

## Translocational changes of localization of synapsin in axonal sprouts of regenerating rat sciatic nerves after ligation crush injury

Ku-birm Kwon, Jin-suk Kim<sup>1</sup>, Byung-joon Chang\*

Department of Anatomy and Histology, <sup>1</sup>Department of Pharmacology and Toxicology, College of Veterinary Medicine, Konkuk University, Seoul 143-701, Korea

**Time-dependent translocational changes of Synapsin I (SyI), a synaptic vesicle-associated phosphoprotein and its involvement in the axonal transport were investigated in the regenerating axonal sprouts. A weak SyI immunoreactivity (IR) was found in the axoplasm of normal axons. Rat sciatic nerves were crush-injured by ligating with 1-0 silk thread at the mid-thigh level and released from the ligation 24 h later. At various times after release, immunocytochemistry was performed. SyI was translocated from the proximal to the distal site of ligation and also involved in the sprouting of regenerating axons. The distribution patterns of SyI IR were changed in the crush-injured nerves. SyI immunoreactive thin processes were strongly appeared in the proximal region from 1 h after release. After 3 h, a very strong IR was expressed. The intense SyI immunoreactive thin processes were elongated distally and were changed the distribution pattern by time-lapse. After 12 h, strong immunoreactive processes were extended to the ligation crush site. At 1 day, a very intense IR was expressed. At 2 days, immunoreactive thin processes extended into the distal region over the ligation crush site and strong IR was observed after 3 days. SyI was accumulated in the proximal region at the early phases after release. These results suggest that SyI may be related to the translocation of vesicles to the elongated membranes by a fast axonal transport in the regenerating sprouts.**

**Key words:** sciatic nerve, regeneration, immunocytochemistry, Synapsin I.

### Introduction

Illustration of the transported substances and the mechanisms involved in the regeneration of the peripheral

nervous system (PNS) may be a great help to cure peripheral nerve injuries and demyelinating diseases [26]. There is a dichotomy between the PNS and the central nervous system (CNS) in their ability to regenerate [1, 26, 27, 35]. The injured CNS neurons can not be regenerated, whereas the injured PNS neurons can be regenerated by reestablishing synaptic connections, thereby resulting in recovering their functions. The ability of nerve regeneration may be attributed to the structural differences in the cellular organization between the PNS and the CNS [26-30, 34]. The CNS has no basal lamina of axons, while the PNS has the basal lamina of Schwann cells which plays an important role in the nerve regeneration [28]. Each fiber of both myelinated and unmyelinated peripheral nerves is lying within a continuous basal lamina tube. A sprout, the early axolemmal extension from the parent axon, extends through the space between the basal lamina and the myelin sheath. It has been reported that the sprout formation was found as early as 5 h after injury [27]. The node can produce multiple sprouts and these sprouts appear to be regenerating axons in the proximal stump and grow to the distal stump as a growth cone. Newly synthesized membrane proteins were added to the axonal growth cones. In the growth cone, materials and membrane vesicles were preferentially provided for the axonal growth [11].

SyI, the collective name for Synapsin Ia and Ib, is a phosphoprotein associated with synaptic vesicles in the nerve terminal [13, 15-17]. There are a slight difference in molecular weight (MW) between synapsin Ia and Ib [13, 15-17]. The MW of Sy Ia is 86 kDa, and that of SyIb is 80 kDa. SyI is present only in the nerve terminals. Within the terminals, it is associated with small synaptic vesicles [5]. It is present virtually in all synapses [13, 15, 16] and appears simultaneously with synapse formation during development [23]. It is a peripheral protein of the cytoplasmic surface of the vesicle. SyI acts as a link protein between the vesicle and cytoskeletal matrix of the terminal. Therefore, it seems like to connect synaptic

\*Corresponding author

Phone: 82-2-450-3711; Fax: 82-2-3437-3661

E-mail: bjchang@konkuk.ac.kr

vesicles and anchor them to the cytoskeletons in the presynaptic terminals. SyI plays a regulatory role in neurotransmitter release [14, 23]. In mature neurons, SyI is concentrated almost exclusively in the presynaptic terminals [9, 10, 23, 24] and colocalized with SV48 [9] and synaptotagmin [10]. The essential function of the synapsins is regulating the traffic of synaptic vesicles [33]. SyI and 200 kDa neurofilament protein, an axonal marker, were colocalized. These indicate that SyI may be involved in the axonal elongation [1, 2]. Immunocytochemical studies on rat brain demonstrated that SyI IR is specifically associated with the neuronal cytoskeleton as well as the synaptic vesicles. Thus, SyI may play a critical role in the dynamics of the cytoskeletal functions and the cytoskeleton-membrane interactions [19].

