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Localization of HPC-1/syntaxin 1 in Developing Rat Cerebellar Cortex

Fumitaka Miya[1,2], Akitsugu Yamamoto[1], Kimio Akagawa[3], Keiji Kawamoto[2], and Yutaka Tashiro[1]

[1]Department of Physiology and Liver Research Center (Cell Biology), [2]Department of Neurosurgery, Kansai Medical University, Osaka 570, and [3]Department of Physiology, School of Medicine, Kyorin University, Mitaka, Tokyo 181, Japan

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ABSTRACT. **In adult rat cerebellum, HPC-1/syntaxin 1 is detected at high density on the plasma membrane of the non-synaptic region of parallel fibers in addition to the synaptic terminal membranes and the synaptic vesicles (Koh, S., Yamamoto, A., Inoue, A., Inoue, Y., Akagawa, Y., Kawamura, Y., Kawamoto, Y., and Tashiro, Y. (1993). J. Neurocytology 22: 995–1005). To assess the possibility that HPC-1/syntaxin 1 participates in the morphogenesis of the nervous system, we examined changes in the localization of HPC-1/syntaxin 1 during postnatal development of the molecular layer of the rat cerebellum. HPC-1/syntaxin 1 appeared in the granule cells in the outer granule cell layer in 3-days-old rat cerebellum when the formation of synapses and the appearance of a synaptic vesicle protein, synaptophysin, had not yet been observed in the molecular layer. At this stage, the granule cells began to form parallel fibers. Confocal laser microscopy and immuno-electron microscopy showed that HPC-1/syntaxin 1 was localized on the extruding plasma membrane of the granule cells to form parallel fibers. In 8-days-old rats, synapses formed between the parallel fibers and the developing dendrites of Purkinje cells, and the HPC-1 immunoreactivity appeared on the axons of parallel fibers and on the synapses. In 21-days-old rats, the HPC-1/syntaxin 1 immunostaining pattern was similar to that of adult rats. These results suggest that HPC-1/syntaxin 1 is involved in the formation of the molecular layer, especially in the axonal growth of the parallel fibers.**

HPC-1/syntaxin 1 was originally identified by Barnstable *et al.* as a neuron-specific protein which was recognized by one of the monoclonal antibodies produced against the membrane fractions of rat hippocampus (3). It has been shown that HPC-1/syntaxin 1 is present not only in the central and peripheral neurons (1, 14, 15, 19) but also in the neuroendocrine cells (16, 20). In rat cerebellum, HPC-1/syntaxin 1 was detected on the plasma membrane of presynaptic boutons and axons, and on the synaptic vesicles, but not on the dendrites (15). Molecular cloning of HPC-1/syntaxin 1 showed that the molecular weight of this protein is ~35 kDa, consisting of 288 amino acid residues, with a hydrophobic membrane anchor region at the carboxy-terminus (5, 13). HPC-1/syntaxin 1 is associated with N-type and P/Q calcium channels and synaptotagmin (17, 30).

Söllner *et al.* (24) identified HPC-1/syntaxin 1, SNAP-25 (synaptosomal associated protein of 25 kD), and VAMP-2 as SNAP (soluble NSF attachment protein) receptors (SNAREs), showed that they form a 20 S complex with NSF (N-ethylmaleimide sensitive factor) and SNAPs (soluble NSF attachment proteins), and proposed the SNARE hypothesis. In this hypothesis, vesicles dock to the target membranes by the specific interaction between v-SNAREs present on the vesicle membranes (e.g., VAMP-2 on the synaptic vesicles) and t-SNAREs present on the target membranes (e.g., HPC-1/syntaxin 1, SNAP-25 on the plasma membrane) respectively. This hypothesis was strengthened by the finding that clostridial neurotoxins inhibit neurotransmitter release by selectively cleaving HPC-1/syntaxin 1, SNAP-25, or VAMP-2 (21).

