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Developmental Changes of Synapsin I Subcellular Localization in Rat Cerebellar Neurons

Akihiro Harada*, Kenji Sobue, and Nobutaka Hirokawa*

**Department of Anatomy and Cell Biology, School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113 and Department of Neuropharmacological Chemistry, School of Medicine, University of Osaka, Osaka, 530, Japan*

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ABSTRACT. **Synapsin I, one of the major synaptic proteins, is thought to associate with synaptic vesicles and to play a regulatory role in neurotransmitter release. In mature neurons, it is concentrated almost exclusively in presynaptic nerve endings. Here, we studied the subcellular localization of synapsin I during the development of rat cerebellar cortices by immunocytochemistry, using anti-synapsin I antibodies and found that during the development of rat cerebellar cortices it tentatively exists in the dendritic growth cones of immature internal granule cells and in the axonal growth cones of mossy fibers as well as mature presynaptic endings. Also, we found that synapsin I, in the axonal and dendritic growth cones does not necessarily associate with vesicles, but rather with fuzzy filamentous structures in the cytoplasm. In search of the structure of synapsin I in vivo, we employed the quick-freeze, deep-etch technique after immunogold labeling. Synapsin I seems to thereby connect synaptic vesicles or anchor them to cytoskeletons in presynaptic endings.**

Synapsin I, an 84/82 kd basic phosphoprotein phosphorylated by calcium/calmodulin dependent kinase and cAMP dependent kinase, is known to localize in efferent (presynaptic) (14, 15, 26, 39, 42) and afferent (16) nerve endings. Biochemically it is known to associate with actin, tubulin, neurofilaments, and synaptic vesicles (3, 4, 5, 6, 7, 9, 19, 30, 31, 33, 34, 38). This protein is considered to play an important role in regulating a neurotransmitter release, since after phosphorylation, the affinity with cytoskeletons and synaptic vesicles becomes lower (3, 9, 28, 34, 39). Its localization during development has been investigated with immunofluorescence or the immunoperoxidase method using mice (29), cat (17), and cultured cells (10, 13). Recently, we studied the molecular structure of synapsin I and showed that synapsin I could really form bridge structures among synaptic vesicles and actin filaments in vivo (22). However, previous researches have not elucidated how this protein is expressed during the development *in vivo*. In this paper, we concentrated our study on internal granule cells and mossy fibers in developing rat cerebellum (0, 4, 7, 10, 14, and 21 day-old postnatally). Here, we revealed precisely the localization of synapsin I in the nerve cells during development. We employed ultrathin cryosection (22, 41) or the freeze-thaw antibody infiltration method (11) following gold immunolabeling besides immunofluorescence microscopy. In addition, we performed antibody decoration combined with the quick-freeze, deep-etch (QF–DE) electron microscopy to study the localization of synapsin I *in vivo*.

MATERIALS AND METHODS

Purification of synapsin I. Synapsin I was prepared from bovine brain membrane fractions according to a method described by Okabe and Sobue (33).

Production and purification of anti-synapsin I antibody. Antibodies against synapsin I were prepared and IgG fractions were obtained by a method described previously (23, 33).

Immunofluorescence microscopy. Cerebella were dissected from 0, 4, 7, 10, 14, and 21-day old postnatal rats and were perfused with 2% paraformaldehyde in a 0.1 M phosphate buffer, pH 7.3. After overnight fixation, the tissues were cryoprotected with a graded concentration series of sucrose solutions in a 0.1 M phosphate buffer, pH 7.3, and frozen sections were prepared for immunohistochemistry. Sections were incubated with antibodies to synapsin I, followed by an incubation with secondary antibodies containing rhodamine-conjugated goat antirabbit IgG (Cappel Laboratories, Malvern, PA). As a control sections were incubated with nonimmune rabbit IgG. (Zymed Laboratories. San Francisco, CA). After immunostaining, lobules II–V were selected under a microscope for observation.

Freeze-thaw immunoelectron microscopy of post-embedded sections. Procedures for immunolabeling of ultrathin cryosections were performed according to Tokuyasu (41) with slight modification (22). Procedures for the freeze-thaw antibody infiltration method were performed according to Black *et al.* (11) with slight modification. The 0, 4, 7, 10, 14, and 21-day old rats were perfused with 2% paraformaldehyde and 0.1% gluraraldehyde in a 0.1 M phosphate buffer, adjusted to pH 7.3. The cerebellum was dissected, fixed overnight, and sectioned parasagitally (100–200 μm) with a vibratome. From the free floating sections, lobules II–V were dissected out with razor blades, then incubated with a 0.1 M phosphate buffer, pH 7.3 containing 4% sucrose for 30 min, followed by incubation with the above solution containing 1 mg/ml $NaBH_4$ for 30 min (at 4°C). After the sections were washed with the above solution without $NaBH_4$ for 30 min, they were cryoprotected with 10 and 20% sucrose in a 0.1 M phosphate buffer for 30 min respectively, stored in 30% sucrose in the same buffer overnight (all at 4°C), and given a rapid freeze-thaw with liquidified Freon 22 chilled in liquid nitrogen. The sections were then transferred to 10% sucrose in a 0.1 M phosphate buffer, pH 7.3, to 4% sucrose in the same buffer, and finally to PBS. Next, after pretreatment with 50 mM glycine in PBS for 20 min and with 5% NGS in 1% BSA PBS (60 min, three changes), they were incubated overnight (at room temperature) in rabbit anti-synapsin I antibody (20 μg/ml in PBS containing 1% BSA). After extensive washing (90 min, six changes) in PBS/0.2% BSA, they were incubated overnight in gold conjugated goat anti-rabbit IgG (Janssen Pharmaceutica, Beerse, Belgium), washed extensively (90 min, six changes), and fixed with 3% glutaraldehyde in a 0.1% cacodylate buffer for 1 hr. Some samples were taken for QF–DE. The others were postfixed with OsO_4, then with uranyl acetate, followed by dehydration in ethanol and propylene oxide, embedded in Epon 812 and processed for conventional ultrathinsection for EM. In serial sectioning, sections (colors: gold-purple) were cut with LKB microtome, picked up with single slot grids, and covered with formvar membrane.