Accumulation of transported materials can be studied at a focal block of axonal transport caused by sever, crush and cold block or ligation. Because we thought pools of SyI may travel at a fast rate, the pattern of anterograde transport of SyI was investigated in the regenerating peripheral nerves. We investigated the time-dependent translocational changes of SyI by axonal transport after ligation crush injury and the involvement of SyI in vesicular transport of membrane elongation in the sciatic nerve regeneration.

## Materials and Methods

### Experimental animals

Forty-eight adult male Sprague-Dawley rats, weighing 250~300 g and aged 12~18 weeks, were used in this study. Feed and water were provided *ad libitum*.

### Operation procedures

The animals were slightly anesthetized with diethyl ether and then deeply anesthetized by a mixture of Ketamine (*Ketara*<sup>®</sup>: 50 mg/kg I.M.) and Xylazine (*Rompun*<sup>®</sup>: 10 mg/kg I.M.) or pentobarbital sodium (*Entobar*<sup>®</sup>: 60 mg/kg I.P.). Left sciatic nerves were exposed at the mid-thigh level through the sciatic notch and simply crushed by a strong ligation with 1-0 silk thread [1, 7, 8, 21, 30, 34]. No ribbon-shaped ligation was performed to avoid rejection reaction of surrounding tissues [8]. After 1 day, the rats were anesthetized again as the same way and the nerves were released from the ligation. Skin was resutured without sideways tearing. No infectious signs were found in the animals operated by this way. Preliminary experiments [28] showed that there was no difference in the results obtained from animals operated either sterilely or non-sterilely.

### Preparation of tissues

Immediately, at 1 h, 2 h, 3 h, 5 h, 6 h, 8 h, 12 h, 18 h, 1 day, 2 days, 3 days, 4 days, 5 days, 7 days, and 14 days after

release, the animals were deeply anesthetized with diethyl ether. Using a probe-ended stainless steel gastric tube, the rats were slowly perfused transcidentally with vascular rinse solution, followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4) [32]. After perfusion, the left sciatic nerve segments including ligated area were excised and postfixed in the same fixative for 4 h at 4°C. After washing in 0.1 M PBS for 1 h, the nerve segments were infiltrated by increasing phosphate buffered sucrose solution for cryoprotection; 10% for 1 day, 20% for 1 day, and finally 30% for 3 days at 4°C. The sunk nerve segments were excised and quickly frozen by a snap-freezing in the isopentane inside of the liquid nitrogen bottle for 10 min. The nerve segments were embedded in the tissue freezing embedding medium, frozen rapidly, and sectioned longitudinally with a 10- $\mu$ m thickness by cryosection. The sections were thaw-mounted on the prepared gelatin-coated slide glasses [32] and air-dried for 1 day at room temperature.

### Light microscopic ABC immunocytochemistry

After air-dried for 1 day at room temperature, sections were washed three times in 0.1 M PBS for 10 min. The sciatic nerve segment sections were transferred into buffer A (0.1 M PBS with 1% BSA and 0.2% saponin) including 0.5% H<sub>2</sub>O<sub>2</sub> for 30 min to block endogenous peroxidase activity. The sections were rinsed with 0.05 M glycine in buffer A for 10 min. After washing with buffer A, sections were incubated in 1.5% normal goat serum (Serotec) in 0.1 M PBS with 10% BSA and 0.2% saponin (buffer B) for 1 h. Sections were sequentially incubated for 1 day at 4°C with rabbit polyclonal anti-synapsin I (Calbiochem-Novabiochem), which cross-reacts with rat SyI and was diluted to 1 : 100 with antibody dilution solution [1]. After washing 3 times with buffer A for 10 min, sections were incubated for 1 h with biotinylated secondary goat anti-rabbit IgG antibody working solution (Vector) with a dilution of 1 : 400 at room temperature by the method as described by Hsu et al. [25].

After washing with buffer A, sections were incubated for 1 h with avidin-biotinylated horseradish peroxidase complex (Vectastain<sup>®</sup> Elite ABC HRP-conjugated reagent, Vector) in buffer A at room temperature [32]. The ABC complex was prepared by the mixture of 1 : 100 dilution of Vectastain A and B reagents in buffer A 30 min prior to use at room temperature. After washing with buffer A, sections were reacted with 0.02% 3,3'-DAB 4 HCl (Sigma) in 0.05 M Tris-buffered saline (TBS) for 30 min. Then the sections were reacted with 0.05% H<sub>2</sub>O<sub>2</sub> in 0.02% DAB in 0.05 M TBS for 10 min. After washing with 0.1 M PBS for 10 min, the sections were mounted with a Histotec permanent aqueous mountant (Serotec). Counterstain was performed separately using 1% cresyl violet. The changes in the distribution of SyI were observed and evaluated. The

immunocytochemical staining procedure for appropriate negative controls was performed with the omission of the primary antibody or the omission of the goat anti-rabbit IgG.