However, HPC-1/syntaxin 1 is present not only on the synaptic portion of the plasma membrane, but also on the preterminal portion of the axon and synaptic vesicles in adult rats (7, 15, 23). This fact suggests a function of this protein other than exocytosis of the synaptic vesicle as a t-SNARE. The dense localization of HPC-1/syntaxin 1 on the plasma membrane of preterminal portion of the parallel fibers raises the possibility that this protein may control the development of the neural processes of the axon and the morphogenesis of the nervous system. In fact, suppression of HPC-1/syntaxin 1A biosynthesis causes neurite-sprouting in cultured

Corresponding Author; Akitsugu Yamamoto, Department of Physiology and Liver Research Center (Cell Biology) Kansai Medical University, Osaka 570, Japan.
Telephone: 06–992–1001 (ext. 2435), Fax: 06–993–5319
e-mail: yamamota@takii.kmu.ac.jp

nerve cells (29).

The formation process of the molecular layer of rat cerebellar cortex has been investigated in detail by Altman (2). Immediately after birth, most granule cells are observed to be aligned outside of the cerebellum. During neonatal development, the granule cells extrude processes, form parallel fibers, and move towards Purkinje cells with a T-shaped configuration. Ultimately, the cell bodies of the granule cells pass through the layer of Purkinje cells to form inner granule layers. During the movement of granule cells, Purkinje cells develop dendrites, and begin to form synapses with parallel fibers from 4 days after birth (8), and the molecular layer develops. At 21 days after birth, formation of the molecular layer is completed, and the cerebellum shows almost the same morphology as that of the adult rat.

On the basis of the above observations by Altman, we investigated whether HPC-1/syntaxin 1 is involved in the developmental process of the molecular layer of cerebellum by examining the localization of HPC-1/syntaxin 1 in developing rat cerebellar cortex using immunohistochemical techniques. We found that HPC-1/syntaxin 1 appeared on the processes of the granule cells extruding parallel fibers before the formation of the synapses in the molecular layer. This result suggests that HPC-1/syntaxin 1 is involved in the formation of the molecular layer, especially in axonal growth of the parallel fibers or in the formation of synapses between the parallel fibers and the dendrites of Purkinje cells. This work was presented in part at the 46th Annual Meeting of the Japan Society for Cell Biology (18).

MATERIALS AND METHODS

Material. Sprague Dawley (Crj: CD (SD)) rats were used at 3, 8, 14, or 21 days of age. β-galactosidase/HPC-1 fusion protein was administered to rabbits to obtain a polyclonal antibody against the HPC-1/syntaxin 1 (13). The antibody against HPC-1/syntaxin 1 recognizes both HPC-1/syntaxin 1A and HPC-1/syntaxin 1B in the membrane fraction of rat cerebellum (14). This antibody was used throughout the present study. Mouse monoclonal antibody against synaptophysin was purchased from Böehringer Mannheim (Germany).

Light microscopic localization of HPC-1/syntaxin 1 by avidin-biotin-peroxidase complex (ABC) method on cryosections. Rats were anesthetized with ether and perfused through the left ventricle with Hanks' solution (pH 7.4) for 3 min, then fixed by perfusion with 4% paraformaldehyde containing 0.1% glutaraldehyde in Hank's solution for 10 min and washed with phosphate buffered saline (PBS) containing 50 mM NH_4Cl for 5 min.

The brains were frozen rapidly in liquid nitrogen. Frozen sections were cut with a cryostat, mounted on gelatin-coated glass slides, and incubated for 30 min with 0.3% H_2O_2 in PBS to inactivate endogenous peroxidase activity. For detection of synaptophysin, this step was omitted, as H_2O_2 treatment significantly reduced immunostaining. Sections were then incubated for 15 min with PBS containing 20% normal goat serum, 0.5% bovine serum albumin (BSA), and 0.3% Triton X-100, and reacted for 2 h with either antiserum against HPC-1/syntaxin 1 (diluted 1:400), immunoglobulin G (IgG) against synaptophysin (2 μg/ml), or non-immunized rabbit serum (diluted 1:400) as a control. Bound antibodies were detected by the ABC method (4, 10). The sections were washed three times in PBS containing 0.3% Triton X-100 for 5 min, subsequently reacted with biotinylated goat anti-rabbit IgG (5 μg/ml) in PBS containing 0.5% BSA and 0.3% Triton X-100 for 30 min, and washed three times in PBS for 5 min.