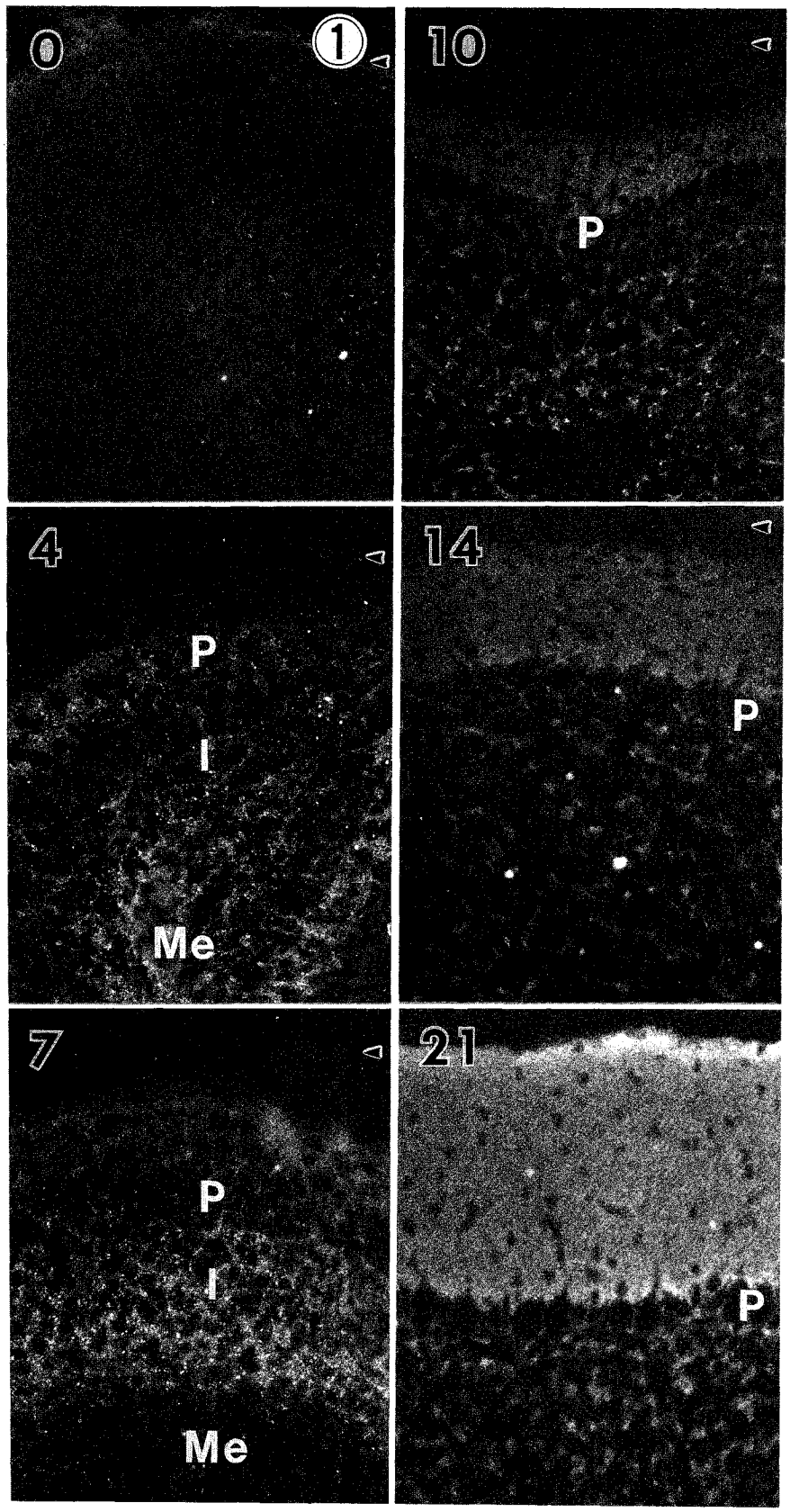
QE–DE method of immunogold reacted thick sections. After treatment of immunogold, some slices were quick-frozen, subjected to QF–DE technique, as described previously (20, 21, 22, 25).

RESULTS

Immunofluorescence. We first conducted an immunofluorescence study with anti-synapsin I antibody on developing rat cerebellar cortices to determine the approximate location of the antigen. Fig. 1 demonstrates that in postnatal 0-day old rats (0d), little immunostaining was found throughout the layers. In 4d, rats however, the area between the external and internal granule layers, where it is presumed to be, the molecular layer was stained with bright dots (1). Coarser immunostaining was also seen below the internal granule layer. In 7d rats, the staining between the internal and external granule layers became broader, and the one between the internal granule cells grew much brighter. In contrast, the area below the internal granule layer became almost free of stain. From 10d on, the staining in the molecular layer became broader as the layer widened, and the one in the internal granule layer became confined to smaller areas between the granule cells by around 20d when cerebellar structure reaches its adult form.

Subcellular localization of synapsin I in synaptic terminals. We chose a glomerulus, a large synaptic complex involving the presynaptic terminal of mossy fibers in the center and the surrounding postsynaptic terminals of internal granule cells and the presynaptic terminals of Golgi cells, as a developmental model of synapsin I expression in a presynaptic terminal since its development begins postnatally at large and its large presynaptic ending is quite easy to identify (Fig. 2C) (1). Two techniques, the ultrathin cryosection (22, 41) and the freeze-thaw method (11), were used to investigate the subcellular localization of synapsin I. Fig 2A and 2B show adult and 14d glomeruli respectively. Fig. 2A, using the ultrathin cryosection, shows 10 nm gold particles accumulated almost exclusively in the presynaptic terminal, suggesting that synapsin I localizes in the presynaptic terminal much more than in the postsynaptic one. At a higher magnification, gold particles were recognized between synaptic vesicles. Figure 2B, using the freeze-thaw method, presents almost the same immunogold (5 nm) localization as Fig. 2A. However, this method shows a better ultrastructural image than the ultrathin cryosection (Figs. 2B, C). At a higher magnification, immunogold particles thereby seem to associate with the filamentous materials between the synaptic vesicles rather than with the synaptic or plasma membranes themselves. We found the same immunogold distribution pattern at a more advanced stage (data not shown). Fig. 2D shows that the use of non-immune rabbit IgG at the same concentration (20 μg/ml) yielded few, evenly distributed particles on any of the subcellular structures. We came across very few non-specific gold aggregates. Next more immature presynaptic terminals are shown in Fig. 3. Fig 3A shows a growth cone in 4d rat presumed to be the immature form of glomerulus because it has an electron lucent cytoplasm and several