## Results

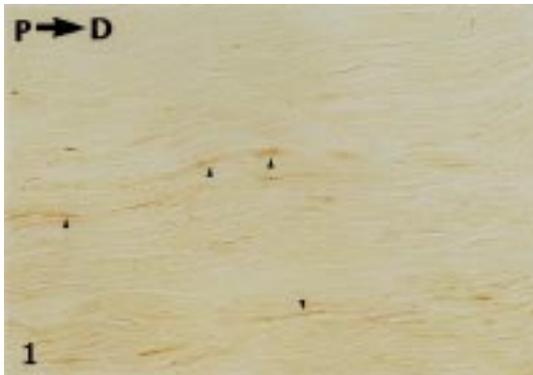
### Normal sciatic nerve (SCN) fibers

In normal rats, SCN fibers immunostained with anti-Synapsin I (SyI) antibody (Ab) showed a weak immunoreactivity (IR) (Fig. 1). A slight immunoreaction appeared throughout the axoplasm in some fibers. IR was also found in the contact site between the axolemma and the myelin sheath. Both the myelinated and unmyelinated nerve fibers showed a weak immunoreactivity.

### Ligation crush-injured SCN fibers

No IR was found in the crush, just proximal, and distal regions in the perfused rat SCN segment after immediate release from ligation (Fig. 2). However, the distribution patterns of IR were changed at 1 h after release. In the SCN segments at 1 h after release from ligation, IR was

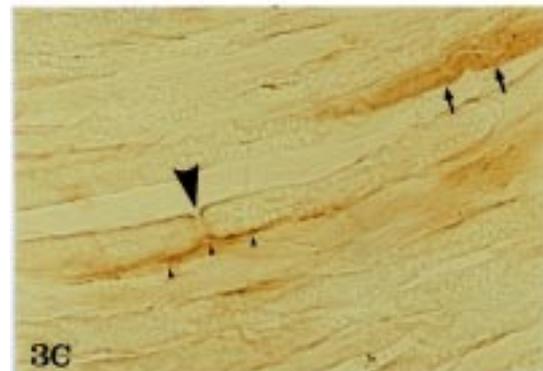
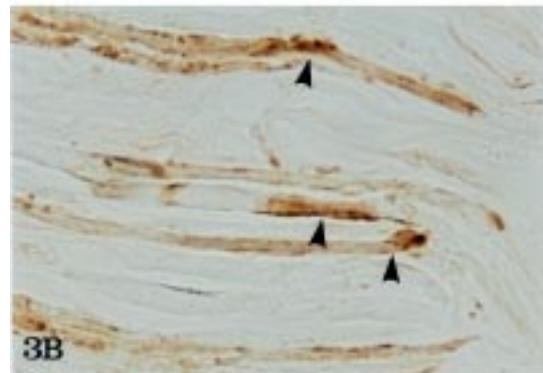
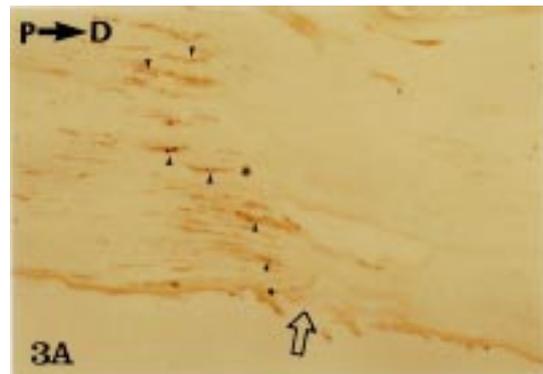
appeared at the proximal region of the ligation crush site. Moderate immunoreactive thin processes were detected in the proximal regions about 2 mm proximal to the site of ligation crush (Fig. 3A). No morphological changes were observed in the more than 4 mm proximal region to the crush site. However, within 2 mm proximal region to the crush site, myelin sheaths were degraded and nerve fibers were remained clearly. Many immunoreactive thin processes were extended along the outer surface of the