The sections were incubated for 30 min with avidin-biotin-peroxidase complex (Vectastain; Vector Labs, Burlingame, CA) in PBS. After the sections were washed three times with PBS for 5 min, peroxidase activity was visualized by incubation with 0.1% 3,3′-diaminobenzidine (DAB) in 50 mM Tris-HCl buffer (pH 7.4) supplemented with 0.02% H_2O_2. The sections were treated in 0.1% aqueous OsO_4 for 1 min and were counterstained with hematoxylin.

Immunofluorescence localization of HPC-1/syntaxin 1 by confocal laser microscopy. Frozen sections, cut with a cryostat and mounted on gelatin-coated glass slides as described above, were incubated for 15 min with PBS containing 20% normal goat serum, 0.5% bovine serum albumin (BSA), and 0.3% Triton X-100, and then reacted for 2 h with antiserum against HPC-1/syntaxin 1 (diluted 1:400). After washing three times in PBS for 3 min, the cells were incubated for 30 min with tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG (E-Y Laboratories, San Mateo, CA). The slides were rinsed, mounted in 80% glycerol and 0.02% azide in 40 mM Tris-HCl buffer (pH 8.0), then examined with a confocal laser scanning microscope (Olympus LSM-GB200, Olympus Optical Co., Japan).

Electron microscopic localization of HPC-1/syntaxin 1 by the IgG-gold technique on frozen ultrathin sections. Frozen ultramicrotomy was performed as described by Tokuyasu (27), with some modifications. Rats were fixed by perfusion as described above. The dissected cerebellum was incubated overnight in 2.3 M sucrose in 0.1 M sodium phosphate buffer (pH 7.4) containing 20% polyvinyl pyrrolidone, and rapidly frozen in liquid propane at −180°C. Frozen ultrathin sections were cut with a Reichert Ultracut-E with a cryoattachment (FC-4D) at −100°C to a thickness of about 80 nm. The sections were picked up on formvar-carbon-coated nickel grids, incubated with 2% gelatin in PBS containing 10 mM glycine, and reacted for 60 min with either antiserum against HPC-1/syntaxin 1 (diluted 1:400) or nonimmunized rabbit serum (diluted 1:400). The sections were then washed six times with gelatin solution and reacted for 15 min with gold particles conjugated with goat IgG against rabbit IgG (5 nm in diameter, OD 525 mm=0.08, ULTRA BIOSOLS, Liverpool, UK). After washing with sodium cacodylate buffer (pH 7.4), sections were post-fixed in 2% glutaraldehyde and then in 1% OsO_4,

stained with uranyl acetate, embedded in LR White resin (26), and observed under a Hitachi H-7000 electron microscope (Hitachi; Tokyo, Japan).

RESULTS

Light microscopic localization of HPC-1/syntaxin 1. In three-day-old rats, there was a thick outer granule cell layer (a proliferative zone and a premigratory zone), and molecular layer had not yet developed. HPC-1/syntaxin 1 immuno-reactivity was already present in the granule cells in the outer granule cell layer (Figs. 1A and 2A). In three-days-old rats, synaptophysin (a synaptic vesicle protein) was not yet detected in the cerebellar cortex (Fig. 2E).

In eight-days-old rats, the granule cells in the outer granule cell layer moved towards the Purkinje cells, and the formation of the molecular layer between granule cells and Purkinje cells had already begun. Strong HPC-1/syntaxin 1 immunostaining appeared on the developing molecular layer in addition to the outer granule cell layer (Figs. 1B and 2B). The staining in the mo-

