid-binding specificity by alternative splicing. A study with saposin B-derived synthetic peptides. *J. Biol. Chem.*, **1994**, *269*, 20528-20532.
[85] Madar-Shapiro, L.; Pasmanik-Chor, M.; Vaccaro, A.M.; Dinur, T.; Dagan, A.; Gatt, S.; Horowitz, M. Importance of splicing for prosaposin sorting. *Biochem. J.*, **1999**, *337*, 433-443.
[86] Cohen, T.; Auerbach, W.; Ravid, L.; Bodennec, J.; Fein, A.; Futerman, A.H.; Joyner, A.L.; Horowitz, M. The exon 8-containing prosaposin gene splice variant is dispensable for mouse development, lysosomal function, and secretion. *Mol. Cell. Biol.*, **2005**, *25*, 2431-2440.
[87] Gallo, J.M.; Jin, P.; Thornton, C.A.; Lin, H.; Robertson, J.; D'Souza, I.; Schlaepfer, W.W. The role of RNA and RNA processing in neurodegeneration. *J. Neurosci.*, **2005**, *25*, 10372-10375.
[88] D'Souza, I.; Schellenberg, G.D. Regulation of tau isoform expression and dementia. *Biochim. Biophys. Acta*, **2005**, *1739*, 104-115.
[89] Chambers, C.B.; Muma, N.A. Tau mRNA isoforms following sciatic nerve axotomy with and without regeneration. *Brain Res. Mol. Brain Res.*, **1997**, *48*, 115-124.
[90] Liu, W.K.; Dickson, D.W.; Yen, S.H. Heterogeneity of tau proteins in Alzheimer's disease. Evidence for increased expression of an isoform and preferential distribution of a phosphorylated isoform in neurites. *Am. J. Pathol.*, **1993**, *142*, 387-394.
[91] Hiraiwa, M.; Campana, W.M.; Mizisin, A.P.; Mohiuddin, L.; O'Brien, J.S. Prosaposin: a myelinotrophic protein that promotes expression of myelin constituents and is secreted after nerve injury. *Glia*, **1999**, *26*, 353-360.
[92] Yan, L.; Otero, D.A.; Hiraiwa, M.; O'Brien, J.S. Prosaptide D5 reverses hyperalgesia: inhibition of calcium channels through a pertussis toxin-sensitive G-protein mechanism in the rat. *Neurosci. Lett.*, **2000**, *278*, 120-122.
[93] Sano, A.; Matsuda, S.; Wen, T.C.; Kotani, Y.; Kondoh, K.; Ueno, S.; Kakimoto, Y.; Yoshimura, H.; Sakanaka, M. Protection by prosaposin against ischemia-induced learning disability and neuronal loss. *Biochem. Biophys. Res. Commun.*, **1994**, *204*, 994-1000.
[94] Kotani, Y.; Matsuda, S.; Wen, T.C.; Sakanaka, M.; Tanaka, J.; Maeda, N.; Kondoh, K.; Ueno, S.; Sano, A. A hydrophilic peptide comprising 18 amino acid residues of the prosaposin sequence has neurotrophic activity *in vitro* and *in vivo*. *J. Neurochem.*, **1996**, *66*, 2197-2200.
[95] Igase, K.; Tanaka, J.; Kumon, Y.; Zhang, B.; Sadamoto, Y.; Maeda, N.; Sakaki, S.; Sakanaka, M. An 18-mer peptide fragment of prosaposin ameliorates place navigation disability, cortical infarction, and retrograde thalamic degeneration in rats with focal cerebral ischemia. *J. Cereb. Blood Flow Metab.*, **1999**, *19*, 298-306.
[96] Lu, A.G.; Otero, D.A.; Hiraiwa, M.; O'Brien, J.S. Neuroprotective effect of retro-inverso prosaptide D5 on focal cerebral ischemia in rat. *Neuroreport*, **2000**, *11*, 1791-1794.
[97] Hozumi, I.; Hiraiwa, M.; Inuzuka, T.; Yoneoka, Y.; Akiyama, K.; Tanaka, R.; Kikugawa, K.; Nakano, R.; Tsuji, S.; O'Brien, J.S. Administration of prosaposin ameliorates spatial learning disturbance and reduces cavity formation following stab wounds in rat brain. *Neurosci. Lett.*, **1999**, *267*, 73-76.
[98] Kotani, Y.; Matsuda, S.; Sakanaka, M.; Kondoh, K.; Ueno, S.; Sano, A. Prosaposin facilitates sciatic nerve regeneration *in vivo*. *J. Neurochem.*, **1996**, *66*, 2019-2025.
[99] Calcutt, N.A.; Freshwater, J.D.; O'Brien, J.S. Protection of sensory function and antihyperalgesic properties of a prosaposin-derived peptide in diabetic rats. *Anesthesiology*, **2000**, *93*, 1271-1278.
[100] Jolivalt, C.G.; Vu, Y.; Mizisin, L.M.; Mizisin, A.P.; Calcutt, N.A. Impaired prosaposin secretion during nerve regeneration in diabetic rats and protection of nerve regeneration by a prosaposin-derived peptide. *J. Neuropathol. Exp. Neurol.*, **2008**, *67*, 702-710.
[101] Jolivalt, C.G.; Ramos, K.M.; Herbetsson, K.; Esch, F.S.; Calcutt, N.A. Therapeutic efficacy of prosaposin-derived peptide on different models of allodynia. *Pain*, **2006**, *121*, 14-21.
[102] Jolivalt, C.G.; Dacunha, J.M.; Esch,F.S.; Calcutt, N.A. Central action of prosaptide TX14[A] against gp120-induced allodynia in rats. *Eur. J. Pain*, **2008**, *12*, 76-81.
[103] Hiraiwa, M.; Campana, W.M.; Wang, C.Y.; Otero, D.A.; O'Brien, J.S. A retro-inverso prosaptide D5 promotes a myelination process in developing rats. *Dev. Brain Res.*, **2001**, *128*, 73-76.

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An alternative spliced mouse presenilin-2 mRNA encodes a novel γ-secretase inhibitor

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ABSTRACT

The γ-secretase, composed of presenilin-1 (PS1) or presenilin-2 (PS2), nicastrin (NCT), anterior pharynx-defective phenotype 1 (APH-1), and PEN-2, is critical for the development of Alzheimer's disease (AD). PSs are autoproteolytically cleaved, producing an N-terminal fragment (NTF) and a hydrophilic loop domain-containing C-terminal fragment. However, the role of the loop domain in the γ-secretase complex assembly remains unknown. Here, we report a novel PS2 isoform generated by alternative splicing, named PS2β, which is composed of an NTF with a hydrophilic loop domain. PS2β disturbed the interaction between NCT and APH-1, resulting in the inhibition of amyloid-β production. We concluded that PS2β may inhibit γ-secretase activity by affecting the γ-secretase complex assembly.

Structured summary:
MINT-7025654: *APH1* (uniprotkb:Q96BI3) *physically interacts* (MI:0218) with *PEN2* (uniprotkb:Q9NZ42), *PS2 beta* (uniprotkb:Q61144-2) and *PS1* (uniprotkb:P49769) by *anti tag coimmunoprecipitation* (MI:0007)
MINT-7025631: *APH1* (uniprotkb:Q96BI3) *physically interacts* (MI:0218) with *NCT* (uniprotkb:Q92542), *PEN2* (uniprotkb:Q9NZ42) and *PS1* (uniprotkb:P49769) by *anti tag coimmunoprecipitation* (MI:0007)

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by memory impairment and amyloid-β protein (Aβ) deposition in the brain. Aβ is generated from the amyloid precursor protein (APP) by two distinct enzymes, β-secretase (or BACE) and γ-secretase. γ-Secretase is an intramembrane aspartyl protease composed of presenilin-1 (PS1) or presenilin-2 (PS2), nicastrin (NCT), presenilin enhancer 2 (PEN-2), and anterior pharynx defective-1 (APH-1) [1]. Presenilins are endoproteolytically processed at the hydrophilic loop domain near transmembrane domain 6 into two fragments that associate as a heterodimer: an N-terminal fragment (NTF) and a loop domain containing C-terminal fragment (CTF). NCT is glycosylated during γ-secretase complex assembly, which occurs during transport from the endoplasmic reticulum (ER) to the Golgi apparatus [2–4]. All four components (PS1 or PS2, NCT, PEN-2, APH-1) are required for γ-secretase activity and NCT maturation [5–7]. The current model for γ-secretase complex assembly progresses in three steps: initial assembly of NCT with APH-1 [8], presenilin binding to the NCT/APH-1 dimer, and PEN-2 incorporation into the PS/NCT/APH-1 trimer. The final step allows endoproteolysis of PS and formation of the complete γ-secretase complex [6], which includes one of each component [9]. Missense mutations in PS1 or PS2 increase the ratio of $A\beta_{1-42}$ to $A\beta_{1-40}$, leading to formation of Aβ aggregates, thereby leading to AD. By analysis of PS1 and PS2 knockout mice, Chen et al. [10] observed the differential effect of PS1 and PS2 on NCT maturation. However, a role of PS2 in the γ-secretase complex assembly remains obscure. In this study, we cloned a novel splicing isoform of PS2, PS2β containing a hydrophilic loop domain, but missing the C-terminal transmembrane domain. The interaction between NCT and APH-1 was inhibited in the presence of PS2β,

Abbreviations: PS, presenilin; PEN-2, presenilin enhancer 2; APH-1, anterior pharynx-defective phenotype 1; NCT, nicastrin; CHAPS, 3-cholamidopropyl dimethylammonio propanesulfonic acid; mRFP, monomeric red fluorescence protein

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leading to a disturbance in NCT maturation and a decrease in γ-secretase activity.

