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Synapsin I Deficiency Results in the Structural Change in the Presynaptic Terminals in the Murine Nervous System

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Abstract. Synapsin I is one of the major synaptic vesicle-associated proteins. Previous experiments implicated its crucial role in synaptogenesis and transmitter release. To better define the role of synapsin I in vivo, we used gene targeting to disrupt the murine synapsin I gene. Mutant mice lacking synapsin I appeared to develop normally and did not have gross anatomical abnormalities. However, when we examined the presyn-

aptic structure of the hippocampal CA3 field in detail, we found that the sizes of mossy fiber giant terminals were significantly smaller, the number of synaptic vesicles became reduced, and the presynaptic structures altered, although the mossy fiber long-term potentiation remained intact. These results suggest significant contribution of synapsin I to the formation and maintenance of the presynaptic structure.

SYNAPSIN I, composed of 86-kD synapsin Ia and 80-kD synapsin Ib, is a basic phosphoprotein expressed specifically in neurons (De Camilli et al., 1990; Südhof et al., 1989). It is localized in the presynaptic terminals (De Camilli et al., 1983a,b). Previous studies indicated that synapsin I binds to actin, tubulin, neurofilaments, and is associated with synaptic vesicles (Böhler and Greengard, 1987; Böhler et al., 1989; Baines and Bennett, 1986; Benfenati et al., 1989; Goldenring et al., 1986; Huttner et al., 1983; McCaffery and DeGennaro, 1986; Navone et al., 1984; Okabe and Sobue, 1987; Schiebler et al., 1986). Quick freeze-deep etch (QF-DE)¹ electron microscopical analysis, combined with immunocytochemistry of in vivo synapses and in vitro reconstruction systems suggested strongly that synapsin I forms cross-bridges among synaptic vesicles and the cytoskeleton, mainly actin filaments (Harada et al., 1990; Hirokawa et al., 1989; Hirokawa, 1990), and showed that synapsin I tends to be located distally from the presynaptic membrane (Hirokawa et al., 1989). The binding of synapsin I and cytoskeletal elements or membranes was reported to be regulated by phosphorylation of synapsin I via calcium-calmodulin-dependent pro-

tein kinase II (Böhler and Greengard, 1987; Bennett and Scheller, 1994; Benfenati et al., 1992; Kelly, 1993; Okabe and Sobue, 1987; Petrucci and Morrow, 1987). In addition physiological studies have proposed that phosphorylation of synapsin I could regulate the release of neurotransmitter (Llinás et al., 1985). But a recent molecular genetic approach showed that mice lacking synapsin I did not manifest gross dysfunction of the nervous system, but exhibited an increase in paired pulse facilitation (PPF), suggesting that synapsin I is not essential for neurotransmitter release, but serves to limit neurotransmitter release caused by elevated calcium concentration after initial stimulation of PPF (Rosahl et al., 1993).

On the other hand, several lines of evidence suggest that synapsins are involved in neuronal morphogenesis, especially in synaptogenesis. The expression of synapsin I correlates with the developmental time course of neurite extension and synapse formation (DeCamilli et al., 1983a; Haas and DeGennaro, 1988; Harada et al., 1990; Mason, 1986; Bixby and Reichardt, 1985). Recent in vitro experiments also support this idea. For example, overexpression of synapsin IIB in the NG108-15 cell line results in the formation of synapse-like contact and in increases in the number of neuritic varicosities and vesicle densities in the varicosities (Han et al., 1991). In *Xenopus* cell culture, injection of purified synapsin I into embryonic spinal neurons led to the functional maturation of neuromuscular synapse (Lu et al., 1992). These experiments suggest that synapsins play a crucial role in synaptogenesis and synaptic maturation. However, contrary to expectations, no overt ab-

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1. *Abbreviations used in this paper:* DT-A, diphtheria toxin A; fEPSP, field excitatory postsynaptic potential; LTP, long-term potentiation; PPF, paired pulse facilitation; QF-DE, quick freeze-deep etch.

normality was found in brains of mice lacking synapsin I by immunocytochemistry using synaptic markers (Rosahl et al., 1993). A recent study showed that double-knockout mice lacking both synapsin I and synapsin II have no gross anatomical abnormalities in their brain, but the number of synaptic vesicles is reduced to ~50% in mutants compared to wild-types (Rosahl et al., 1995). However, it is still not clear how the loss of individual synapsins contributes to this phenotype. Since detailed structural data of mice lacking synapsin I had not been reported, we generated mice lacking synapsin I and investigated their synaptic morphology in detail in order to assess the function of synapsin I in synaptogenesis and in the dynamics of synaptic vesicles in vivo. We focused on hippocampal mossy fiber giant terminals and nerve terminals of cerebellar granule cells. We present evidence to suggest that synapsin I itself is important for the formation and maintenance of the pre-synaptic structure, that the function of synapsin I in the formation of nerve terminals cannot be completely compensated for by other molecules including synapsin II.