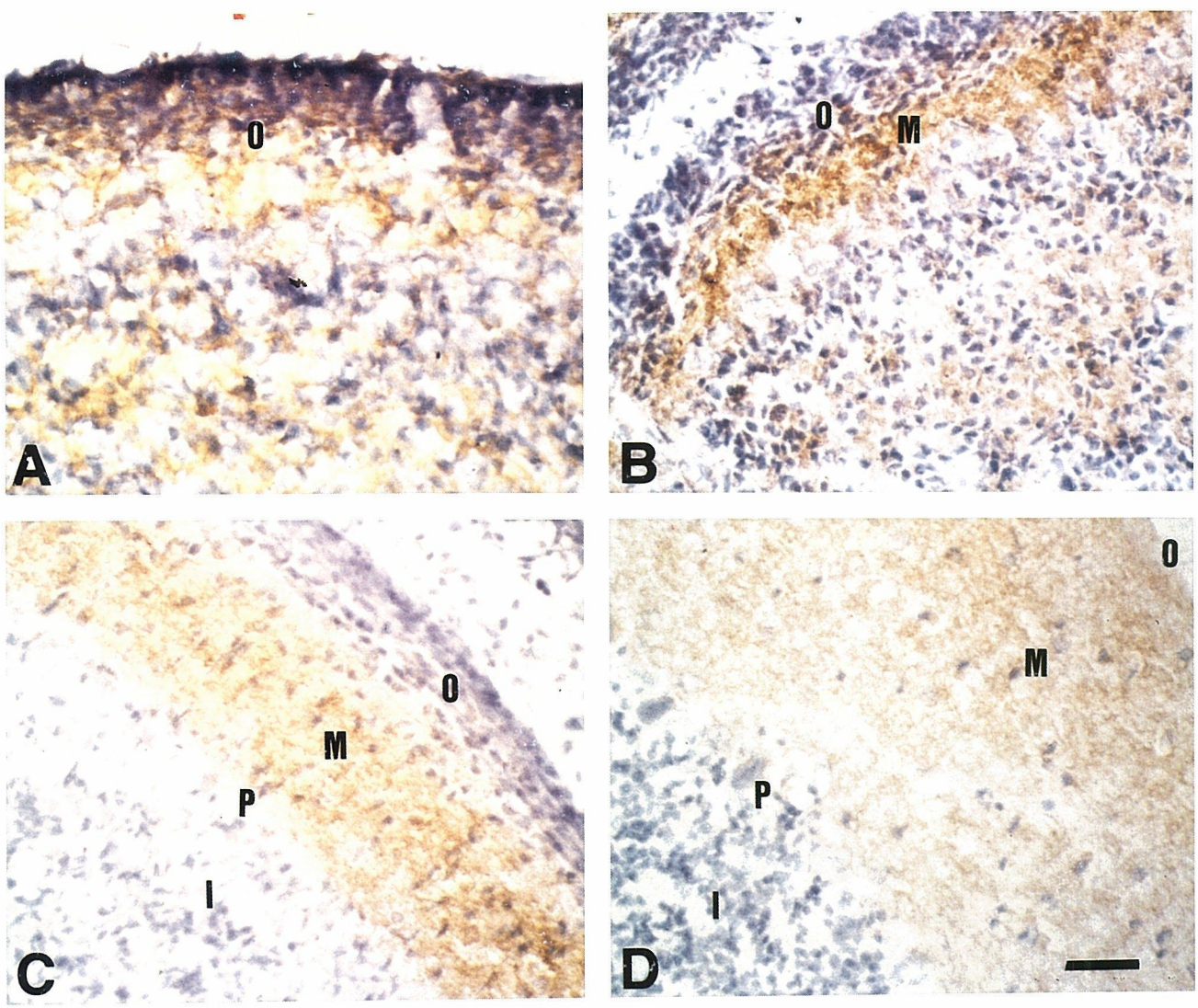

Fig. 1. Light microscopic localization of HPC-1/syntaxin 1 during development of the cerebellar cortex at a low magnification. Cryosections of cerebella of neonatal rats were processed for immunolocalization of HPC-1/syntaxin 1 using the ABC method. O: outer granule cell layer, M: molecular layer, P: Purkinje cells, I: inner granule cell layer. (A). At 3 days after birth, reaction product (brown color) showing the presence of HPC-1/syntaxin 1 is already detected in the outer granule cell layer. (B). At 8 days after birth, formation of the molecular layer is observed. HPC-1/syntaxin 1 is localized in the molecular layer in addition to the outer granule cell layer. (C). At 14 days after birth, the molecular layer is increased in width, and the staining is localized to the molecular layer. (D). At 21 days after birth, HPC-1/syntaxin 1 is localized in the well developed molecular layer. Most of the outer granule cells has moved to the inner granule cell layer from the outer granule cell layer. Bar: 50 μm.

lecular layer presumably corresponded to the parallel fibers and synapses, as the formation of synapses between Purkinje cells and parallel fibers occurs from 4 days after birth (8). In 8-days-old rats, synaptophysin was detected in the molecular layer corresponding to the formation of the synapses in this layer (Fig. 3F).

On postnatal day 14, the molecular layer further expanded in width (Fig. 1C). Both HPC-1/syntaxin 1 and synaptophysin were detected in the molecular layer (Figs. 2C and 2G).