2. Materials and methods

2.1. Antibodies

Anti-PS2β antibody was generated in rats by serial immunization with the synthetic peptide CESKGA conjugated to KLH (Biogate Co., Ltd., Japan). Anti-FLAG antibody was purchased from Sigma (Saint Louis, MO).

2.2. DNA constructs

Mouse presenilins (PS1, PS2, PS2β) and human PEN-2, APH-1, and NCT cDNAs were tagged with HA (PS1, PS2, PS2β, and PEN-2), FLAG (APH-1), Myc (PS1), V5 (NCT), Xpress (PS2β), and monomeric red fluorescence protein (mRFP) (PS1, PS2β). FLAG, Myc, and V5 are tagged at C-terminal and the others are tagged at N-terminal. Human mannosidase II cDNA was inserted into pEGFP (Clontech, Mountain View, CA) as the Golgi apparatus marker [11]. The transmembrane domain (100–134 amino acid residues) of cb5 was fused to GFP and mRFP to generate the ER markers, ER-GFP and ER-mRFP, respectively.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR

RT-PCR was performed according to a previously described procedure [12]. PCR was carried out for 30 cycles with an annealing temperature of 65 °C. The PCR primer pairs were as follows: mouse PS2, 5′-AGGAGCTGGAGGAGGAGGAG-3′ (PS2W-1), 5′-ATGTAGAGCTGGTGGGAGGC-3′ (PS2W-2); mouse PS2β, 5′-AGGAGGAGGAAAGTAAGGGG-3′ (PS2BETA-2); mouse β-actin, 5′-GTTTGAGACCTTC-

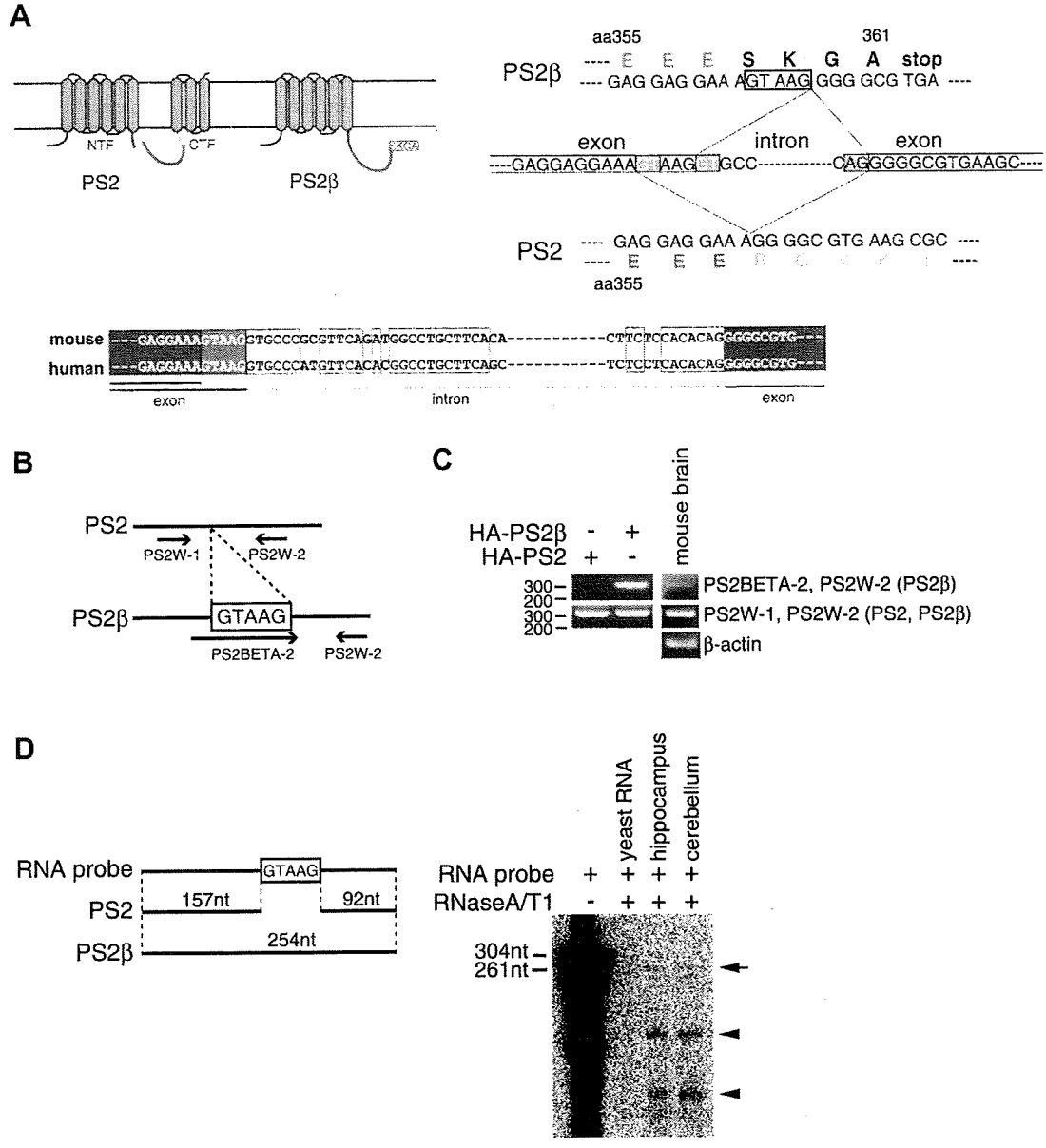

Fig. 1. Expression of an alternatively spliced isoform of mouse PS2. (A) Predicted structure of the PS2β (left). Alternative splicing of PS2 (right). PS2 gene (middle); PS2β cDNA (top); PS2 cDNA (bottom). Alignment between the mouse and human PS2 (NG_007381) genes (bottom). (B) Position of PCR primer. (C) PCR using plasmid as template (left) and RT-PCR (right). (D) RNase protection assay. The level of PS2β mRNA is about 12% of that of PS2 mRNA in hippocampus and cerebellum of adult mouse brain. RNA probe and protected fragment (left). arrow: PS2β; arrowhead: PS2.

AACACC-3′, 5′-GTGGTGGTGAAGCTGTAG-3′. Real-time PCR was performed using SYBR Green by Thermal Cycler Dice (Takara, Japan) according to the manufacturer's protocol. The PCR primer pairs have been described previously [13].

2.4. PS2β/SC100 transformant and ELISA

Xpress-tagged PS2β and pTK-Hyg (Clontech) were co-transfected into HEK293 cells stably expressing SC100 [13], selected with 600 μg/ml of hygromycin, by calcium-phosphate and DNA precipitation. ELISA was performed as described previously [13] using Aβ ELISA Kits (Wako, Japan). All measurements were performed in three independent experiments.

2.5. Immunoprecipitation

HEK293 cells were lysed in lysis buffer [50 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% 3-cholamidopropyl dimethylammonio propanesulfonic acid (CHAPS) (Dojindo, Japan)] supplemented with protease inhibitor (Roche Applied Science, Mannheim, Germany) and centrifuged at 12 000×g for 30 min. After the supernatant was precleared with protein A, immunoprecipitation was performed using an ANTI-FLAG M2 affinity gel (Sigma) according to the manufacturer's protocol.

2.6. Western blot

Subcellular fractionation was performed as described previously [12]. Cells and tissues were lysed in RIPA buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% Na deoxycholate, 0.1% SDS) or TTS buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 5 mM EDTA) containing protease inhibitors. Equal amounts of cell or tissue lysates were separated on SDS–PAGE and transferred to PVDF membranes (Immobilon P, Millipore, Billerica, MA).

2.7. Microscopic analysis

Cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) at 24 h after transfection and mounted on Perma Fluor™ (Thermo Shandon, Pittsburgh, PA). Samples were examined with confocal microscopy (LSM 510, Carl Zeiss, Oberkochen, Germany).

2.8. RNase protection assay

RNase protection assay was performed according to a previously described procedure [14]. Briefly, 0.6 μg of mRNA was hybridized with antisense RNA probe and then samples were digested by RNase A/T1 using RPA III™ Ribonuclease Protection Assay Kit (Ambion, Austin, TX). Antisense RNA probe was synthesized using the MAXIscript In Vitro Transcription Kit (Ambion) with [α-^{32}P]UTP (800 Ci/mmol, MP Biomedicals Inc., Solon, OH). The reactions were assessed on a 5% polyacrylamide/8M urea gel. Signal was calculated using Image J, corrected by number of UTP.