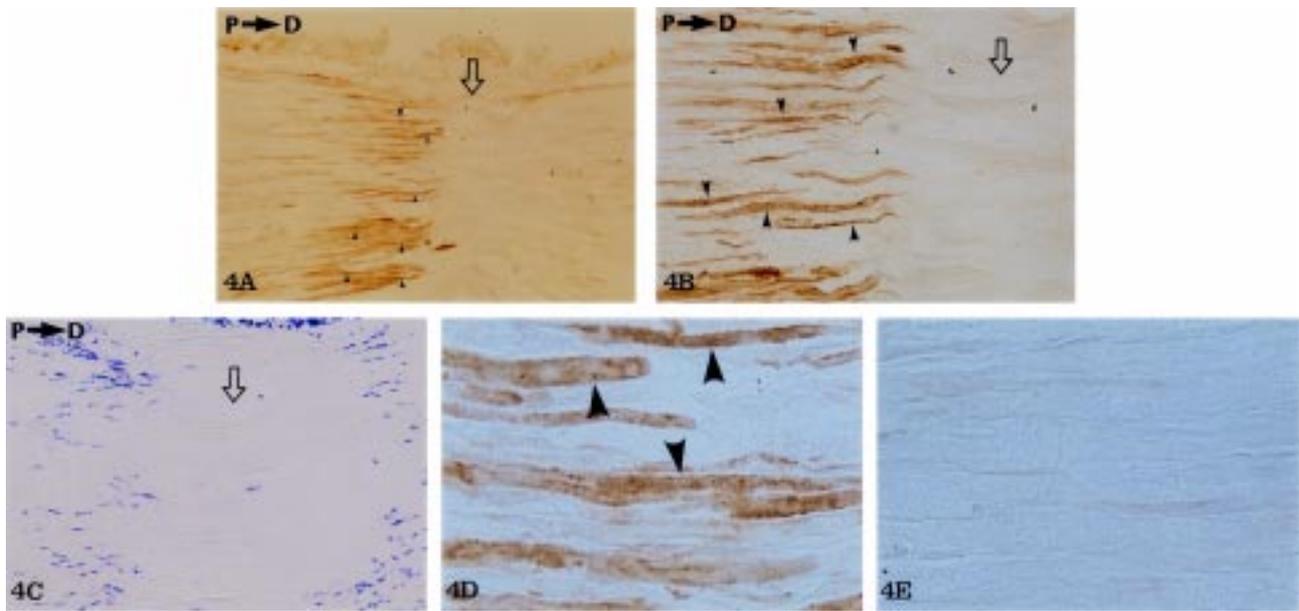
**Fig. 1.** Normal rat SCN fibers immunostained with anti-SyI Ab. Weak immunoreactivity (IR) was found. Slight immunoreaction (arrowheads) was expressed throughout the axoplasm.  $\times 120$



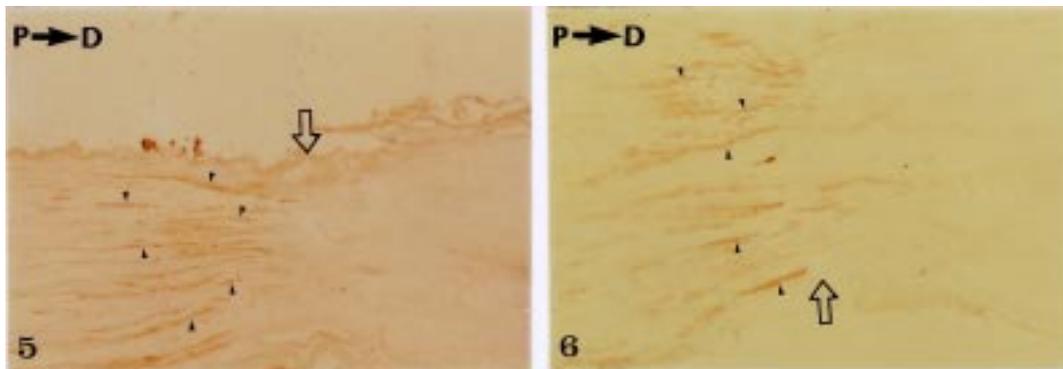
**Fig. 2.** Immediately perfused SCN immunostained with anti-SyI Ab. No IR was found.  $\times 120$



**Fig. 3.** SCN segment at 1 h after release. (A) IR was appeared proximal to the crush (arrowheads).  $\times 50$  (B) Immunoreactive thin processes (arrowheads) were shown in the proximal region.  $\times 475$  (C) IR was extended from the nodal region (large arrowhead) of axon. IR was expressed in the axolemma (small arrowheads) and throughout the axoplasm (arrows). IR was exhibited to both proximal and distal directions from the node in the proximal region (arrowheads).  $\times 475$



**Fig. 4.** SCN segment at 3 h after release. (A and B) Strong immunoreactive thin processes (arrowheads) were shown proximal to the crush. No immunoreactivity were shown in the crush and distal regions. (A)  $\times 50$  (B) Higher magnification of Fig 4A.  $\times 120$  (C) Counter cresyl violet staining.  $\times 120$  (D) Strong immunoreactive thin processes were exhibited in the proximal region (arrowheads). IR was expressed throughout the axoplasm.  $\times 475$  (E) No IR was shown in the distal region.  $\times 475$