In 21-days-old rats, most of the granule cells had completed their movement from the outer granule cell layer to the inner granule cell layer which was located deep to the Purkinje cells. The immunostaining patterns of HPC-1/syntaxin 1 and synaptophysin were similar to those of adult rats (Figs. 1D, 2D, and 2H).

Confocal laser microscopic localization of HPC-1 /syntaxin 1. To examine the localization of HPC-1 /syntaxin 1 during the molecular layer formation more precisely, we prepared cryosections of three-days-old rat cerebellum for immunofluorescence staining to observe them under a confocal laser scanning microscope.

In three-days-old rats, HPC-1/syntaxin 1 was detected in both the cell bodies and the fiber-like processes of granule cells corresponding to the appearance of parallel fibers in the process of axonal growth (Fig. 3A). In Fig. 3B, relative intensity of the immunofluorescence staining in the same area as Fig. 3A is shown in pseudocolor.

Electron microscopic localization of HPC-1/syntaxin 1 by the IgG-gold technique on frozen ultrathin sections. At three days after birth, although synapse formation was not yet recognized, neural processes were already formed. Many gold particles indicating the presence of HPC-1/syntaxin 1 were detected on the processes (Fig. 4A). At postnatal day 8 when synapses and synaptic vesicles already appeared, gold particles bound to presynaptic membranes and synaptic vesicles (Fig. 4B) in addition to the preterminal region of the parallel fibers as in the cerebellum of the adult rat (15).

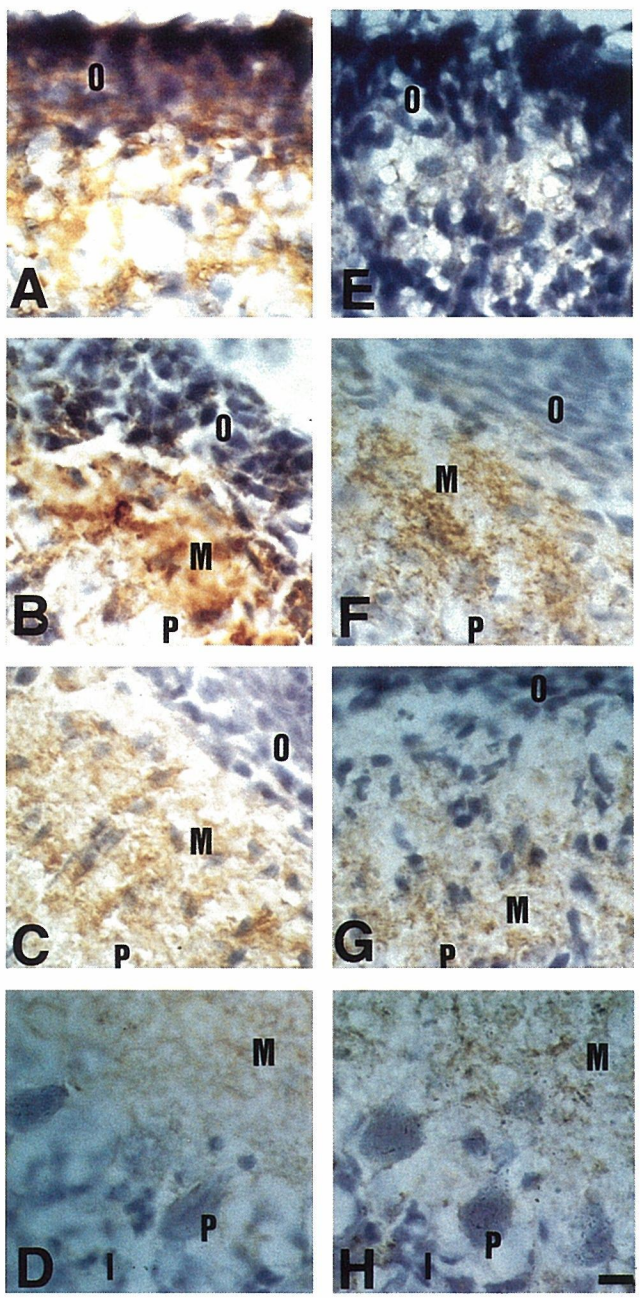

Fig. 2. Light microscopic localization of HPC-1/syntaxin 1 and synaptophysin during development of the cerebellar cortex at a higher magnification. Cryosections of cerebella of neonatal rats (A, E: 3 days, B, F: 8 days, C, G: 14 days, E, H: 21 days) were processed for immunostaining for HPC-1/syntaxin 1 (A–D) or synaptophysin (E–H) using the ABC method. O: outer granule cell layer, M: molecular layer, P: Purkinje cells, I: inner granule cell layer. Bar, 10 μm.