3. Results

3.1. Molecular cloning and expression of PS2β

To understand the role of PS2 in γ-secretase complex formation, we amplified the full-length mouse PS2 cDNA from mRNA of the mouse eye by RT-PCR. By coincidence, we cloned a novel isoform of PS2, whose molecular weight was smaller than the predicted size. It had an extra five nucleotides (GTAAG), leading to the translation of an additional four out-of-frame residues (SKGA) at the loop domain (Fig. 1A). By cloning the intron at the insertion point, we found that the exon was extended. Interestingly, the mouse and human splicing site sequences were highly conserved, especially at the boundary of the 5′ end of the intron (5′ splicing site). This splicing isoform, named PS2β, was composed of NTF and a hydrophilic loop domain, but it lacked the seventh transmembrane domain and downstream C-terminal regions. Under highly stringent conditions, the PS2β transcript was detected by RT-PCR (Fig. 1C) and RNase protection assay (Fig. 1D) from mouse brain mRNA, and the PS2β protein was enriched in the microsomal fraction detected by anti-ESKGA antibody, which specifically reacted with the PS2β protein (Fig. 2A and B). The PS2β loop domain-cleaved product was not observed in lysates from HEK293 cells stably expressing PS2β as observed with the active site mutated PS1 in the seventh transmembrane domain (data not shown). We performed confocal microscopy analysis using ER and Golgi apparatus markers to

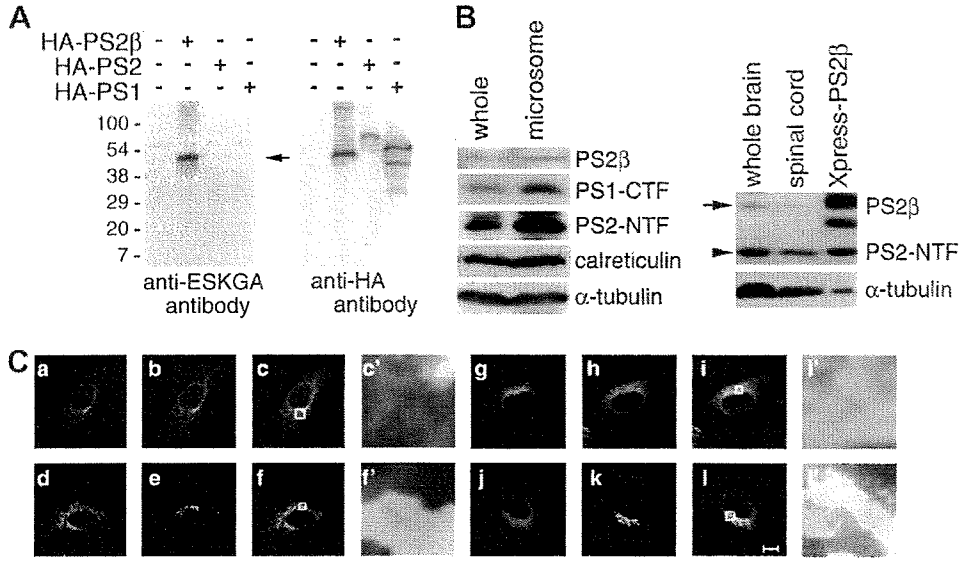

Fig. 2. Expression of PS2β protein. (A) Specificity of anti-ESKGA antibody. (B) Endogenous PS2β protein in the microsomal fraction of mouse brain (left). PS2β was detected by anti-PS2NTF antibody (right). Arrow: PS2β; arrowhead: PS2. (C) Localization of PS2β. (a, d) monomeric red fluorescence protein (mRFP)–PS2β; (g, j) mRFP–PS1; (b, h) ER–GFP; (e, k) mannosidase II-GFP; (c, f, i, l) merge; (c′, f′, i′, l′) higher magnification of the region marked by the square in c, f, i, l, respectively. Scale bars represent 5 μm.

examine the subcellular localization of PS2β (Fig. 2C). PS1 localized at the Golgi as well as ER (Fig. 2Cg–l), whereas PS2β mainly localized at the ER (Fig. 2Ca–f). Thus, we conclude that PS2β, which is composed of NTF and a hydrophilic loop domain, is expressed in the mouse brain and mainly localized at the ER.

3.2. PS2β inhibits the interaction between NCT and APH-1, leading to disturbance of NCT maturation

To examine the role of PS2β in NCT maturation, we determined whether PS2β was integrated into the γ-secretase complex. After transfection of PS2β with four γ-secretase components (NCT-V5, PS1-Myc, HA-PEN-2, APH-1-FLAG), an immunoprecipitation assay with the anti-FLAG antibody was carried out using lysis buffer containing 0.1% CHAPS (Fig. 3A). We found that the association between APH-1 and NCT was significantly inhibited in the presence of PS2β. The interaction of PS1 or PEN-2 with APH-1 was not disturbed. Interestingly, the NTF of PS2 (PS2N, Met^{1}-Lys^{306}) did not disturb the interaction between NCT and APH-1 (Fig. S1). To investigate whether γ-secretase complex was affected by the presence of PS2β, blue native polyacrylamide gel electrophoresis (BN-PAGE) was performed after transfection of all four γ-secretase components (NCT, PS1, PEN-2, APH-1) into parental HEK293 cells and HEK293 cells stably expressing PS2β. The anti-NCT antibody recognized a 440 kDa protein complex, which was observed in the other three components (PS1, PEN-2, and APH-1) by western blot (Fig. S2). The signal for the endogenous as well as exogenously expressed NCT band was drastically reduced, indicating that the incorporation of NCT into the γ-secretase complex was disturbed in the presence of PS2β (Fig. 3B). Next, we examined whether transportation of NCT was affected. A Western blot analysis revealed that brefeldin A, which disturbs Golgi function, decreased the upper (possibly glycosylated mature) NCT (Fig. 3C). The maturation of endogenous NCT was consistently inhibited in HEK293

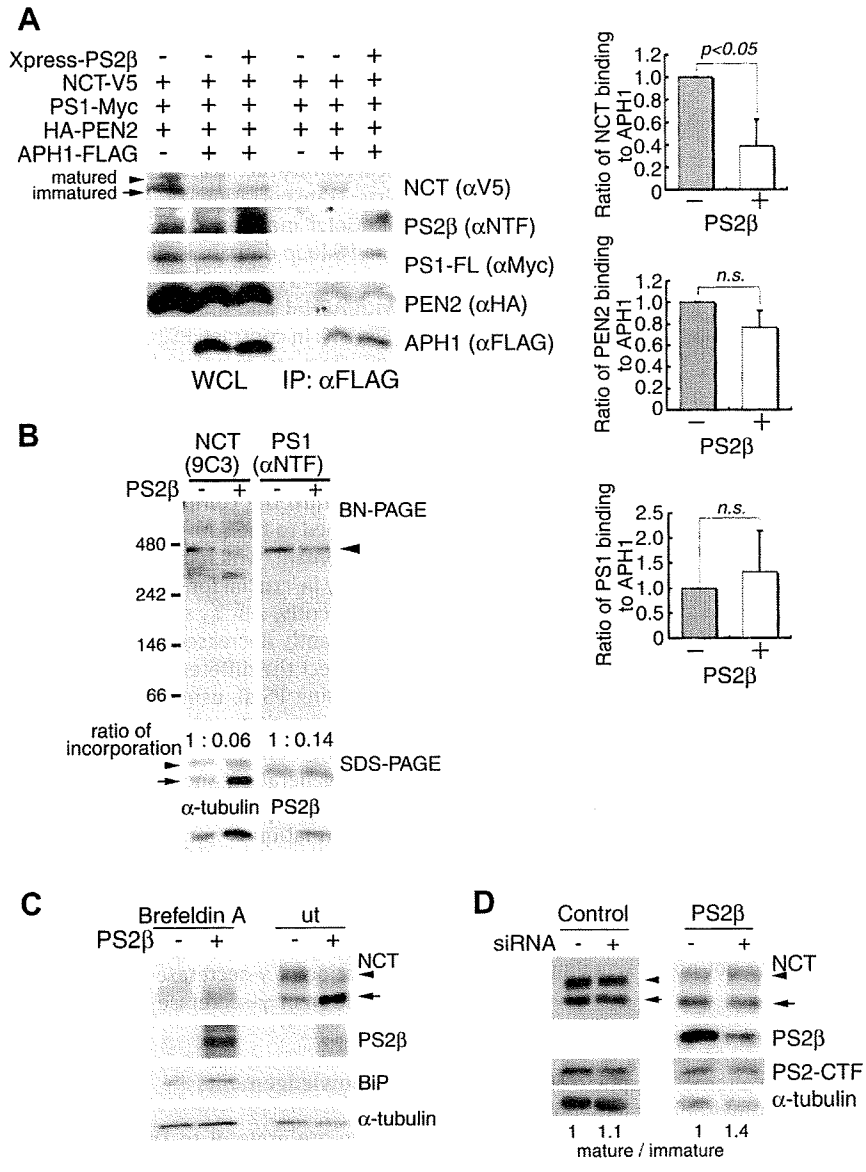

Fig. 3. PS2β disturbed γ-secretase complex assembly. (A) Immunoprecipitation of γ-secretase components with anti-Flag antibody (APH-1) after co-transfection into HEK293 cells. Data represent mean ± S.E.M in three independent experiments corrected by the APH-1 protein (right). (B) PS2β inhibited the incorporation of NCT into the γ-secretase complex in HEK293 cells stably expressing PS2β (arrowhead). top, BN-PAGE; middle and bottom, SDS-PAGE. (C) 10 μg/ml of brefeldin A treatment. (D) Matured NCT increased in HEK293 cells stably expressing PS2β after transfection of PS2 siRNA. arrowhead, matured form of endogenous NCT; arrow, immature NCT. Control: parental HEK293 cells; PS2β: HEK293 cells stably expressing PS2β.