Materials and Methods

Construction of Synapsin I Targeting Vector

Six independent clones derived from the 129/Sv mouse genomic library containing the first exon of synapsin I gene were subcloned into pBlue-scriptII. The overlapping region was used to construct vector-1 and -2. The upstream 6.3-kb homologous sequence and the downstream 1.1-kb homologous sequence were separately subcloned (p5'syn and p3'syn). Oligonucleotide containing the stop codon was introduced in frame to *EagI* site in the first coding exon (5'syn-oligo). The diphtheria toxin A fragment (DT-A) cassette (Yagi et al., 1993) was inserted in the downstream polylinker site of p3'syn-DT-A. 3'syn-DT-A fragment was ligated to the downstream polylinker site of p5'syn-oligo (p5'syn-oligo-3'syn-DT-A). The PGK-neo cassette (PGK-neoA), derived from pKJ1 (McBurney et al., 1991), or PGK- β -geopA (fusion protein of β gal and neo with PGK promoter (Friedrich and Soriano, 1991), kindly provided by P. Soriano) was introduced in the same transcriptional orientation to the *Sall* site between 'oligo' and '3'syn.'

ES Cell Culture

The J1 line of ES cells used for these experiments was cultured essentially as described previously (Li et al., 1992). After linearized, vector-1 and -2 were electroporated into J1 cells essentially as described (Thomas and Capecchi, 1987). J1 cultures were dispersed into single cells and resuspended at a density of 4×10^7 cells/ml in electroporation buffer and 25 μ g/ml of targeting vector. J1 cells were electroporated in a volume of 0.8 ml at 400 V and 25 μ F using a Gene Pulser (Bio-Rad Laboratories, Cambridge, MA). Cells were immediately seeded onto 100 mm gelatinized plates in the presence of G418-resistant embryonic fibroblasts, in ES medium supplemented with 1,000 U/ml recombinant leukemia inhibitory factor (AMRAD). After 36 h, the medium was supplemented with 175 μ g/ml (active) G418. The cells were screened for the presence of a disrupted synapsin I gene by Southern blot analysis. ES cells carrying the disrupted synapsin I gene were injected into C57BL/6 embryos at the blastocyst stage using standard techniques (Bradley, 1987).

Preparation of Crude Extracts and Immunoblotting

Whole brains were dissected out and homogenized with 25 mM Tris 2% SDS, followed by boiling to lower the viscosity. Crude extracts were made by centrifuging the homogenates at 20,000 g for 15 min at 4°C. Equal amounts of crude extracts were separated by polyacrylamide gel (Laemmli, 1970). Immunoblotting was done as described previously (Harada et al., 1994). The following antibodies were used for the first antibodies: anti-synapsin I antisera (Hirokawa et al., 1989) for synapsin I; 171B5 (Obata et al., 1986) for p38; polyclonal antibody of a synthetic peptide for dynamin (Nakata et al., 1991); NN18 (Sigma Chemical Co., St. Louis, MO) for NF-M.

Conventional Electron Microscopy and Morphometric Analysis

Adult mice matched by age and sex were perfused with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer. Tissues (hippocampus and cerebellum) were dissected out and fixed overnight, processed for the conventional electron microscopy, and viewed under a JEOL 1200EX electron microscope at 100 kV. The thickness of ultra-thin sections was controlled by the interference color and we used data from sections of similar thickness for statistical analysis. Matching areas from hippocampal CA3b subregion and cerebellar molecular layer were examined. The mossy fiber giant terminals adjacent to bundles of mossy fiber axons and cerebellar granule cell terminals contacting with Purkinje cell dendritic spines were photographed. The numbers of synaptic vesicles were determined by direct counting from the micrographs. These numbers were then converted into vesicle numbers per area (μm^2) by dividing the vesicle counts by the nerve terminal areas.

Anterograde Labeling of the Mossy Fibers

Adult synapsin I-null mutant and age and sex-matched wild-type control mice were fixed by perfusion of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). After the perfusion procedure, the brains were dissected out from the skull en bloc, and immersed in the aforementioned fixative. The hippocampal regions were cut from the whole brains on the frontal plane, followed by serial sectioning with a vibratome (thickness: ~200 μ m). Very small crystals of the carbocyanine dye DiI (1,1'-diocetadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; Molecular Probes, Eugene, OR) were placed on the surface of the sliced brain by means of electrolytically sharpened tungsten needles in the vicinity of the granule cell layer of the dentate gyrus. 3–8 h after incubation at 27°C diffusion of the dye enabled us to identify mossy fibers by laser confocal microscopy (Bio-Rad).