Fig. 1. Immunolabeled frozen section of rat cerebellar cortex stained with anti-synapsin I antibody. The figure in the left upper corner in each picture represents the day after birth. (0d) Almost no staining was detected. (4d) Bright staining appears in medulla (Me) and around Purkinje cells (P). (7d) Staining in medulla decreased and the one in the inner granule layer (I) and in the molecular layer (M) increased in width and in intensity. (10d) (14d) (21d) Staining localized between granule cell somas in the inner granule cell layer and in the molecular layer. The outermost end is shown by arrowheads.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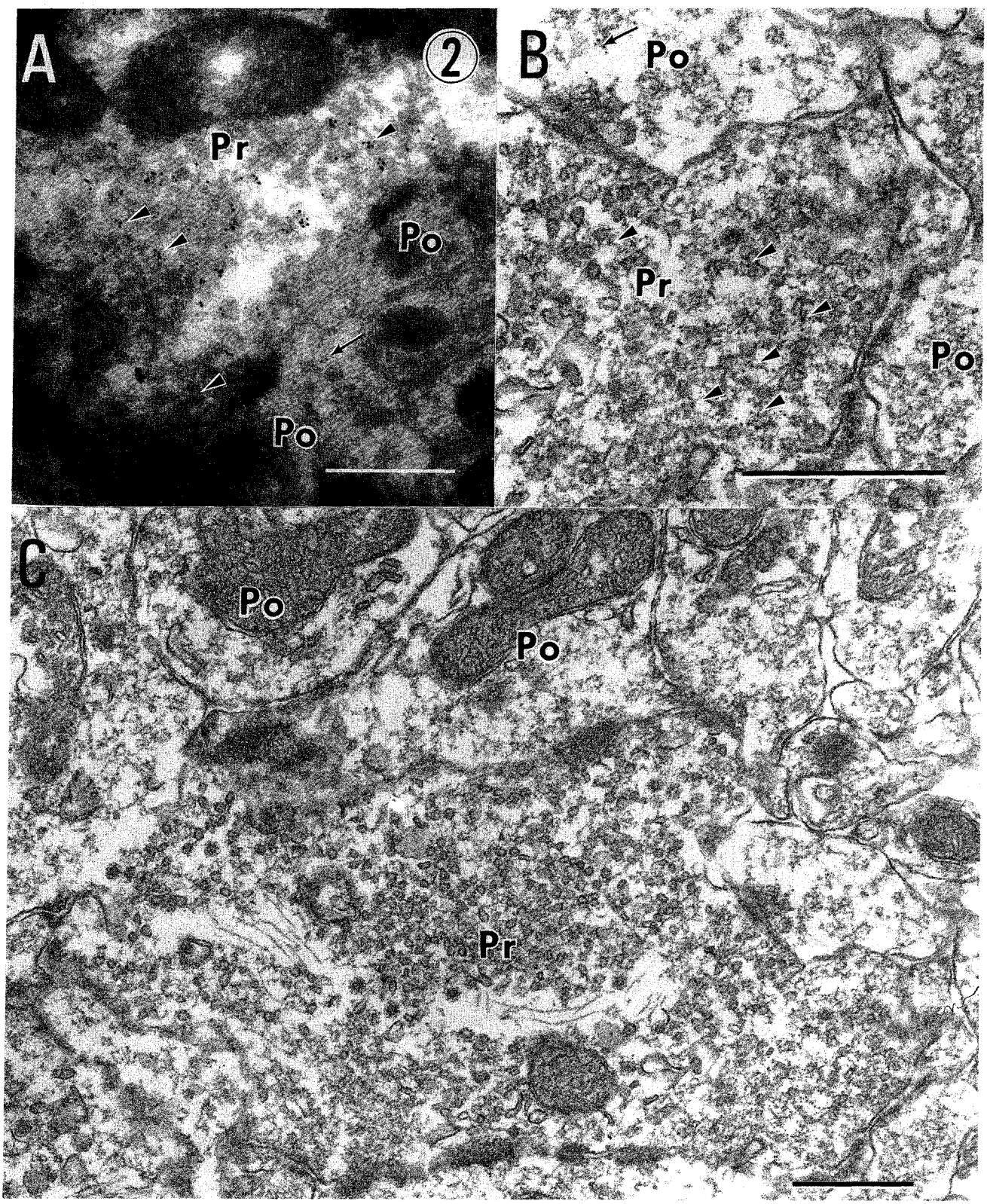
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synaptic vesicle-like or growth cone vesicle-like vesicles and it is just as large as the developed glomeruli (compare 3A with 2C) (12, 18, 24, 43). Higher magnification (Fig. 3B) shows that immunolabeling does not necessarily concentrate around small, synaptic vesicle-like vesicles but distributes diffusely over fuzzy filamentous materials in the cytoplasm. In more advanced presynaptic terminals, we obtained similar results (Fig. 3C, D). However, a part of the labels seemed to be localized around vesicles. In 7d rats we observed another type of synaptic structure in the internal granule layer. In Figs. 4A, B, different places in the same axon are shown. Judging from the large diameter of the axon and from its location (i.e. in the internal granule layer), it was considered to be an immature en passant synapse or precursors of synaptic vesicles in massive transport (35, 37) in a mossy fiber. Fig. 4A, demonstrates diffusely distributed labels over cytoplasm just like the one seen in 4d-old rats (Fig. 3). On the other hand, as shown in Figs. 4B and 4C, gold labels were found around fuzzy structures associated with synaptic vesicles as well as the ones in the cytoplasm. In Fig. 4C, at a higher magnification, immunogold particles were obviously observed between the vesicles.