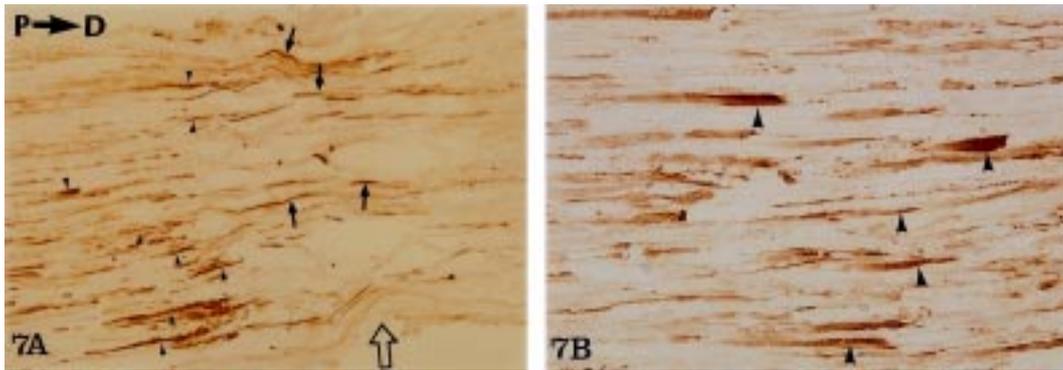
**Fig. 5 and 6.** SCN segment at 5 h (Fig. 5) and 8 h (Fig. 6) after release. Immunoreactive thin processes (arrowheads) were shown the proximal to the crush.  $\times 50$

damaged myelinated nerve fibers in this region (Fig. 3B). IR was extended from the node of Ranvier (Fig. 3C). IR was observed in the axolemma and throughout the axoplasm. IR was exhibited to both proximal and distal directions from the node in the proximal region. Similar patterns were observed in the SCN at 2 h after release.

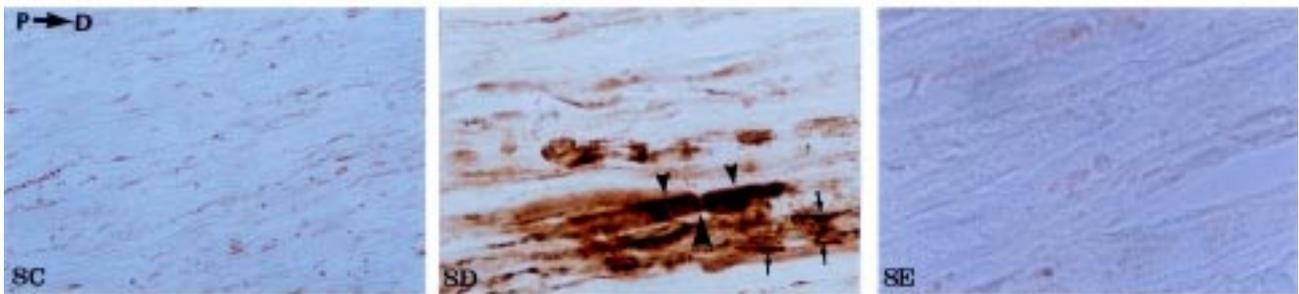
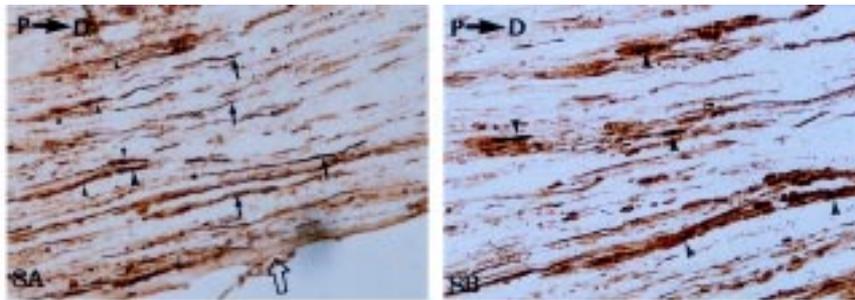
In the SCN segment at 3 h after release, many strongly immunoreactive thin processes were shown just in the proximal to the ligation crush site (Fig. 4A & 4B). Using the counter cresyl violet staining (Fig. 4C), the strongly immunoreactive thin processes were exhibited, assumed them as regenerating axonal sprouts (Fig. 4D). In contrast, there was no IR in the crush and distal regions (Fig. 4B & 4E). In the SCN segment at 5 h (Fig. 5), 6 h, and 8 h (Fig. 6) after release from the ligation, a similar pattern of IR

was observed. Strong SyI immunoreactive thin processes were shown in the proximal region.