DISCUSSION

A large body of experimental evidence supports the idea that HPC-1/syntaxin 1 plays an essential role in the exocytosis of synaptic vesicles as a t-SNARE (6, 21, 24). However, Koh *et al.* (15) showed that HPC-1/syntaxin 1 is localized on the plasma membrane of non-synaptic region and synaptic vesicles in addition to the active zone of the synapse. This peculiar localization of HPC-1/syntaxin 1, which were confirmed by various authors, suggests a complex role for this protein (7, 22, 23, 28). Walch-Solimena *et al.* (28) proposed that HPC-1/syntaxin 1 located on the synaptic vesicles undergoes recycling in the nerve terminal. In adrenal chromaffin cells, some HPC-1/syntaxin 1 is associated with chromaffin granules, suggesting its role as a v-SNARE (25).

In rat cerebellum, HPC-1/syntaxin 1 is most densely localized on the plasma membrane of the preterminal

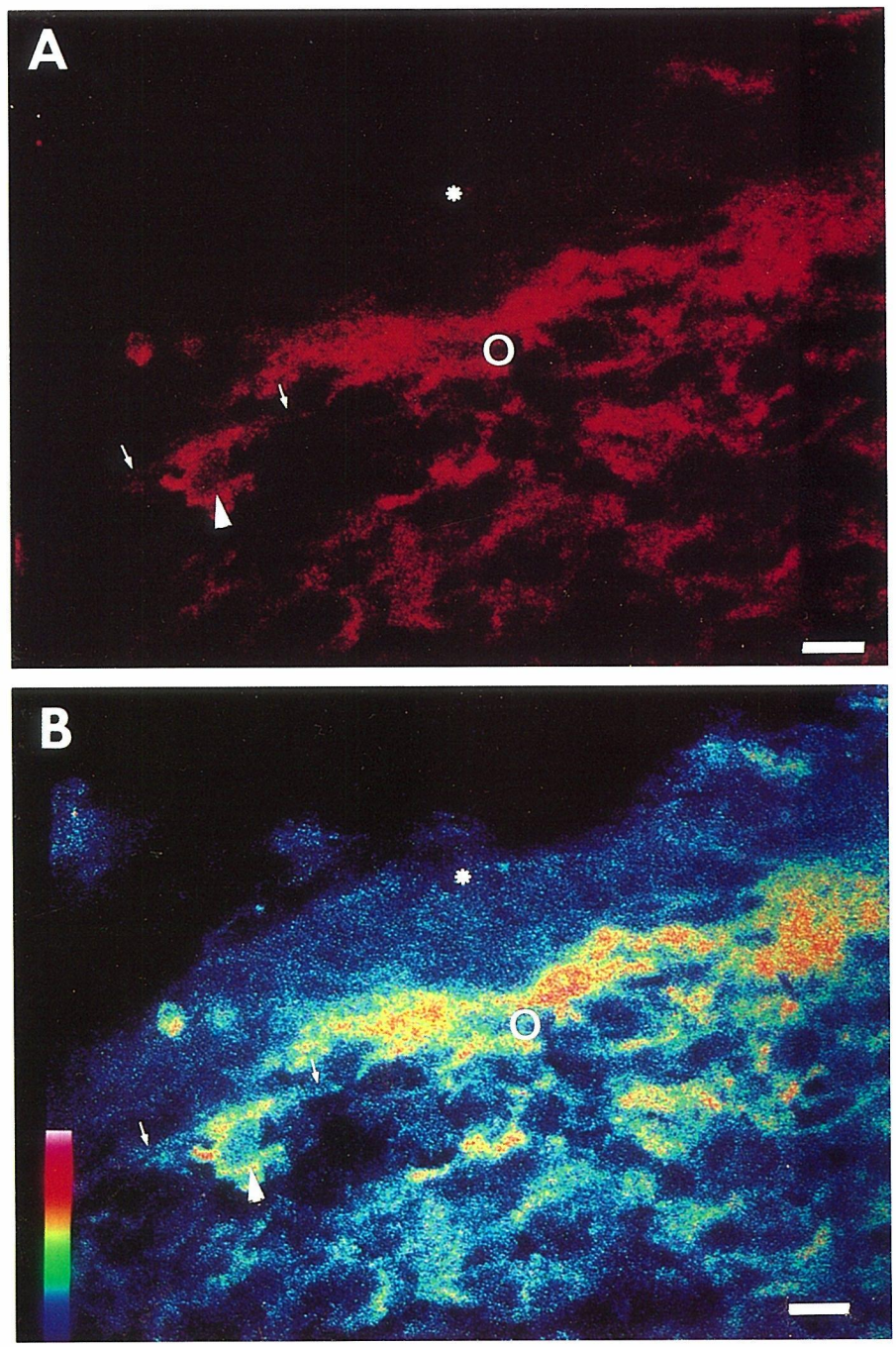

Fig. 3. Confocal laser microscopic immunolocalization of HPC-1/syntaxin 1. Cryosections of rat cerebella from a 3-days-old rat were reacted with antibody against HPC-1/syntaxin 1 and a second antibody labelled with rhodamine, and observed under a confocal laser microscope. (A) HPC-1/syntaxin is localized on the cell bodies (arrow head) and fiber-like processes (arrows) of granule cells in the outer granule cell layer (O). (B) Relative intensity of the immunofluorescence staining in the same area as Fig. 3A is shown in pseudo-color. Asterisks show the outer most end of the cerebellum. Bars: 10 μm.

Fig. 4. Immuno-gold localization of HPC-1/syntaxin 1 on ultrathin cryosections of neonatal rat cerebellum. (A) At 3 days after birth, many gold particles (5 nm) bind to the processes of the plasma membrane of the granule cells. (B) At 8 days after birth, gold particles bind to presynaptic terminal membranes and synaptic vesicles (arrows) in addition to the preterminal axon of the granule cells. S: synapse. Bar: 200 nm.