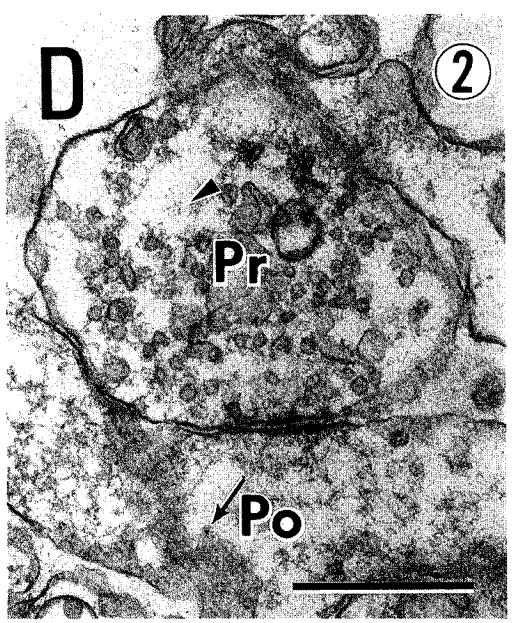


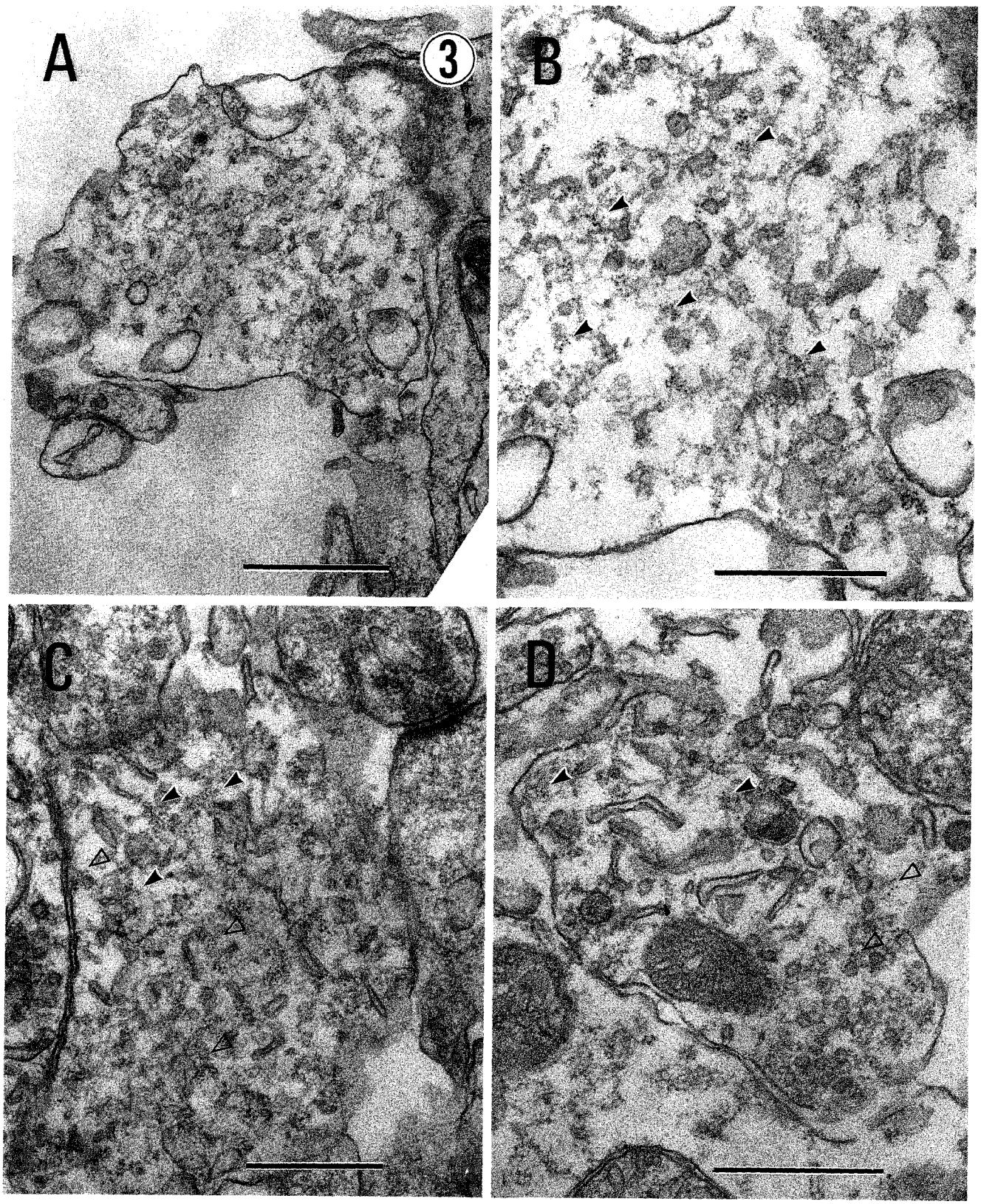
Fig. 2. The view of glomeruli immunolabeled with anti-synapsin I antibodies using the ultrathin cryosection (Fig. 2A) or the freeze-thaw method (Figs. 2B, C). (A–D); 14d-old rats 10 nm gold and 5 nm gold were used in Figs. 2A and 2B.C, respectively. In Figs. 2A, B, gold particles were observed around synaptic vesicles (arrowheads) in the presynaptic ending (Pr). Few particles were recognized in the postsynaptic endings (Po) (arrows). Bars 0.5 μm. In Fig. 2C, the low magnification view of Fig. 2B shows the good preservation of the tissue. Fig. 2D is a control for Figs. 2A, C. The same concentration of nonimmune rabbit IgG shown slight nonspecific labeling (arrow and arrowhead). Bar 0.5 μm.

Subcellular localization of synapsin 1 in immature internal granule cells. We investigated samples from 4 day-old rats. Since it is difficult to distinguish axons and dendrites in one section at this early stage of development, we collected samples from every section for electron microscopy and reconstructed the images serially. In Figs. 5A–C, the neuronal process stretching downward(*) (away from the molecular layer) received synaptic inputs (arrowheads in Figs. 5A–C), where postsynaptic density was observed (Figs. 5A–C, F, G). We also observed a neurite extending in the opposite direction (i.e., toward the molecular layer) (arrows in Figs. 5D, E). In the previous report (32), almost all granule cell axons are small in diameter and are extending towards the molecular layer. Therefore, this neurite appears to be an axon. From these observations (receiving synaptic inputs and its direction), we regarded the neurite(*) in Figs. 5A–C as a dendrite. In this immature dendrite, generally, many gold particles were observed in the cytoplasm. Near the synaptic complex (Figs. 5F, G), a larger number of particles (arrows) were found compared with mature postsynaptic terminals. In addition, near another tip of this dendrite which was not making synaptic contacts (Figs. 5H, I), we found more gold particles than the postsynaptic parts (as in Figs. 5F, G) in the same stage. Similarly, in the paranuclear area (Fig. 5J), we found many particles associated not with vesicles and Golgi bodies but with fuzzy materials around them (arrows).

When we used the same concentration of rabbit nonimmune IgG as the first antibody, we found very few particles throughout the dendrites (data not shown).

QF-DE after immunolabeling. We chose a 4 day-old rat as a sample for the QF-DE technique. In the presynaptic terminals (Figs. 6A, B) filled with vesicles, we found many gold particles near the fine, short, filamentous structures between synaptic vesicles (arrows) rather than directly synaptic vesicles or membranous structures. Sometimes we came across well-developed synaptic terminals (Figs. 6C, D). In agreement with the data obtained by thin section, a larger number of gold particles were seen in the presynaptic terminals (arrows) than