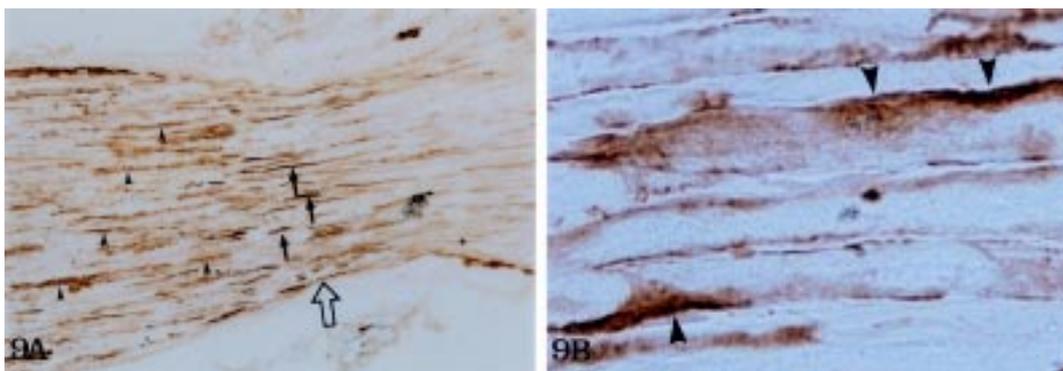
In the SCN segment at 12 h after release from the ligation, SyI immunoreactive thin processes were extended to the ligation crush site (Fig. 7A). Strong immunoreactive thin processes were exhibited in the proximal region (Fig. 7B). Regenerating axonal sprouts were extended to the crush region. A distinct SyI IR was seen in the proximal region. In the SCN segment at 1 day after release from the ligation, very strong SyI immunoreactive thin processes were shown in the proximal region and extended to the crush region (Fig. 8A). Many strong immunoreactive thin processes were shown in the proximal region (Fig. 8B). However, no IR was shown in the distal region (Fig. 8C & 8E). The IR at 1 day was much intenser than the former



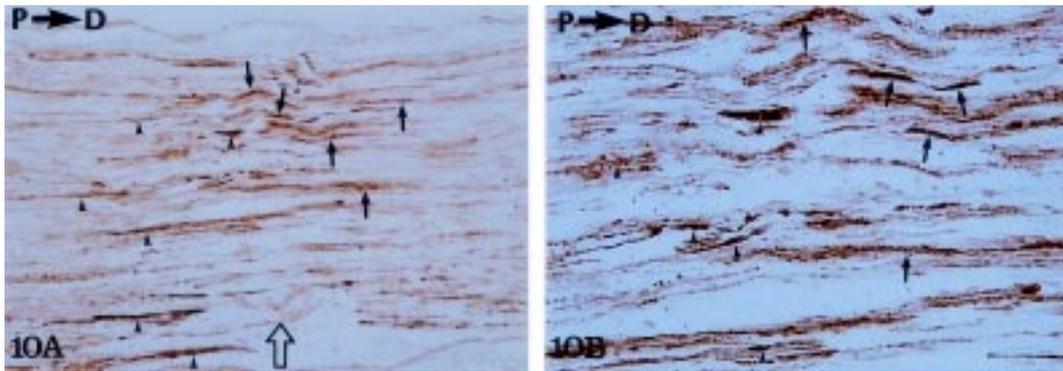
**Fig. 7.** SCN segment at 12 h after release. (A) Strong immunoreactive thin processes (arrowheads) were shown in the proximal region and extended to the crush site (arrows).  $\times 50$  (B) Strong immunoreactive thin processes were exhibited in the proximal region (arrowheads).  $\times 120$



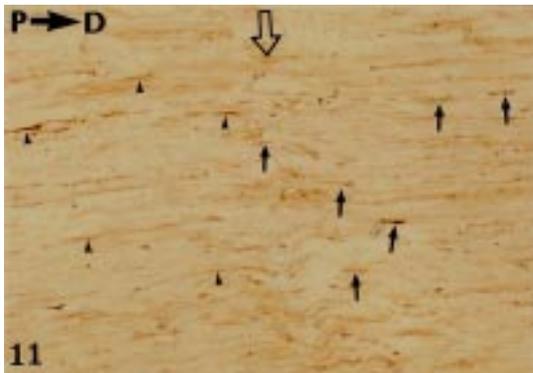
**Fig. 8.** SCN segment at 1 day after release. (A) Strong immunoreactive thin processes were shown in the proximal region (arrowheads) and extended to the crush region (arrows).  $\times 50$  (B) Many strong immunoreactive thin processes were shown in the proximal region (arrowheads).  $\times 120$  (C) No IR was shown in the distal region.  $\times 120$  (D) Strong immunoreactive thin processes were shown in the proximal region and extended from the node of Ranvier (large arrowhead). IR was expressed in the axolemma (arrows) and throughout the axoplasm (arrowheads).  $\times 475$  (E) No IR was shown in the distal region.  $\times 475$