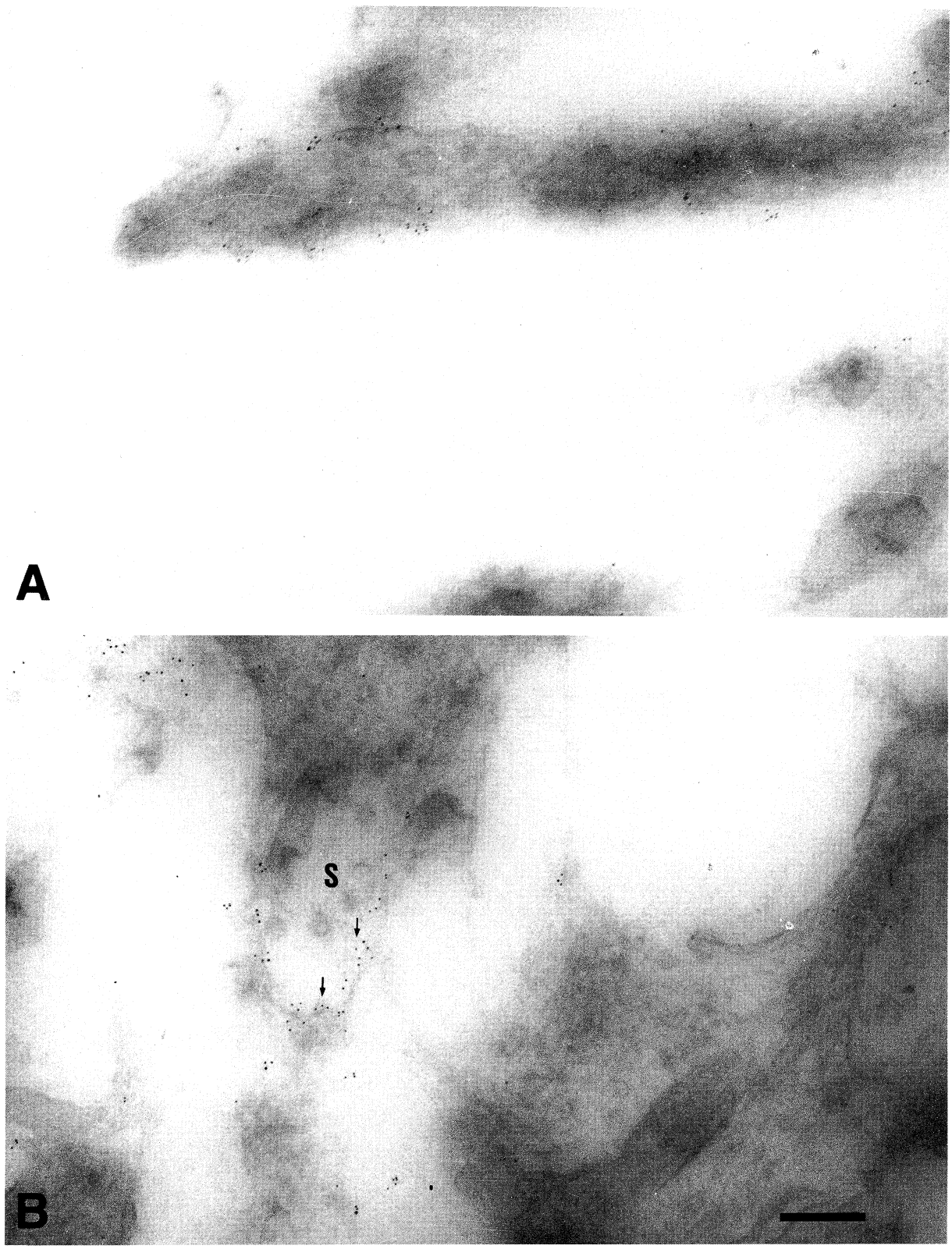

Fig. 4.

portion of the parallel fibers (15). This raises the possibility that HPC-1/syntaxin 1 controls the morphogenesis of the axon. In the present study, we investigated the changes in the localization of HPC-1/syntaxin 1 during the development of the molecular layer of rat cerebellum to examine whether HPC-1/syntaxin 1 is involved in the morphogenetic processes. We found that HPC-1 /syntaxin 1 appears in the granule cells in the outer granule cell layer already at 3 days after birth, when formation of the synapses in the molecular layer has not yet begun. At this stage, the granule cells start to form parallel fibers, extruding the processes. Confocal laser microscopy and immuno-electron microscopy showed that HPC-1/syntaxin 1 was localized on the expanding plasma membrane of the parallel fibers during axonal growth. These results suggest that HPC-1/syntaxin 1 is involved in the formation of the molecular layers, especially in the axonal growth of the parallel fibers or in the formation of synapses between the parallel fibers and the dendrites of Purkinje cells.

Our results are consistent with those of a recent report by Igarashi *et al.