Fig. 3. Gold-labeled immature presynaptic endings. (A–C); 4d, (D); 7d-old rats (A) An immature presynaptic ending was identified at low magnification. (B) At higher magnification, vesicles of various sizes existed in the cytoplasm. Gold particles were associated with fuzzy materials in the cytoplasm (arrowheads) rather than with the vesicles. (C), (D) More mature presynaptic ending. Small, round vesicles increased in number. Fuzzy material decreased and appeared to concentrate around vesicles. Some labels concentrated around vesicles (open arrowheads), others did not (filled arrowheads). Bars (A): 1 μm; (B–D): 0.5 μm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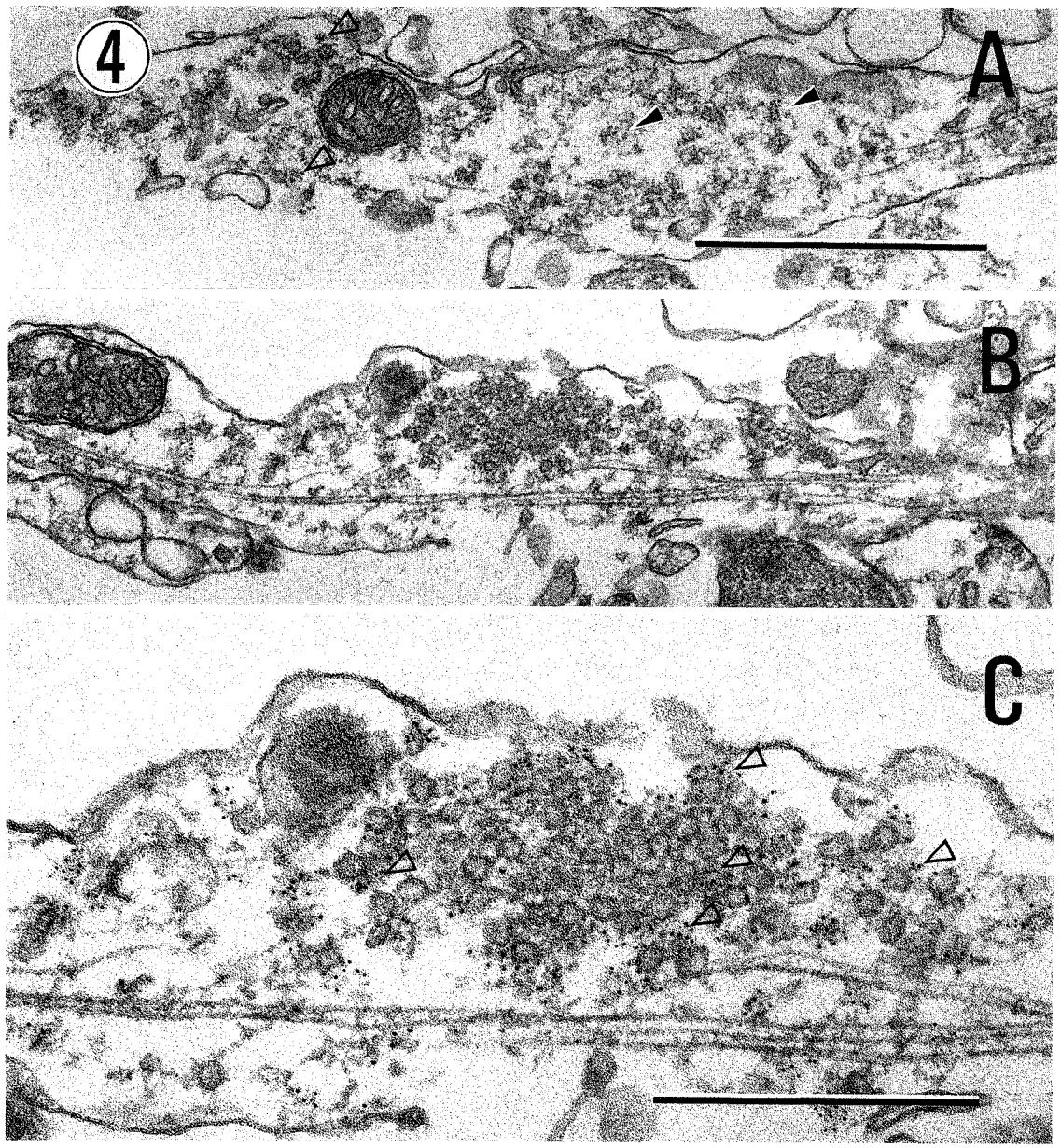
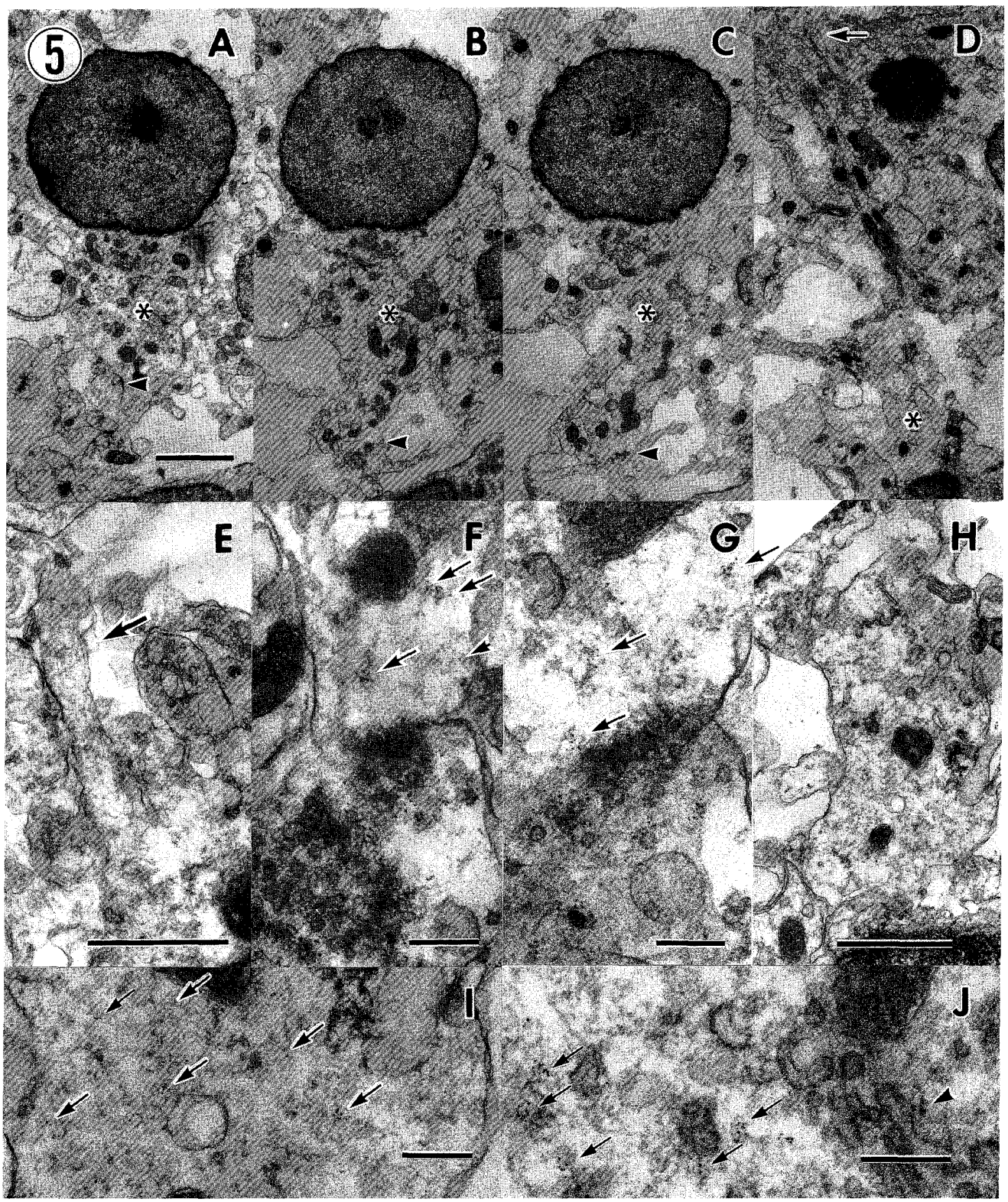