**Fig. 9.** SCN segment at 2 days after release. (A) Strong immunoreactive thin processes were shown in the proximal region (arrowheads) and extended to the crush region (arrows).  $\times 50$  (B) Strong immunoreactive thin processes were shown in the proximal region. IR was expressed in the periphery of the axoplasm (arrowheads).  $\times 475$



**Fig. 10.** SCN segment at 3 days after release. (A) Strong immunoreactive thin processes were shown in the proximal (arrowheads), crush region (arrows) and extended to the distal region (arrows).  $\times 50$  (B) Strong immunoreactive thin processes were shown in the proximal and crush region (arrowheads), and extended to the distal region (arrows).  $\times 120$



**Fig. 11.** SCN segment at 7 days after release. Immunoreactive thin processes were shown in the proximal (arrowheads) and crush region (arrows), and extended to the distal region (arrows). Crush part was slightly swelled.  $\times 50$

groups. The IR was strong especially in the proximal region to the ligation crush site. The IR was strongly expressed in the axolemma and throughout the axoplasm in the proximal region. Very strong immunoreactive thin processes were extended from the node of Ranvier within 2 mm proximal to the ligation site (Fig. 8D). A similar distribution pattern of IR was shown in the SCN segment at 2 days after release (Fig. 9A). A weak IR was expressed in the distal region. A strong IR was expressed in the periphery of the axoplasm in the proximal and the crush regions (Fig. 9B).

In the SCN sections at 3 days after release, strong SyI immunoreactive thin processes were extended over the site of ligation crush and most of sprouts were extended into the distal region of the crush (Fig. 10A & 10B). Strong immunoreactive thin processes were also shown in the distal to the crush site. No distinct SyI IR was seen in the degenerating parent axons in the distal region (Fig. 10B).

Similar distribution patterns were observed in the SCN segment at 7 days (Fig. 11), 14 days, and 28 days after release from the ligation. But after 7 days of release, the

crush site was slightly swelled. IRs at the various time intervals were weaker than the previous groups. The negative control sections reacted with normal serum were not stained.

## Discussion

Significant amounts of proteins and materials are transported from the site of their synthesis for axonal regeneration since the axon itself is unable to synthesize them [20]. In addition, axonal lipids and proteins are produced in the neurons for the regeneration of injured nerves. These materials are transported to the distal site of injury over the injured part by a slow or fast axonal transport [26]. The axoplasmic transport and the molecular mechanisms by which the synapsins are conveyed from cell bodies to nerve terminals still remain to be elucidated. The fast axonal transport in the nerve regeneration contributes to the insertion of the regenerating sprout of glycoprotein into the axolemma. SyI is synthesized in the neuronal cell bodies and conveyed to the synaptic terminals by the process of axonal transport together with most axonal and synaptic proteins. The normally transported SyI accumulates at the nerve endings [31]. Recently it has been demonstrated that the transport mechanism of synaptic vesicles in the presynaptic terminal can be applied to the regeneration [1, 2]. Slow axonal transport provides the bulk of the axoplasmic and cytoskeletal proteins, whereas fast axonal transport contributes to the conveyance of elements for the axolemma. Because SyI is a surface membrane protein, it is likely related to vesicular accumulation and fast axonal transport [20].

SyI is one of the proteins that are highly specific to the nerve terminals. SyI had been referred to for several years as protein I [6, 18], until its virtually ubiquitous and specific localization at synapses was known [16]. The SyI binds to neurofilament1,2, small synaptic vesicles [5], actin [22], and tubulin [3]. The colocalization of SyI and

neurofilament has implicated that SyI-immunoreactive processes occur in the axons but not in the Schwann cells and other non-neural cells [35].  $\text{Ca}^{2+}$  influx through the presynaptic  $\text{Ca}^{2+}$  channel activates  $\text{Ca}^{2+}$ -calmodulin-dependent protein kinase, which phosphorylates SyI, then detaches from synaptic vesicles, and is released from the actin, microtubules, and other synaptic vesicles [24]. SyI plays an important role in the movement of vesicles to the active sites in the presynaptic membrane, thus plays a regulatory role for neurotransmitter release [12]. In addition, SyI may be involved in the elongation of regenerating axons in the PNS regeneration [1, 7, 8, 12]. However, the involvement of SyI in the PNS regeneration is still controversial.