Quick-freeze, Deep-etch Electron Microscopy

After fixation by perfusion of 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), brains were dissected out. In some cases slices of fresh brains were also prepared. We processed them for quick-freeze, deep-etch electron microscopy as described previously (Hirokawa, 1982; Hirokawa et al., 1989).

Electrophysiology

6–10-wk-old male mice were decapitated under halothane anesthesia and the hippocampi were quickly removed. Transverse hippocampal slices (400- μ m thick) were cut using a tissue slicer (Dosaka). The superfusing solution was composed of (mM): 119 NaCl, 2.5 KCl, 1.3 MgSO_4 , 2.5 CaCl_2 , 26.2 NaHCO_3 , 1.0 NaH_2PO_4 , 11 glucose, equilibrated with 95% O_2 /5% CO_2 . A tungsten bipolar stimulating electrode was placed in the granule cell layer of the dentate gyrus and a recording glass microelectrode filled with 1 M NaCl (2–5 M Ω) was placed in the stratum lucidum of area CA3. The field excitatory postsynaptic potential (fEPSP) was recorded by stimulating mossy fibers at 0.1 Hz. Mossy fiber inputs were routinely identified by the large paired-pulse facilitation (greater than twofold) and also, in some cases, by the reversal of the waveform on changing the recording site from s. lucidum to s. radiatum. LTP was induced by two trains of 100 Hz stimulation for 1 s separated by 10 s (tetanic stimulation) at base-line stimulus strength. At the end of each experiment, 6-cyano-7-nitroquinoxaline-2,3-dione (20 μ M) was added to the bath to assess a fiber volley component. A patch clamp amplifier (EPC-7, List) was used to record fEPSP. Records were filtered at 1 kHz, digitized at 10 kHz, and collected on a computer (466/MXV; Dell, North Bergen, NJ). All experiments were carried out at room temperature (24–26°C).

Results

Targeting the Synapsin I Gene

We generated synapsin I deficient mice by gene targeting. Two types of replacement-type targeting vectors, vector-1 and -2, were constructed (Fig. 1, A and B). In vector-1, PGK-neo polyA cassette was inserted between *EagI* site