Fig. 4. (A), (B); An en passant synapse in the inner granule layer in 7d-old rats. Gold particles associated with fuzzy material between small vesicles (open arrowheads). Some of them were recognized in the cytoplasm (filled arrowheads). (C); High magnification view of Fig. 4B. Bars (A, B): 1 μm; (C): 0.5 μm

Fig. 5. (A) (B) (C) (D) Part of serially reconstructed sections of one granule cell from 4d-old rats. From (A) to (C), photomicrophages were taken every three sections. The molecular layer and immature white matter were located above and below the figures, respectively. Photograph (D) was taken nine sections after (C). (E) High magnification view of Fig. 5D. Axon-like neurite (arrow) was extending upward (towards the molecular layer). (F) (G) Each represents synaptic complex in Figs. 5B, C, respectively at high magnification. Many gold particles were observed (arrows) in the postsynaptic areas. (H) One of the tips of dendritic branches which was not making synaptic contacts located two sections after Fig. 5D. (I) The middle part of Fig. 5H at high magnification. More particles (arrows) were found in the cytoplasm compared with Figs. 5F, G. (J) High magnification view of paranuclear area in Fig. 5C. Fewer particles were observed among the vesicular structures (arrowhead) than the filamentous structure around them (arrows). Bars (A–D): 2 μm; (E, H): 1 μm; (F, G, I, J): 0.2 μm.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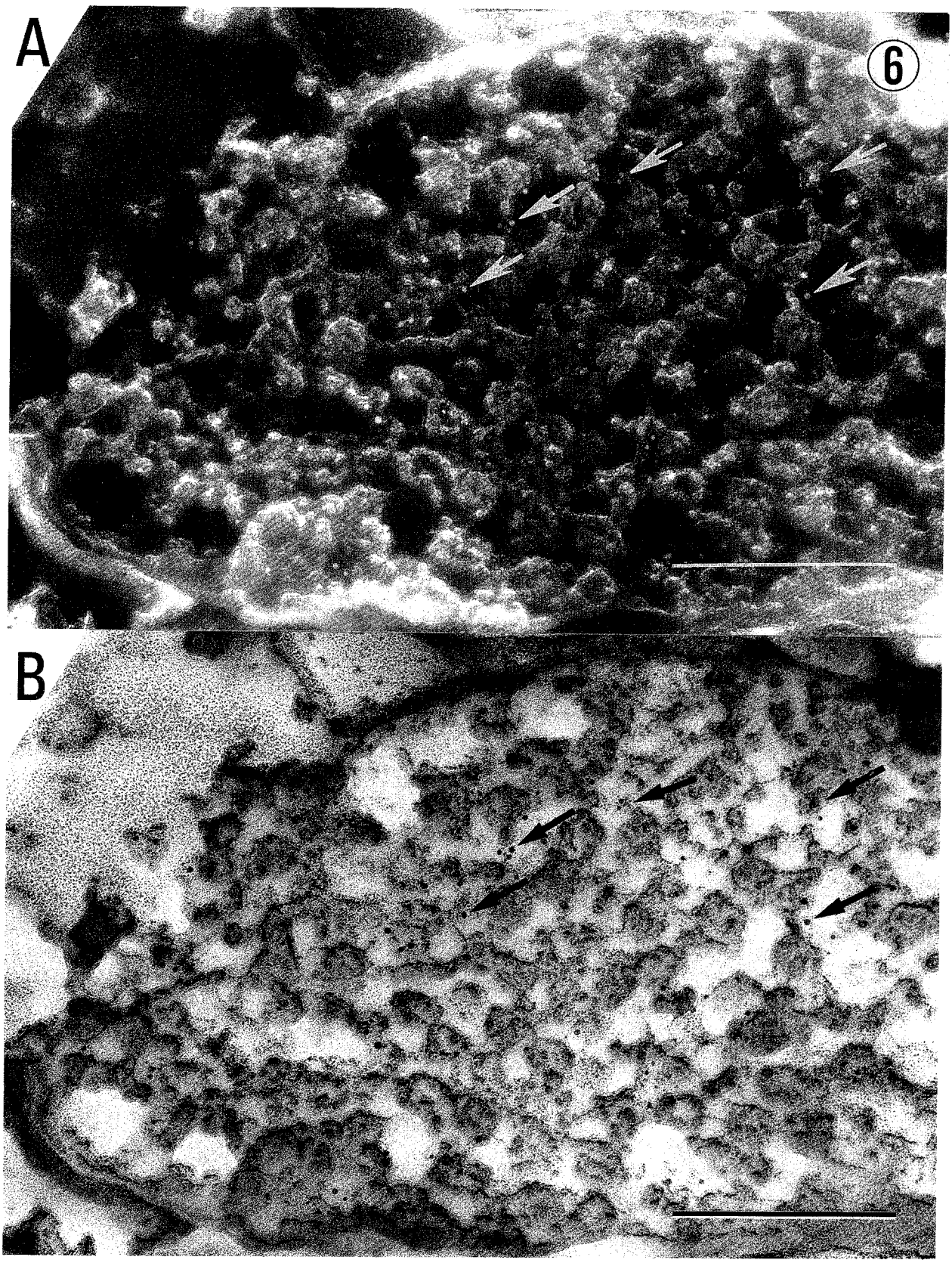
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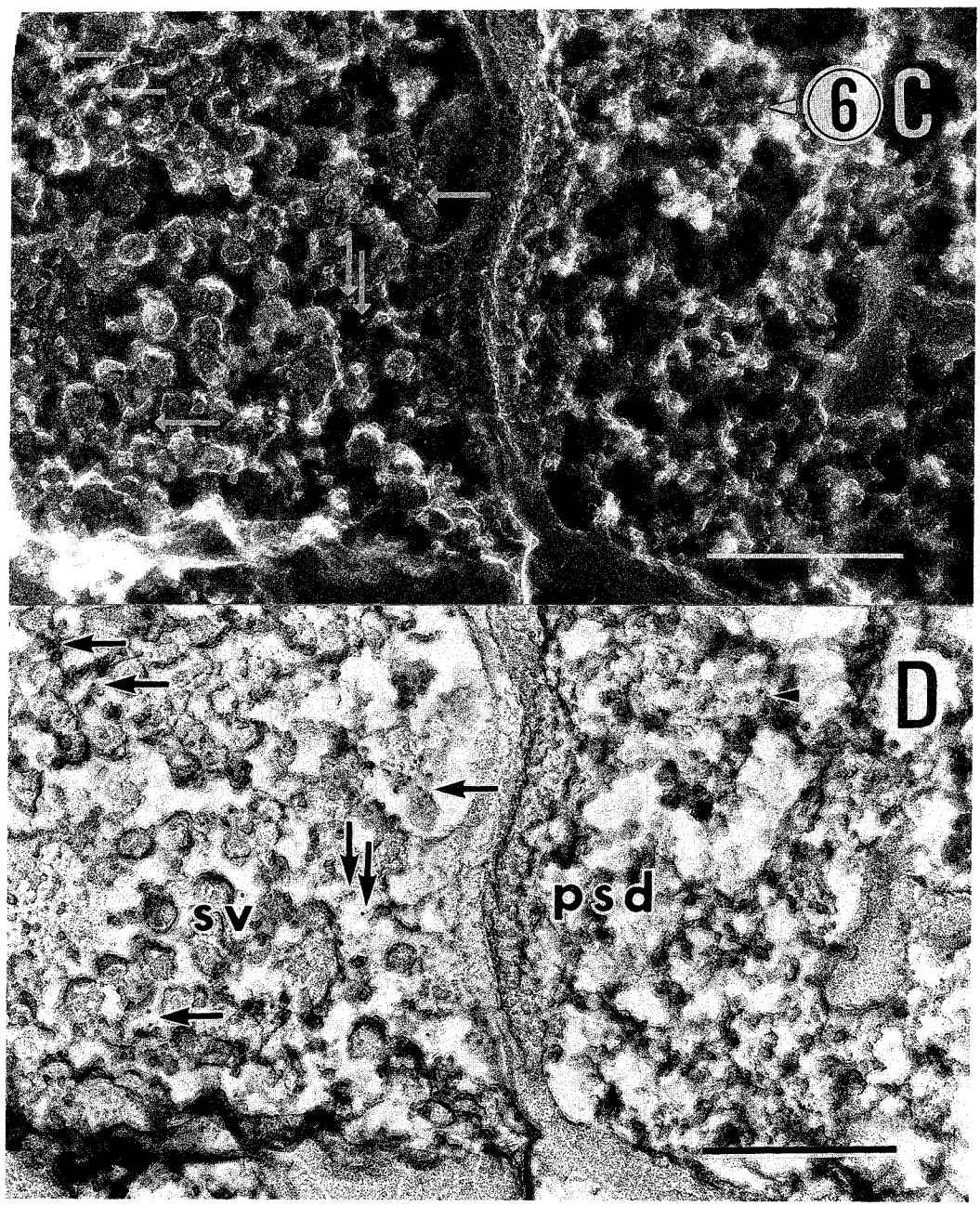