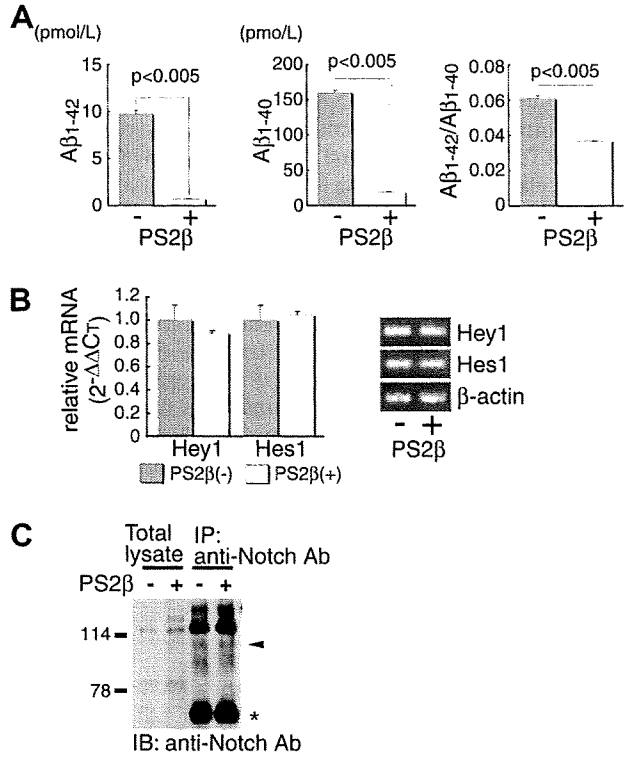

Fig. 4. PS2β disturbed γ-secretase activity. (A) Secreted $Aβ_{1-42}$ and $Aβ_{1-40}$ were measured by ELISA. (B) Real-time PCR of downstream target genes of notch. (C) NICD was similarly detected by western blotting after immunoprecipitation with anti-Notch antibody in both parental HEK293 cells and HEK293 cells stably expressing PS2β (arrowhead) PS2β(−), HEK293 cells; PS2β(+), HEK293 cells stably expressing PS2β. *: heavy chain.

cells stably expressing PS2β, which was rescued by PS2β knockdown (Fig. 3D). Thus, these findings suggest that the loop domain may be critical for γ-secretase complex assembly.

3.3. PS2β inhibits γ-secretase activity

To determine the role of PS2β in relation to γ-secretase activity, we first analyzed Aβ secretion by ELISA using HEK293 cells stably expressing PS2β, which also constitutively expressed the C-terminal region of APP SC100. $Aβ_{1-42}$ and $Aβ_{1-40}$ secretion was significantly inhibited in the presence of PS2β (Fig. 4A). In HEK293 cells, the expression of the downstream notch target genes hes1 and hey1 was regulated by γ-secretase activity [13]. In contrast, in HEK293 cells stably expressing PS2β, real-time PCR showed that hes1 and hey1 expression was not inhibited (Fig. 4B). Moreover, immunoprecipitation with anti-Notch antibody indicated that NICD in HEK293 cells stably expressing PS2β was detected similarly to that in HEK293 cells (Fig. 4C). These data suggest that PS2β mainly affect A production, but not notch signaling.

4. Discussion

In this study, we showed that an alternative splicing isoform of mouse PS2, composed of NTF and a hydrophilic loop domain, disturbs the interaction between NCT and APH-1 leading to inhibition of γ-secretase activity.

PS2β is produced by extension of the mouse PS2 gene exon 9 (NM_011183.2), which causes a frameshift mutation that creates a stop codon in the following exon and encodes for translation of four additional out-of-frame residues (SKGA). Sequences around the splicing sites were conserved between mouse and human (intron 14, NG_007381), suggesting that the extended exon may not be specific to mouse PS2 (Fig. 1A). A PS2β fragment containing GTAAG was detected in the mouse brain even under highly stringent PCR conditions, although its expression level was low (Fig. 1C and D). The PS2β protein was enriched in the microsomal fraction of mouse brain (Fig. 2B). Consistent with a previous study that endoproteolysis in the PS1 mutation of active sites (D385A in TM7) does not occur [15], endoproteolytic cleavage of PS2β was not observed by Western blot. Thus, PS2β existed in cells without endoproteolytic processing at the conventional site of the loop domain.

PS1 CTF, especially the C-terminal residues, interacts with NCT [16] through its transmembrane domain [17], which may be the trimeric assembly intermediate (PS/NCT/APH-1) during γ-secretase complex assembly [8]. In our study, PS2β interfered with the binding of NCT to APH-1 and with NCT incorporation into the γ-secretase complex even in the presence of PS1. However, the NTF of PS2 (PS2N) did not affect binding of NCT to APH-1 (Fig. S1). PS2β also inhibited NCT maturation (Fig. 3C), which was not due to disturbance of transportation, because wild-type CFTR protein was detected at the plasma membrane in the presence of PS2β (Fig. S3). Following treatment with brefeldin A, an inhibitor of intracellular protein transport at the ER–Golgi junction and in trans-Golgi, the upper band of NCT (arrowhead in Fig. 3C) disappeared, but the size of the lower band did not change, suggesting that transportation of NCT was specifically prevented at the ER–Golgi junction by PS2β which is mainly localized at the ER. The PS loop domain is dispensable for PS localization and γ-secretase activity [18,19]. Consistently, NTF of PS2 (PS2 N) interacted with APH-1 and did not affect the γ-secretase complex formation (Fig. S1). In contrast, PS2β, which is PS2N fused with the C-terminal loop domain, prevented the interaction of NCT to APH-1. Since APH-1 stabilize the PS holoprotein in the γ-secretase complex and endoproteolytic processing of PS is occurred after PEN-2 is joined to the complex[6], the holoprotein of PS may be critical for complex formation although the cleaved loop domain does not disturb the complex after all four components of γ-secretase are joined to the complex. These results indicate that a hydrophilic loop domain, which is fused to the C-terminal and not the NTFs of PS, may be crucial for completion of γ-secretase complex assembly. Consistently, $Aβ_{1-42}$ secretion and the ratio of $Aβ_{1-42}/Aβ_{1-40}$ were significantly decreased in the presence of PS2β. However, we could not detect the difference of Notch signaling in HEK293 cells stably expressing PS2β, using two different methods, NICD detected by immunoprecipitation and expression of Notch target genes by real-time PCR. This result suggests the differential effect of PS2β on Aβ generation and Notch signaling. We did not determine the precise mechanism, but one possibility is that the γ-secretase complex formation is not completely destroyed by PS2β (Fig. 3B). The other possibility is that PS2β may regulate γ-secretase activity as TMP21, which differentially regulates γ-secretase activity [20]. However, it requires further experiments to address the precise mechanism. Based on these data, we conclude that PS2β prevented formation of γ-secretase complex assembly at the ER, resulting in inhibition of γ-secretase activity.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.04.014.