In this study, we elucidated the involvement of SyI in the PNS regeneration by immunocytochemistry with special emphasis on a fast axonal transport. SyI has not previously been detected immunocytochemically in the axons of normal nerves [16] until Akagi et al. [1] have demonstrated the presence of SyI in both the normal myelinated and unmyelinated axons. Battering et al. [4] have shown that the bulk of SyI is transported at a velocity of 6 mm/day, while a small amount of SyI is transported at a more rapid velocity up to 240 mm/day. In the normal nerve fibers, the morphological result from the present study is corresponded to the biochemical data. Synapsin I-like immunoreactive materials were accumulated only in the proximal to the crush site, while SV2 and p38-like materials were accumulated bidirectionally in the axons with all sizes. The transmembrane components, SV2 and p38, were retrogradely transported, while SyI was not retrogradely transported. SyI is also known to be transported with the fast axonal transport in the non-autonomic axons like rat sciatic nerve [8].

In this study, the changes in the distribution of SyI in the injured peripheral nerve were observed using an experimental animal model for PNS regeneration. Although the axons and myelin sheaths were injured by a ligation crush, the continuity of axons remained. Therefore, the transport of SyI in the regenerating axons were observed more in detail by immunocytochemistry [21]. The ligation crush method is better than the forcep or hemostat crush method in confirming an exact crush site and observing the transported distribution of SyI [13].

In the kinetics of the axonal transport, three pools of SyI present biochemically. The first pool of newly synthesized SyI departs from the cell body immediately after synthesis. The second and third pools enter the axon after delay of more than one day. We performed ligation of the nerve by 1-0 silk thread and released it after 1 day to observe the pools of SyI *in vivo*. Booj et al. [7] have reported that SyI rapidly accumulates in parallel with synaptic vesicle-specific integral membrane proteins proximal to the crush site. The integral membrane proteins of synaptic vesicles,

but not SyI, accumulate distally to the crush [7, 13]. These indicate that the synaptic vesicle membranes moving retrogradely from the nerve terminal to cell bodies do not carry appreciable amounts of SyI. SyI travels down the axon only anterogradely. Therefore, the translocation of SyI can be observed in the longitudinal section.

Previous studies have shown that some synaptic vesicle-associated proteins like synaptophysin [30] and synaptotagmin [34] were localized in the regenerating axonal sprouts emanating from the nodes of Ranvier. Synaptophysin and synaptotagmin were localized in the proximal region at 1 day after release. Recently, SyI has been reported to express in the regenerating axonal sprouts and growth cones. The immunoreactive regenerating sprouts appeared in the proximal region at 1 day and in the distal region at 3 days. This result suggests that SyI travels along the axon by a slow axonal transport [1]. In contrast, our study showed that SyI immunoreactive processes appeared at very early stages. The result indicates that SyI may be involved in the PNS regeneration and that the changes in the early accumulation of SyI may be related to the fast axonal transport. Dahlström et al. [12] have reported that four different synapsins, SyIa, Ib, IIa, and IIb33, are accumulated in the crushed nerve. A large amount of Sy Ib and a small amount of Sy Ia are accumulated in the parent axons proximal to the crush site up to 8 h after crushing. They concluded that Sy Ib may be transported rapidly in association with membranous organelles, while Sy Ia may be carried slowly in the axoplasm. Akagi et al. [1] have found that SyI IR in the regenerating axons was mainly associated with vesicular organelles. They suggested that SyI IR found on the vesicular organelles might represent Sy Ib in the early sprouts and growth cones of the regenerating axons.

In this study, the SyI, including both Sy Ia and Ib, accumulates at very early stages after release. The material localized at the early stages may represent mainly Sy Ib. De Camilli et al. [13] insisted that the effect of SyI on the axonal transport was not likely to occur *in vivo* since it would require concentrations of SyI normally present only in the nerve terminals but not in axons. However, the result is not consistent with ours. SyI is likely to be axonally transported from the cell body to the terminals. It is strongly expressed especially in the regenerating axonal sprouts. In our study, the distribution of SyI supports the results done by Akagi et al. [1] and Booj et al. [21]. SyI immunoreactive thin processes appeared from the proximal region to the crush site and extended into the distal region after time-lapse. SyI immunoreactive processes were expressed in the proximal region until 8 h after release. This result is consistent with that of Booj et al. [7], but not with that of Akagi et al. [1]. They showed that SyI IR was expressed in the proximal region at 1 day after release on the vesicular organelles. In this study, SyI IR was strongly

expressed from proximal to crush region at 1 day after release. An electron microscopic study may be necessary to elucidate the involvement of SyI on the vesicular organelles on the ultrastructural level.

In conclusion, the distribution patterns of SyI IR were changed. SyI was accumulated in the proximal region at very early stages after release. SyI was translocated from the proximal to distal site of ligation by the time lapse. These results suggest that SyI may be involved in the PNS regeneration in addition to a role as a regulator of neurotransmitter release. In addition, the early accumulation of SyI suggests that SyI may be related to the translocation of vesicles to elongated membrane by a fast axonal transport in the regenerating sprouts.

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