Fig. 6. (A) (B) A presynaptic ending after QF–DE. Gold particles associated with filamentous material between vesicles or protrusions from the vesicles (arrows). Bars 0.2 μm. (C) (D) A pre- and a postsynaptic ending making synaptic contact. Postsynaptic density (psd) and synaptic vesicles (sv) were identified. A larger number of gold particles existed in the presynaptic terminal (left; arrows) than in the postsynaptic terminal (right; arrowheads). The gold particles in the presynaptic terminal were located in the same manner as in Figs. 6A, B. Figs. 6B, D are the negative images of 6A, C, respectively. Though 4d-old rats are used in Figs. 6A–D, the synaptic contact shown in Figs. 6C and 6D is a well established one. Bars 0.2 μm.

in the postsynaptic terminals (arrowheads). In Figs. 6C, D, gold particles seem to associate with almost the same structure as in Figs. 6A or 6B.

DISCUSSION

Developmental changes in synapsin I localization observed with immunofluorescence. Synapsin I immuno-

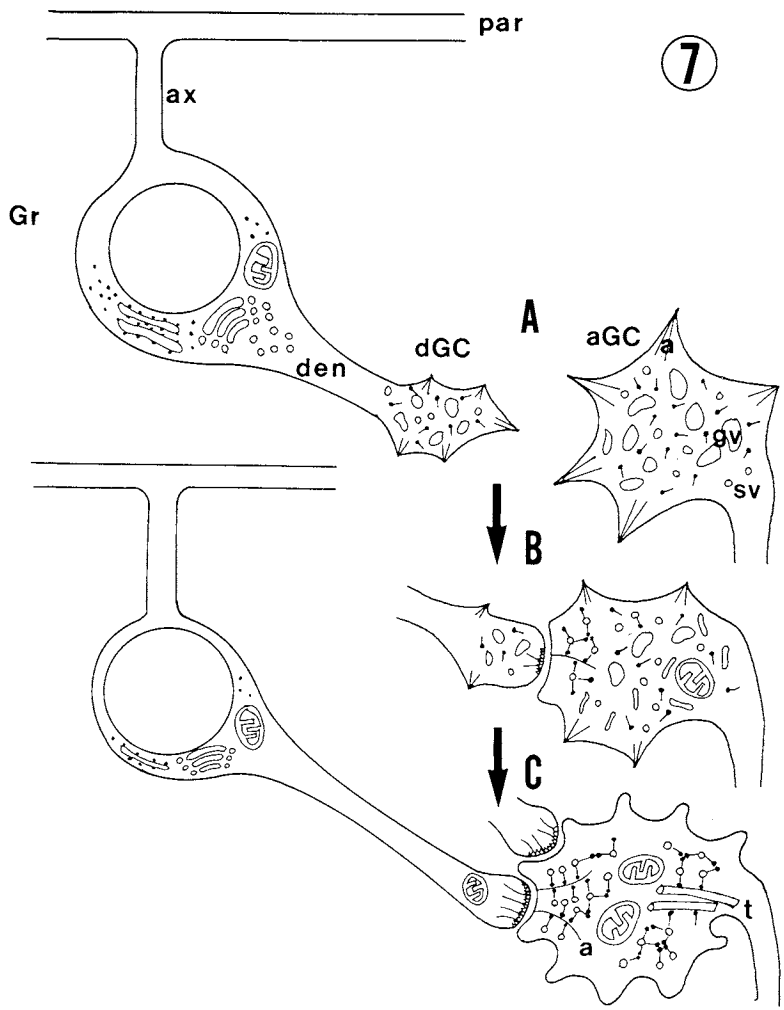

Fig. 7. Schematic illustration of developmental changes in synapsin I distribution. Cytoskeletons in the axons of internal granule cells (Gr) and mossy fibers, and the dendrites of internal granule cells are omitted in the figure; par represents parallel fiber. (A) When the internal granule cell begins to grow out its dendrite(s), a relatively large amount of synapsin I (tadpole-like structure) distributes throughout dendrites. Parts of them were located in the dendritic growth cone (dGC) (Fig. 5). In the axonal growth cone (aGC), synapsin I associates with filamentous materials (omitted in the figure) in the cytoplasm (Fig. 3). Both growth cones contain small vesicles (sv) (similar to synaptic vesicles in size) and larger vesicles (gv) frequently observed in growth cones. Actin filaments (a) occupy the inside of filopodia (straight lines in sharp protrusions from growth cones). (B) The presynaptic and the postsynaptic endings begin to make contacts. In the postsynaptic ending, immature postsynaptic densities are formed, and vesicles and synapsin I decrease in amount (Fig. 4D). In contrast, small vesicles and synapsin I increase and synapsin I molecules tend to accumulate around small vesicles (Figs. 4C, D). Larger vesicles decrease or take a tubular shape (Figs. 4C, D). (C) In the mature synapse, few, if any, synapsin I molecules exist in the postsynaptic side, whereas synapsin I in the presynaptic side associates with synaptic vesicles, actin filaments, and microtubules (based on the in vitro reconstruction data by Hirokawa *et al.* (22) (Fig. 2, Fig. 6).