References

[1] Selkoe, D.J. and Wolfe, M.S. (2007) Presenilin: running with scissors in the membrane. Cell 131, 215–221.
[2] Yang, D.S. et al. (2002) Mature glycosylation and trafficking of nicastrin modulate its binding to presenilins. J. Biol. Chem. 277, 28135–28142.
[3] Kimberly, W.T., LaVoie, M.J., Ostaszewski, B.L., Ye, W., Wolfe, M.S. and Selkoe, D.J. (2002) Complex N-linked glycosylated nicastrin associates with active gamma-secretase and undergoes tight cellular regulation. J. Biol. Chem. 277, 35113–35117.
[4] Yu, G. et al. (2000) Nicastrin modulates presenilin-mediated notch/glp-1 signal transduction and betaAPP processing. Nature 407, 48–54.
[5] Edbauer, D., Winkler, E., Regula, J.T., Pesold, B., Steiner, H. and Haass, C. (2003) Reconstitution of gamma-secretase activity. Nat. Cell Biol. 5, 486–488.
[6] Takasugi, N., Tomita, T., Hayashi, I., Tsuruoka, M., Niimura, M., Takahashi, Y., Thinakaran, G. and Iwatsubo, T. (2003) The role of presenilin cofactors in the gamma-secretase complex. Nature 422, 438–441.
[7] Kimberly, W.T., LaVoie, M.J., Ostaszewski, B.L., Ye, W., Wolfe, M.S. and Selkoe, D.J. (2003) Gamma-secretase is a membrane protein complex comprised of presenilin, nicastrin, Aph-1, and Pen-2. Proc. Natl. Acad. Sci. USA 100, 6382–6387.
[8] Shirotani, K., Edbauer, D., Prokop, S., Haass, C. and Steiner, H. (2004) Identification of distinct gamma-secretase complexes with different APH-1 variants. J. Biol. Chem. 279, 41340–41345.
[9] Sato, T. et al. (2007) Active gamma-secretase complexes contain only one of each component. J. Biol. Chem. 282, 33985–33993.
[10] Chen, F. et al. (2003) Presenilin 1 and presenilin 2 have differential effects on the stability and maturation of nicastrin in Mammalian brain. J. Biol. Chem. 278, 19974–19979.
[11] Velasco, A., Hendricks, L., Moremen, K.W., Tulsiani, D.R., Touster, O. and Farquhar, M.G. (1993) Cell type-dependent variations in the subcellular distribution of alpha-mannosidase I and II. J. Cell Biol. 122, 39–51.
[12] Hayakawa, Y., Itoh, M., Yamada, A., Mitsuda, T. and Nakagawa, T. (2007) Expression and localization of Cayman ataxia-related protein, Caytaxin, is regulated in a developmental- and spatial-dependent manner. Brain Res. 1129, 100–109.
[13] Mitsuda, T., Hayakawa, Y., Itoh, M., Ohta, K. and Nakagawa, T. (2007) ATF4 regulates gamma-secretase activity during amino acid imbalance. Biochem. Biophys. Res. Commun. 352, 722–727.
[14] Nakagawa, T., Okano, H., Furuichi, T., Aruga, J. and Mikoshiba, K. (1991) The subtypes of the mouse inositol 1,4,5-trisphosphate receptor are expressed in a tissue-specific and developmentally specific manner. Proc. Natl. Acad. Sci. USA 88, 6244–6248.
[15] Wolfe, M.S., Xia, W., Ostaszewski, B.L., Diehl, T.S., Kimberly, W.T. and Selkoe, D.J. (1999) Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and gamma-secretase activity. Nature 398, 513–517.
[16] Bergman, A., Laudon, H., Winblad, B., Lundkvist, J. and Naslund, J. (2004) The extreme C terminus of presenilin 1 is essential for gamma-secretase complex assembly and activity. J. Biol. Chem. 279, 45564–45572.
[17] Kaether, C., Capell, A., Edbauer, D., Winkler, E., Novak, B., Steiner, H. and Haass, C. (2004) The presenilin C-terminus is required for ER-retention, nicastrin-binding and gamma-secretase activity. Embo J. 23, 4738–4748.
[18] Kaether, C., Lammich, S., Edbauer, D., Ertl, M., Rietdorf, J., Capell, A., Steiner, H. and Haass, C. (2002) Presenilin-1 affects trafficking and processing of betaAPP and is targeted in a complex with nicastrin to the plasma membrane. J. Cell Biol. 158, 551–561.
[19] Saura, C.A. et al. (2000) The nonconserved hydrophilic loop domain of presenilin (PS) is not required for PS endoproteolysis or enhanced abeta 42 production mediated by familial early onset Alzheimer's disease-linked PS variants. J. Biol. Chem. 275, 17136–17142.
[20] Chen, F. et al. (2006) TMP21 is a presenilin complex component that modulates gamma-secretase but not epsilon-secretase activity. Nature 440, 1208–1212.

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EXERCISE INDUCES METALLOTHIONEINS IN MOUSE SPINAL CORD

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Abstract—Regular exercise has displayed a beneficial effect on the progression of amyotrophic lateral sclerosis (ALS). However, the mechanism is poorly understood. We here present that regular exercise on a treadmill induces metallothioneins (MTs: MT-1, MT-2, and MT-3) in spinal cords of mice. As MTs are strong scavengers of reactive oxygen species and have some neurotrophic activities, exercise may have some beneficial effects on spinal motor neurons in patients with ALS owing to the induction of MTs. The running exercise on a treadmill for 30 min/day increased the mRNA expression levels of MT-1, MT-2, and MT-3 up to 193%, 298%, and 196%, respectively, of the control value 12 h after the start of exercise. After two weeks of daily exercise, Western blotting of the MTs proteins showed that the expression levels of MT-1/2 and MT-3 reached 173% and 146%, respectively, compared with those in sedentary mice. Running exercise on a treadmill for 2 weeks led to the gradual accumulation of MT proteins in the spinal cords of the mice. In addition, MT-1/2 and MT-3 immunoreactivities were enhanced in astrocytes particularly in the gray matter of the spinal cord. We revealed that regular exercise induced transient increases in the expression levels of MT mRNAs and resulted in accumulation of MT proteins in the spinal cords of the normal mice.

Key words: metallothionein, growth inhibitory factor, amyotrophic lateral sclerosis, neurodegenerative disease, astrocyte, treadmill.

Amyotrophic lateral sclerosis (ALS) is a progressive and fatal neurodegenerative disease characterized by selective motor neuron degeneration. The majority of ALS cases are sporadic amyotrophic lateral sclerosis (SALS), and approximately 10% of ALS cases are familial amyotrophic lateral sclerosis (FALS), 10–20% of which are the result of mutations in the Cu/Zn superoxide dismutase (SOD1) gene. The combination of genetic, pathological and biochemical studies has fueled four primary hypotheses for mechanisms that provoke or contribute to the disease, which are oxidative damage, axonal strangulation from neurofilamentous disorganization, toxicity from intracellular aggregates and/or failure of protein folding or degradation, repetitive motor neuron firing and subsequent excitotoxic death due to mishandling of glutamate (Cleveland and Rothstein, 2001; Hozumi et al., 2004). Many other mechanisms have been reported including heavy metal toxicity and dysfunction in neurotrophic factors (Boillée et al., 2006). Despite those studies, the etiology of SALS remains unclarified. We have recently shown that the expression of metallothioneins (MTs: MT-1, MT-2 and MT-3) was diminished in the spinal cords of patients with SALS. The study suggests that MTs are profoundly involved in the pathogenesis of SALS (Hozumi et al., 2008b).

MTs are small (6–8 kDa) cysteine-rich, metal (Cu/Zn)-binding proteins that scavenge reactive oxygen species (ROS). MT-1 and MT-2 exist together in all tissues and are abbreviated as MT-1/2. MT-1 suppresses CNS inflammation and neurodegeneration, and prevents cell death following kainic acid–induced epileptic seizures (Penkowa et al., 2005). MT-3 exists mainly in the CNS (Hozumi et al., 2008a). MT-3 inhibits neurite extension and protects cortical neurons from the toxicity of amyloid beta peptides and hydroxyl radicals (Uchida et al., 1991). Moreover, MT-3 knockout (KO) mice were more susceptible to seizures induced by kainic acid and subsequently exhibited greater neuronal injury in the CA3 field of the hippocampus. Conversely, transgenic mice containing high levels of MT-3 were more resistant to CA3 neuronal injury induced by seizures (Erickson et al., 1997). MT-3 prevents neurodegeneration and may have some effect on neurogenesis after brain damage (Sakamoto et al., 2003; Hozumi et al., 2006). Crossing G93A (glycine at position 93 substituted with alanine) SOD1 mice with MT-1/2 or MT-3 KO mice accelerated the onset or progression of ALS in the progeny (Nagano et al., 2001; Puttaparthi et al., 2002). The expression levels of MT-3 mRNA were decreased in the spinal cords of patients with SALS (Ishigaki et al., 2002). MT-1/2 can be induced by various factors: heat shock, heavy metals such as Zn, Cd, Mg, and Cu (Aschner et al., 1997; Hidalgo et al., 2001), but MT-3 has been shown to be induced only by brain injury and sleep deprivation (Hozumi et al., 1995; Cirelli and Tononi, 2000).

Recent studies suggest that physical exercise has a beneficial effect on the progression of ALS (McCrate et al., 2008). Regular exercise can increase the life span of a transgenic mouse model of FALS (G93A SOD1) and delay the onset (Kirkinezos et al., 2003). However, the mechanism by which exercise affects motor neurons is poorly understood. Here we examined how regular exercise affects the expression of MTs in the spinal cords of normal mice.

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Abbreviations: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; FALS, familial amyotrophic lateral sclerosis; GFAP, glial fibrillary acidic protein; G93A, glycine at position 93 substituted with alanine; KO, knockout; mAb, monoclonal antibody; MT, metallothionein; NGF, nerve growth factor; NT, neurotrophin; ROS, reactive oxygen species; RT-PCR, reverse transcriptase–polymerase chain reaction; SALS, sporadic ALS; SOD1, Cu/Zn superoxide dismutase.

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EXPERIMENTAL PROCEDURES

Animals and exercise

C57BL/6J male mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Mice were housed in a temperature-controlled (23±3 °C) room with a 12-h light/dark cycle. Food and water were provided *ad libitum* throughout this study. The treadmill running exercise program was administered as described previously (Kirkinezos et al., 2003). Briefly, the male mice in the exercise groups ran on a treadmill machine (MK-680 S, Muromachi, Tokyo, Japan) at 13 m/min and 30 min/day. The exercise program was carried out from 9 to 10 o'clock. A control group remained sedentary. The exercise was started at 7 weeks of age. For MT-1/2 or MT-3 protein expression level analysis, mice were anesthetized and sacrificed, and their spinal cords were removed 24 h after the exercise program for a week or 2 weeks. For MT mRNA expression level analysis, mice were anesthetized and sacrificed, and their spinal cords were removed 6 h, 12 h, and 24 h after 30 min of an exercise stimulation. For immunohistochemistry, mice were anesthetized and sacrificed, and their spinal cords were removed 24 h after the exercise program for 2 weeks. The experimental designs and all procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the guidelines on the handling of experimental animals issued by the Japanese Association for Laboratory Animal Science and the Animal Experimental Committee of Gifu University. Every effort was made to minimize the number of animals used and their suffering. This study was approved by the Animal Experimental Committee of Gifu University.