* (11), who demonstrated using botulinum neurotoxin C1 that syntaxin is involved in the axonal growth of chick dorsal root ganglion cells *in vitro* culture. During axonal growth, the expanding plasma membranes are presumably supplied with membranes by vesicle fusion. HPC-1/syntaxin 1 located on the tips of the parallel fibers may participate in the vesicle fusion during axonal growth as a t-SNARE. It is also possible that HPC-1/syntaxin 1 may participate in supplying presynaptic membranes with the components required for forming synapses through exocytosis.

On the other hand, Schulze *et al.* (22) showed that in Drosophila, complete absence of HPC-1/syntaxin-1A causes only subtle morphological defects in the nervous system although it causes complete failure in the evoked neurotransmitter release. This finding suggests that the roles of HPC-1/syntaxin 1 in the morphogenesis of the nervous system in Drosophila is quite different from that in the mammals. Further work is needed to determine the exact significance of the presence of HPC-1 /syntaxin 1 during the morphogenesis of the nervous system.

HPC-1/syntaxin 1 has significant homology to epimorphin/syntaxin 2 (9, 12), and both proteins belong to the syntaxin family (6). Interestingly, epimorphin /syntaxin 2 was originally identified as a factor inducing epithelial morphogenesis of undifferentiated epithelial tissue by Hirai *et al.* (9). It would be very interesting to know whether or not epimorphin/syntaxin 2 participates in the epithelial morphogenesis through exocytosis as a t-SNARE.

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