staining in cerebellum is virtually confined to presynaptic endings in this study. In the molecular layer, numerous, fine immunofluorescent dots were observed since 4d when Purkinje cells and parallel fibers are beginning to make small synaptic contacts. Below the internal granule layer (i.e. in the immature white matter or medulla) at 4d, however, larger and coarser staining was recognized below the internal granule layer. This staining moved up into the area between the inner granule cells at 7d. This observation is consistent with behavior of large presynaptic endings of mossy fibers, the major source of presynaptic endings in the internal granule layer, which moved up from the medulla to the area between internal granule cells around this stage (2, 36).

Developmental changes in synapsin I localization in presynaptic terminals. Our data suggests that in synaptic endings, synapsin I appears and associates diffusely over fuzzy filamentous materials in the immature 'growth cone' stage (Figs. 3B, C). However, as synaptic vesicles accumulate, that is, as they begin to mature,

synapsin I tends to gradually be confined around synaptic vesicles (Figs. 2A, B, 3C, D). Association of synapsin I with fuzzy filamentous materials, most probably cytoskeletons (F-actin, microtubule, neurofilament, etc.) as the beginning of synapsin I transport, was supported by biochemical experiments (3, 4, 6, 8, 9, 19, 38) and by an *in vitro* reconstruction experiment (22). In particular, the latter experiment showed that synapsin I had high affinity to the cytoskeletons and could crossbridge them in vitro. Cytoskeletal architecture of growth cones seems to reinforce this assumption. In growth cones, a large amount of F-actin localizes and is considered to play a role in maintaining their characteristic shape and function (27). It is thus quite possible that synapsin I molecules are associated with cytoskeletons, in particular F-actin in the growth cones. Baitinger and Willard (7) reported that a large part of synapsin I molecules are transported at the same rate as actin and fodrin in mature axons. This could occur in the immature axons as well. The idea of translocation of synapsin I from cytoskeletons to vesicles is supported by the previous in vitro studies on synapsin I affinity with other elements, revealing that synapsin I molecules have higher affinity (Km = 10–52 nM) with the vesicles than with existing cytoskeletons (Km = 0.5–5 μM) (3, 6, 34, 39). The reduction of cytoskeleton in content as the maturation of presynaptic endings may also be important in this translocation. Several previous studies using culture cells have already mentioned colocalization of synapsin I with another integral vesicle protein, implying a similar developmental sequence. One of them, carried out by Burry *et al.* (13), pointed out that synapsin I expression preceded that of the integral vesicle protein, SV48, in rat cerebellar culture cells. They also found specific immunostaining at a very early stage of development. In another study by Bixby *et al.* (10) using chick ciliary ganglion cells, however, synapsin I and the integral protein p65 were expressed almost simultaneously and in the same distribution pattern, suggesting synapsin I usually associates with the vesicles. This apparent discrepancy may reflect the sequence of synapsin I expression and the synaptic vesicle formation. Since synaptic vesicles or its integral proteins work as a synapsin I receptor (4, 8, 9, 30, 31, 34, 38), synapsin I will concentrate around the vesicles soon after the vesicles are formed. Therefore, since p65 was detected sooner than synapsin I by protein blot, synapsin I colocalizes with p65 if p65 or the vesicles integrating p65 have affinity with synapsin I. The differences in their systems (cell types, use of polylysine, etc.) should also be considered.

Developmental changes in synapsin I localization in postsynaptic terminals. We observed a high density of immunogold particles in internal granule cell dendrites, in particular in distal tips and paranuclear areas. That synapsin I localized in the distal ends of dendrites may be associated with cytoskeletal elements, such as F-actin, as seen in the presynaptic terminals (22). In contrast, we observed few gold particles associated with vesicles, Golgi bodies, and ERs in the paranuclear area. Therefore, the protein does not seem to be synthesized and integrated in rERs like synaptophysin, a major integral protein (40), but to be synthesized by free ribosomes.

Transport and final location of synapsin I in neural development. Based on our observation of synapsin I distribution in axons and dendrites, we assume that the distribution process in the course of development is as follows; 1) Synapsin I distributes in axons and dendrites in probably the same density. Some part may associate with actin filaments in both axonal and dendritic growth cones (Fig. 7A). 2) As axons mature, more synapsin I gets to the axon terminals with synaptic vesicles or precursors of synaptic vesicles. Moreover, synaptic vesicles themselves can work as receptors for synapsin I, and thus retain synapsin I among them (Fig. 7B). 3) In contrast, in dendrites, for lack of supplement and receptors with high affinity, synapsin I may be diffused, degraded, or retrograde-transported during maturation (Fig. 7C). As to the supplement of synapsin I after synaptic maturation in axons, it may be transported distally along with cytoskeletons (e.g. actin and fodrin) as Baitinger and Willard have pointed out. However, the possibility of transport with synaptic vesicles should not be excluded. This assumption must be reinforced by studies using simpler systems, e.g. cultured cells.

Interaction of synapsin I with synaptic vesicles. We obtained a more detailed view of the synapsin I association with synaptic vesicles by QF–DE immunocytochemistry in the present study than with the conventional thin section. We think the location of immunogold well reflects that of synapsin I, because the location well coincides with the thin section and in vitro reconstruction data (22). Because our previous and present studies show 30–60 nm crosslinks between synaptic vesicles and the present study frequently show the gold labels on the structures between synaptic vesicles, we assume that many of the bridges (30–60 nm) between synaptic vesicles in vivo could be composed of synapsin I. This fits with our previous structural data of synapsin I molecules (−47 nm in length) and the recent data (4, 8, 9) which indicate that both collagenase resistant and sensitive domains could bind to the lipids and proteins of synaptic vesicle membranes. Taken together, both single synapsin I and several synapsin I molecules could crosslink adjacent synaptic vesicles thus forming 30–60 nm crosslinks.

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