Antibodies

Western blotting. A mouse anti-MT-3 monoclonal antibody (mAb) was prepared as described previously (Tokuda et al., 2007; Hozumi et al., 2008a). A mouse anti-MT-1/2 mAb (E9) (Dako, Produktionsvej, Denmark), a mouse anti-α-tubulin mAb (1:2000, Sigma Aldrich, St. Louis, MO, USA), and a sheep anti-mouse IgG Ab (GE Healthcare, Buckinghamshire, UK) were used in this study.

Assay for protein levels of MTs

The spinal cords of the mice were homogenized in PBS containing protease inhibitor cocktail set (10 μM AEBSF, 0.8 μM aprotin, 50 μM bestatin, 15 μM E-64, 20 μM leupeptin, and 10 μM pepstatin A). After centrifugation at 12,000×*g* for 15 min at 4 °C, the supernatant was collected. Proteins (50 μg) were separated by 5–20% linear gradient SDS-PAGE and transferred to a PVDF membrane sheet (Bio-Rad Laboratory, CA, USA). Protein concentration was determined using the BCA Protein Assay Reagent (Thermo Fisher Scientific Inc., IL, USA). The blots were developed with ECL plus (Amersham, Piscataway, NJ, USA) and analyzed using Science Lab (2005) Multi Gauge Ver3.0 (Fujifilm, Tokyo, Japan).

Real-time reverse transcriptase–polymerase chain reaction (RT-PCR) analysis

The gene expressions of MT-1, MT-2, and MT-3 were quantified by real-time RT-PCR analysis as described previously (Wakida et al., 2006). Briefly, total RNAs were isolated from spinal cords using RNeasy Lipid Tissue Mini Kit (Qiagen Sciences, MD, USA). cDNA synthesis and real-time PCR were carried out using SYBR PrimeScript RT-PCR Kit (TaKaRa Bio Inc., Shiga, Japan) and the Thermal Cycler Dice Real Time System (TaKaRa). The following primer pairs were used as follows: the *mt-1* sense primer 5′-GGTCCTCTAAGCGTCACCAC-3′ and antisense primer 5′-GAGCAGTTGGGGTCCATTC-3′; the *mt-2* sense primer 5′-CCTGTGCCTCCGATGGAT-3′ and antisense primer 5′-ACTTGTTCGGAAGCCTCTTTG-3′; the *mt-3* sense primer 5′-CTGAGACCTGCCCCTGTC-3′ and antisense primer 5′-TTCTCGGCCTCTGCCTTG-3′. As a housekeeping gene, *β-actin* was amplified using the sense primer 5′-GATCTGGCACCACACCTTCT-3′ and antisense primer 5′-GGGGTGTTGAAGGTCTCAAA-3′.

Immunohistochemistry

Under ether anesthesia, mice were sacrificed, and the spinal cords were removed and immersed in 4% paraformaldehyde (pH 7.4) overnight. The lumbar spinal cords were embedded in paraffin, sectioned (5 μm), and then deparaffinized using xylene and a graded series of alcohols. Sections were then washed in PBS, treated with 0.3% hydrogen peroxide for 10 min, and then washed again. Immunohistochemical analysis was performed using an immunoperoxidase reagent kit, Histofine SAB-PO (M) Kit (Nichirei Corporation, Tokyo, Japan), following the instructions of the manufacturer. The primary antibody used was a mouse anti-MT-1/2 or -MT-3 mAb. The sections on slides were dehydrated through graded alcohols, coverslipped with Permount, and then viewed under a Zeiss Axioskop 2. The sections were double-stained with an anti–glial fibrillary acidic protein (GFAP) antibody (DAKO, Produktionsvej, Denmark) to confirm the MT-immunopositive cells.

Statistical analyses

Data were assessed by one-way analysis of variance (ANOVA), followed by Scheffe's post hoc test. When applicable, an unpaired Student's *t*-test was used. The significance was set at $P<0.05$.

RESULTS

Changes of MT mRNAs in spinal cords after treadmill running exercise

The levels of MTs mRNA in spinal cords of mice with single 30 min-exercise or after 2-week-exercise were analyzed with real time RT-PCR. With 30 min of exercise stimulation, the expression levels of MT mRNAs were significantly increased 12 h after the beginning of the exercise training. The MT-1 and MT-3 mRNA expression levels were upregulated to approximately 1.93-fold and 1.96-fold, respectively, compared with sedentary levels. The MT-2 mRNA expression level was markedly more increased up to 2.98-fold compared with sedentary level (Fig. 1A). The levels of MT-1 and MT-2 mRNA showed a tendency of upregulation even after 2 weeks' exercise but lower than those after single 30-min-exercise at first. The levels of MT-3 showed no significant change after 2 weeks' exercise (Fig. 1B).

MT-1/2 and MT-3 protein expression levels in spinal cords after treadmill running exercise

The levels of MT-1/2 and MT-3 protein in spinal cords of 2-week-exercised (9 weeks old) and 2-week-sedentary mice (9 weeks old) were analyzed by Western blotting. The MT-1/2 and MT-3 protein expression levels in the spinal cords of 2-week-exercised mice were 1.73-fold ($P=0.043$) and 1.46-fold ($P=0.040$) higher than those of sedentary mice, respectively (Fig. 2A, B). The levels of MTs proteins were chronologically analyzed at control (7 weeks old mice), and 7-day-exercised and 14-day-exercised mice in

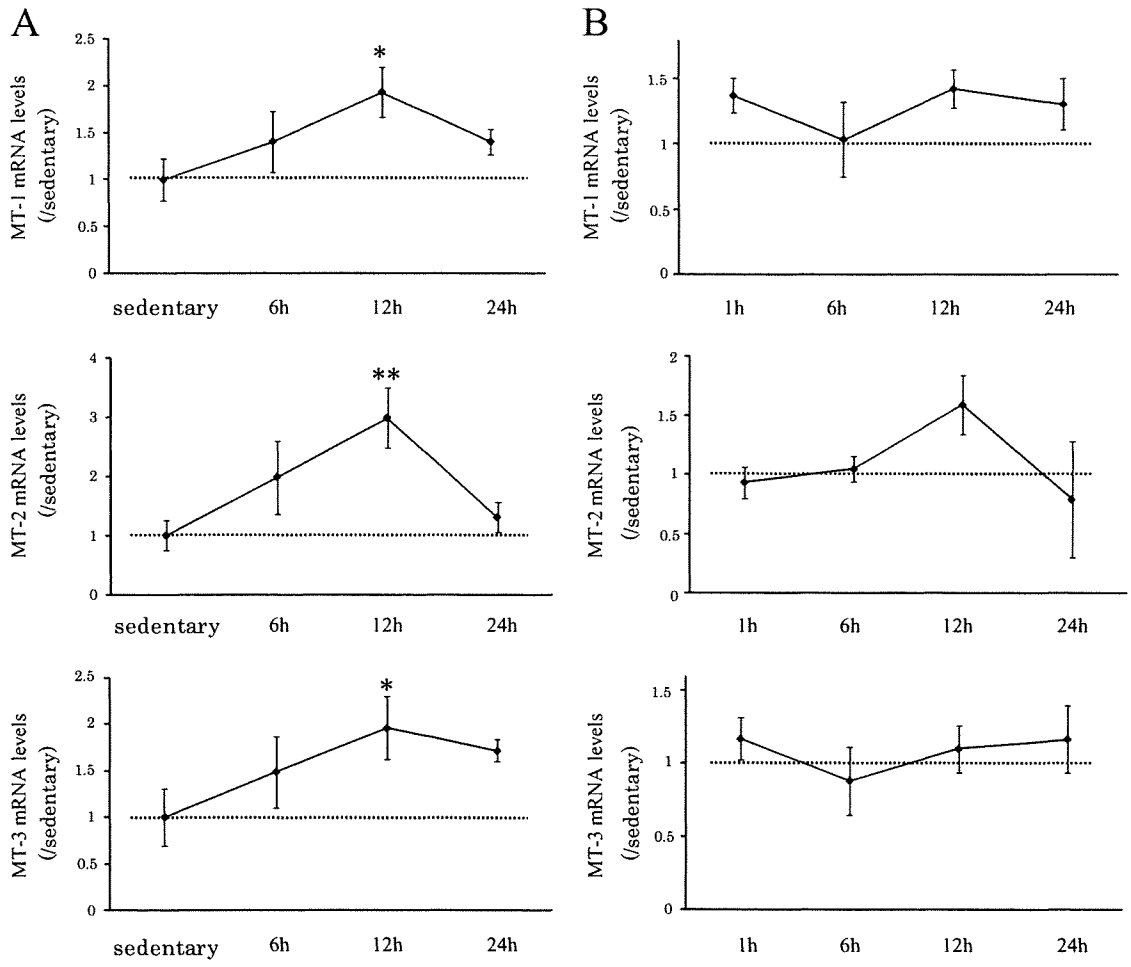

Fig. 1. Regulation of MT mRNA expression levels in spinal cords after treadmill running exercise. The expression levels of MTs mRNAs were significantly increased 12 h after the beginning of the exercise training. After 2 weeks of exercise the levels of MT-1 and MT-2 mRNA still showed a tendency of upregulation. Thirty minutes of exercise stimulation was administered and MT mRNA expression levels in the spinal cords were examined at 6 h 12 h, and 24 h after the beginning of exercise (A, $n=3$). Two weeks of exercise was administered and MT mRNA expression levels in the spinal cords were examined at 1 h, 6 h, 12 h, and 24 h after the beginning of last exercise (B, $n=3$). One-way ANOVA revealed significant effects in the interaction between MT mRNA expression levels and exercise stimulations. Scheffe's post hoc test ** $P<0.01$, * $P<0.05$ vs. control.

the spinal cord by Western blotting. The levels of MTs in the spinal cord in mice were not significantly changed at least between 7 and 9 weeks old in the experiment. The time course study showed that the MT-1/2 protein expression level was gradually increased for 14 days, reaching 2.04-fold that of the control value (0 day). The MT-3 protein expression level also gradually increased, reaching 1.39-fold that of the control value. However, the increase in MT-3 protein expression level was slower and smaller than that in MT-1/2 protein expression level (Fig. 3).

Localization of MT proteins in spinal cords after treadmill running exercise

Localization and immunoreactivity of MT proteins in spinal cords after 2 weeks of exercise were analyzed by immunohistochemistry analyses. In the lumbar spinal cords of sedentary mice, MT-1/2 immunoreactivity was observed in astrocytes and their processes particularly in the white matter, and it was faintly recognized in small cells in the gray matter, whereas MT-3 immunoreactivity was recognized in small cell bodies in the white matter. In the lumbar spinal cord after the 2-week-exercise, MT-1/2 immunoreactivity was enhanced in astrocytes particularly in the gray matter, whereas MT-3 immunoreactivity was also enhanced in small cells particularly in the gray matter (Fig. 4). The MT-positive cells were confirmed to be astrocytes by co-staining with an anti-GFAP antibody immunostaining (Fig. 5).

DISCUSSION

We demonstrated that regular exercise on a treadmill physiologically induced MTs in the spinal cords of normal mice in this study. MT-1/2 and MT-3 immunoreactivities were enhanced in astrocytes particularly in the gray matter of the spinal cords. MTs are low-molecular-weight, cys-

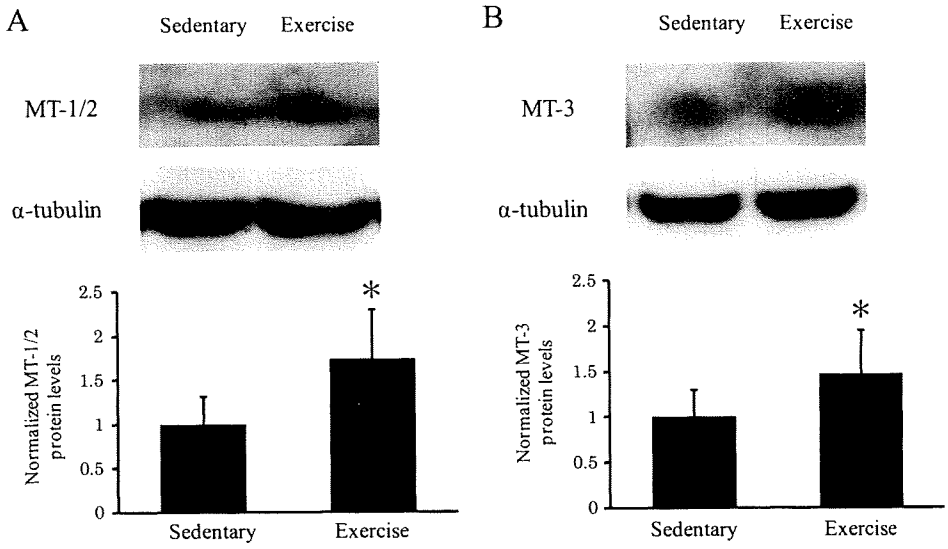

Fig. 2. Differences in MT-1/2 or MT-3 protein expression levels in spinal cords after 2 weeks of treadmill running exercise. Both MT-1/2 (A) and MT-3 (B) protein expression levels in the spinal cords of 2-week-exercised mice were significantly higher than those of sedentary mice. Immunoblotting was performed using the anti-MT-1/2 antibody (A, left, $n=5$) or the anti-MT-3 antibody (B, right, $n=7–8$). The density levels were analyzed and presented as fold induction compared with that of the sedentary group (bottom graph). Unpaired Student's t-test * $P<0.05$.

teine-rich, and metal (Cu/Zn)-binding proteins with multifunctions including ROS. Therefore, this study provides a promising clue to developing a new treatment for neurodegenerative diseases such as ALS.

The group of ALS patients that exercised three times weekly showed significantly better clinical functional scores than the group receiving conventional care (Bello-Haas et al., 2007). Kirkinezos et al. (2003) showed in the G93A-SOD1 FALS model mice that regular exercise had a beneficial effect on the progression, the mechanism of which remains to be elucidated. In G93A SOD1 mice, the onset and survival time are different among reported studies. Moreover, the effects of exercise are different among reported studies. Kirkinezos et al. (2003) showed a beneficial effect, especially in male mice. Liebetanz et al. (2004) have reported that extensive exercise showed a positive trend for the survival time and no significant differences between sexes for the onset and survival time. Veldink et al. (2003) showed that exercise had a beneficial effect on the onset time of the low-copy G93A SOD1 female mice

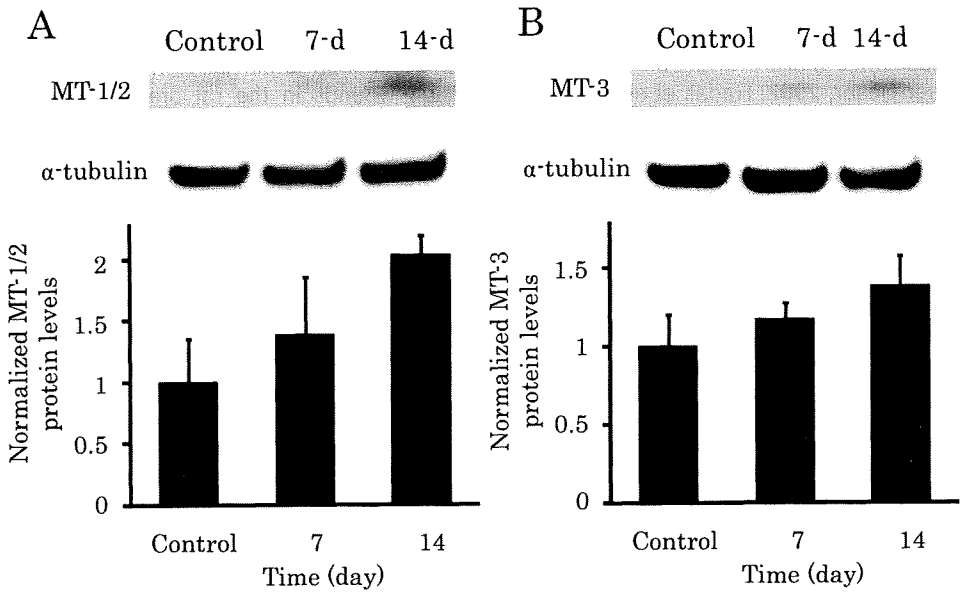

Fig. 3. Time course of MT-1/2 or MT-3 protein expression levels in spinal cords. MT-1/2 (A) and MT-3 (B) protein expression level was gradually increased with time for 14 days. Exercise stimulation was administered for the duration indicated and spinal cords were examined after 24 h. Immunoblotting and density analysis was performed as described in the legend to Fig. 1.

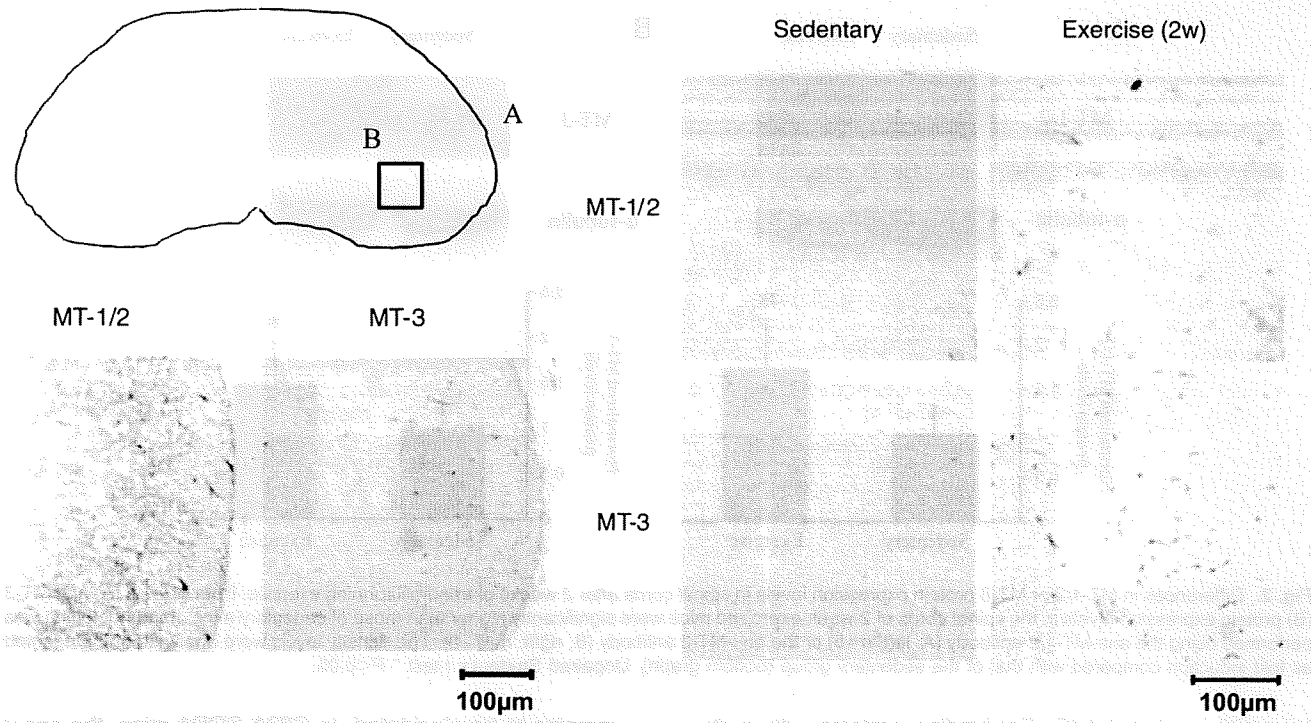

Fig. 4. MT immunoreactivities in lumbar spinal cords after 2 weeks of treadmill running exercise. Immunohistochemical analysis was performed using the anti-MT-1/2 antibody or the anti-MT-3 antibody. (A) White matter; (B) ventral horn of lumbar region.

and the survival time of the high-copy G93A SOD1 female mice. In the study on the wild type mice, the difference of effects by exercise between sexes has not been examined. Regular exercise has a wide range of beneficial effects on physical and mental functions. The molecular mechanism of exercise in detail is still unknown. Physical exercise may induce neurogenesis via neurotrophic factors, decrease oxidative damage, and protect brain function, while excessive exercise may increase proteolytic degradation by proteasomes (Adlard and Cotman 2004; Cotman and Engesser-Cesar, 2002; Radak et al., 2001).

In CNS, neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) affect the maintenance of neurons and the interconnectedness of synapses. A previous study showed that exercise affected the expression of BDNF, NGF and other factors particularly in the hippocampus but not in the spinal cord (Hennigan et al., 2007). In the spinal cord only BDNF, neurotrophin (NT)-3 and NT-4 expressions have been reported to be affected by exercise (Skup et al., 2000; Gómez-Pinilla et al., 2001). Although IGF-1 and GDNF did not change in the spinal cord (Engesser-Cesar et al., 2007; Siamilis et al., 2009), interestingly, synergy of IGF-1 and exercise has been reported to have a benefit in ALS mice (Kaspar et al., 2005). To our knowledge other factors related with exercise have not been reported yet. Intriguingly, exercise acutely induced BDNF mRNA expression after a single bout of exercise (170%) and a week of exercise (138%), and increased the protein expression level of BDNF only 2 h later (Gómez-Pinilla et al., 2001). NT-3 and NT-4 mRNA expressions and immunoreactivities in the spinal cord were enhanced acutely by wheel voluntary exercise but not by treadmill exercise (Ying et al., 2003).

In our study, interestingly, regular exercise on a treadmill induced not only MT-1 and MT-2 but also MT-3. The change in the expression of the MT-3 protein was smaller and slower in reaction compared with that of the MT-1/2 protein. The levels of protein in MTs are thought to be accumulated little by little by single bout of exercise. This phenomenon is similar to the finding observed following stab wounds in the rat brain (Hozumi et al., 1995). In addition, MT-2 mRNA was prominently induced in the spinal cord. This is also similar to the phenomenon observed in the rat brain with focal cerebral ischemia (Wakida et al., 2006). The induction of MT isoforms seems to be similarly regulated by various stimuli and stresses.

Histologically, both MT-1/2 and MT-3 immunoreactivities were enhanced in astrocytes particularly in the gray matter. Similarly, MT-1/2 immunoreactivity reportedly has been enhanced in the G93A-SOD1 FALS model mice (Gong and Elliott, 2000). Crossing G93A-SOD1 mice with either MT-1/2 or MT-3 KO mice markedly accelerated the expression of paralysis and shortened survival (Nagano et al., 2001; Puttaparthi et al., 2002). The MTs-positive cells cannot be recognized except in astrocytes in this experiment, although MT-immunoreactivities were recognized in a few neurons in human autopsy materials in the previous study. In some pathological conditions, we speculate that other cells except astrocytes may be MT-positive. The immunoreactivity of MT-3 in astrocytes in the gray matter of the lumbar spinal cord in human ALS was negatively

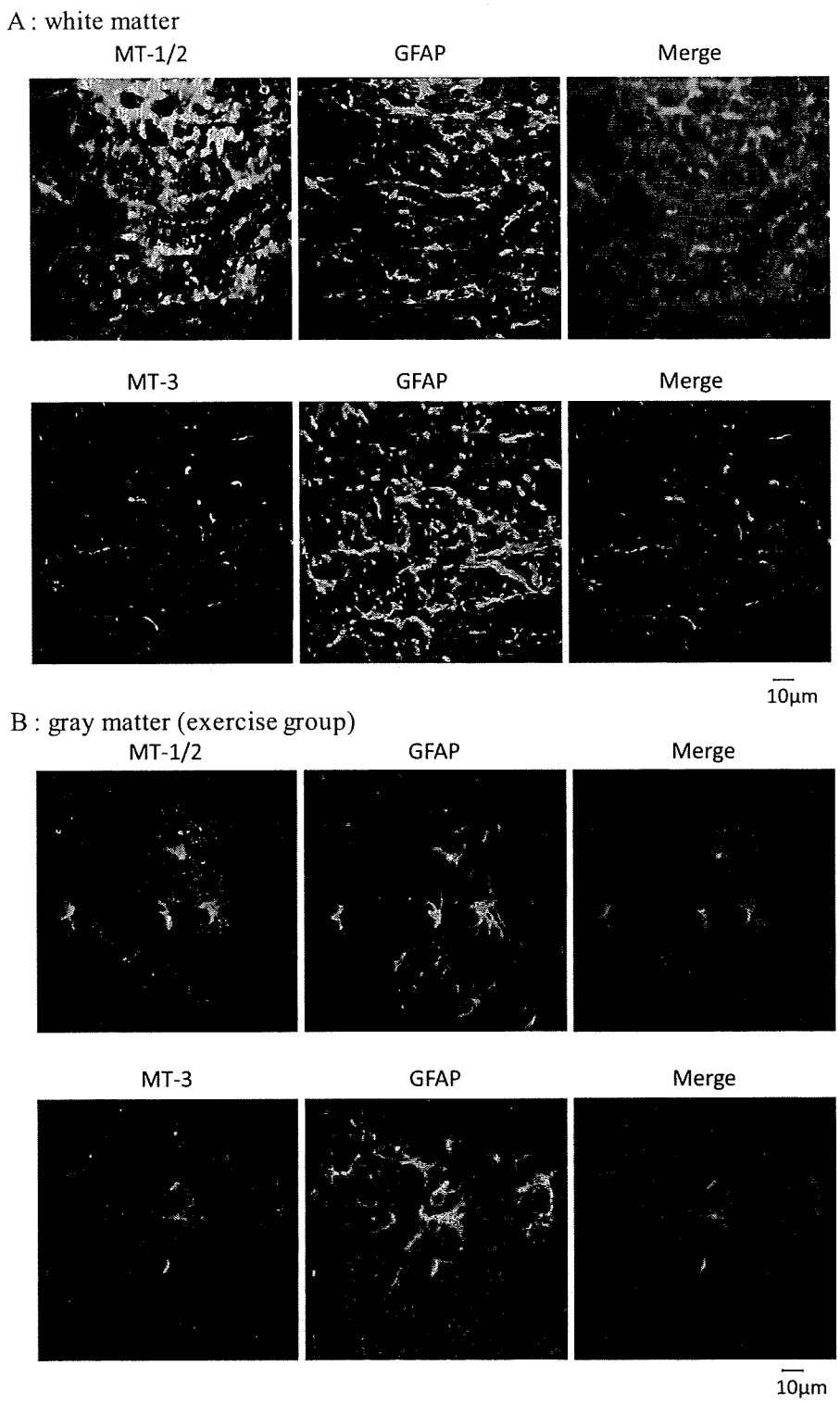

Fig. 5. MT co-localization in astrocytes in the white matter (A) and the gray matter (B) of the lumber spinal cords after 2 weeks of treadmill running exercise and in the gray matter of the sedentary mice (C) as a control. Immunohistochemical analysis was performed using the anti-MT-1/2 antibody or the anti-MT-3 antibody, and anti-GFAP antibody. MT-1/2-positive cells were confirmed to coincide with GFAP-positive cells. MT-3-positive cells were also confirmed to coincide with GFAP-positive cells. Both MT-1/2 and MT-3 immunoreactivities were enhanced in astrocytes particularly in the ventral horn of the gray matter of the lumbar region after the treadmill running exercise for 2 weeks.

correlated with the duration of ALS (Hozumi et al., 2008b). Recently, the role of astrocytes in the spinal cord has been further stressed in the pathogenesis of ALS in G93A-SOD1 mice (Bilsland et al., 2008; Nagai et al., 2007). Ishigaki et