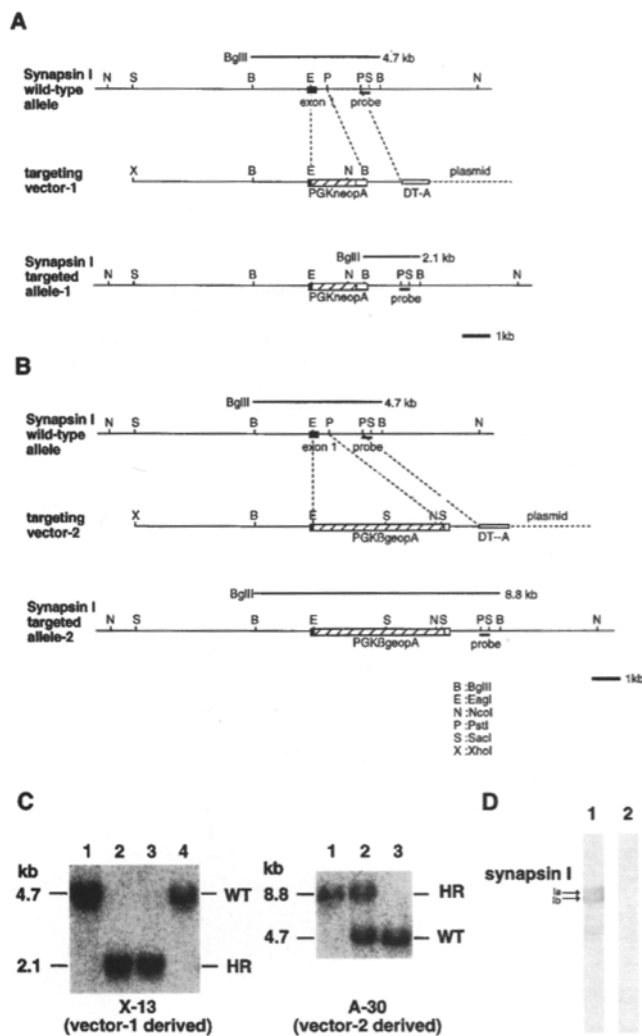


Figure 1. Targeted inactivation of the synapsin I gene in ES cells and mice. (A and B) Targeting strategies: wild-type allele containing the first coding exon of synapsin I, replacement type-targeting vectors, and targeted allele after homologous recombination are shown. (C) Genotype of F2 mice in X-13 line and A-30 line. Tail DNA was isolated and analyzed by Southern blotting using the 3'-flanking probe (A and B, probe), after digestion with BglII. In X-13 line, lanes 1 and 4 are from wild-type mice, and lanes 2 and 3 are from synapsin I-null mutant mice. In A-30 line, lanes 1 is from synapsin I-null mutant mouse, lane 2 from heterozygous mutant mouse, and lane 3 from wild-type mouse. WT, wild-type band; HR, homologous recombinant band. (D) Immunoblot analysis of crude extracts from brain with the polyclonal antibody against synapsin I. Lane 1, wild-type brain; Lane 2, synapsin I-null mutant brain.

in the first exon and PstI site downstream of the first exon for positive selection, and DT-A was added onto the 3' end of the construct for negative selection. To construct vector-2, we used β gal-neo fusion gene with PGK promoter (PGK- β geo) instead of the PGK-neo in vector-1 for the purpose of tagging targeted cells. These two vectors contained 6.3 kb of 5' homology and 1.1 kb of 3' homology. The linearized targeting vectors were electroporated into the J1 ES cells, followed by selection of G418. Genomic DNA from resistant colonies was digested with BglII and analyzed individually on Southern blots for the homo-

gous recombination events. Hybridization was performed with a probe from the 3' end of the synapsin I gene, a genomic region that was not included in the recombination constructs. This probe (Fig. 1, A and B, probe) detected a 2.1-kb BglII fragment after insertion of vector-1 and a 8.8-kb fragment of vector-2. The analysis of G418^R clones revealed a targeting frequency of approximately 2/310 in vector-1 electroporation and 11/121 in vector-2. Homologous recombination in these cell lines was further confirmed using NcoI (data not shown). J1 clones carrying a targeted synapsin I gene were injected into C57Bl/6 blastocysts and transferred into the uteri of pseudopregnant recipients. X-13 cell line derived from vector-1 and A-30 cell line derived from vector-2 generated chimeric mice with ES cell contributions ranging from 50–100% as judged by the proportion of agouti coat color. These chimeric mice transmitted the ES cell genome through the germ line, as indicated by the agouti coat color of the offspring. Female agouti pups, heterozygous for the mutant synapsin I allele, were indistinguishable from their wild-type male agouti littermates and displayed no discernible abnormalities. To determine the phenotype of synapsin I-null mutant mice, we interbred F₁ heterozygous female animals and F₁ wild-type males, and genotyped the litters after weaning at 4 wk of age (Fig. 1 C). The predicted 1:1:1 distribution of wild-type male, wild-type female, heterozygous female, hemizygous male was found at this age. Synapsin I-null mutant animals were phenotypically indistinguishable at a gross level from their heterozygous and wild-type littermates, and exhibited no significant differences in weight and size. Moreover, they had no gross anatomical changes in brains and other organs.

Expression of Synaptic Vesicle-associated Proteins in Synapsin I-Null Mutants

Immunoblotting with anti-synapsin I polyclonal antibody did not detect synapsin I in brain crude extracts from synapsin I-null mutant mice (F2 hemizygous male animals) (Fig. 1 D). To investigate whether inactivation of synapsin I gene affects the expression of other synaptic-vesicle associated proteins, we compared the level of p38 (synaptophysin) and dynamin in wild-type and synapsin I-null mutant mice. Crude extracts from adult brain were prepared and the relative amount of p38 and dynamin were measured. The amount of p38 was decreased to about 80% in adult mutant mice (data not shown). The level of dynamin in mutants was similar to that in controls (data not shown).

Synaptic Vesicle Density Was Reduced in Hippocampal and Cerebellar Synapses

To investigate the morphological change in synapses lacking synapsin I, we examined hippocampal and cerebellar tissues. The gross cytoarchitecture and cell morphology of synapsin I-deficient sections were indistinguishable from control sections (data not shown). We examined two types of nerve terminals by electron microscopy, hippocampal mossy fiber terminals in the CA3 field (Figs. 2 and 3) and cerebellar granule cell terminals making synaptic connection with dendritic spines of Purkinje cells (Fig. 4). We found the number of synaptic vesicles per area of synaptic terminals to be markedly reduced in mutant synaptic but-

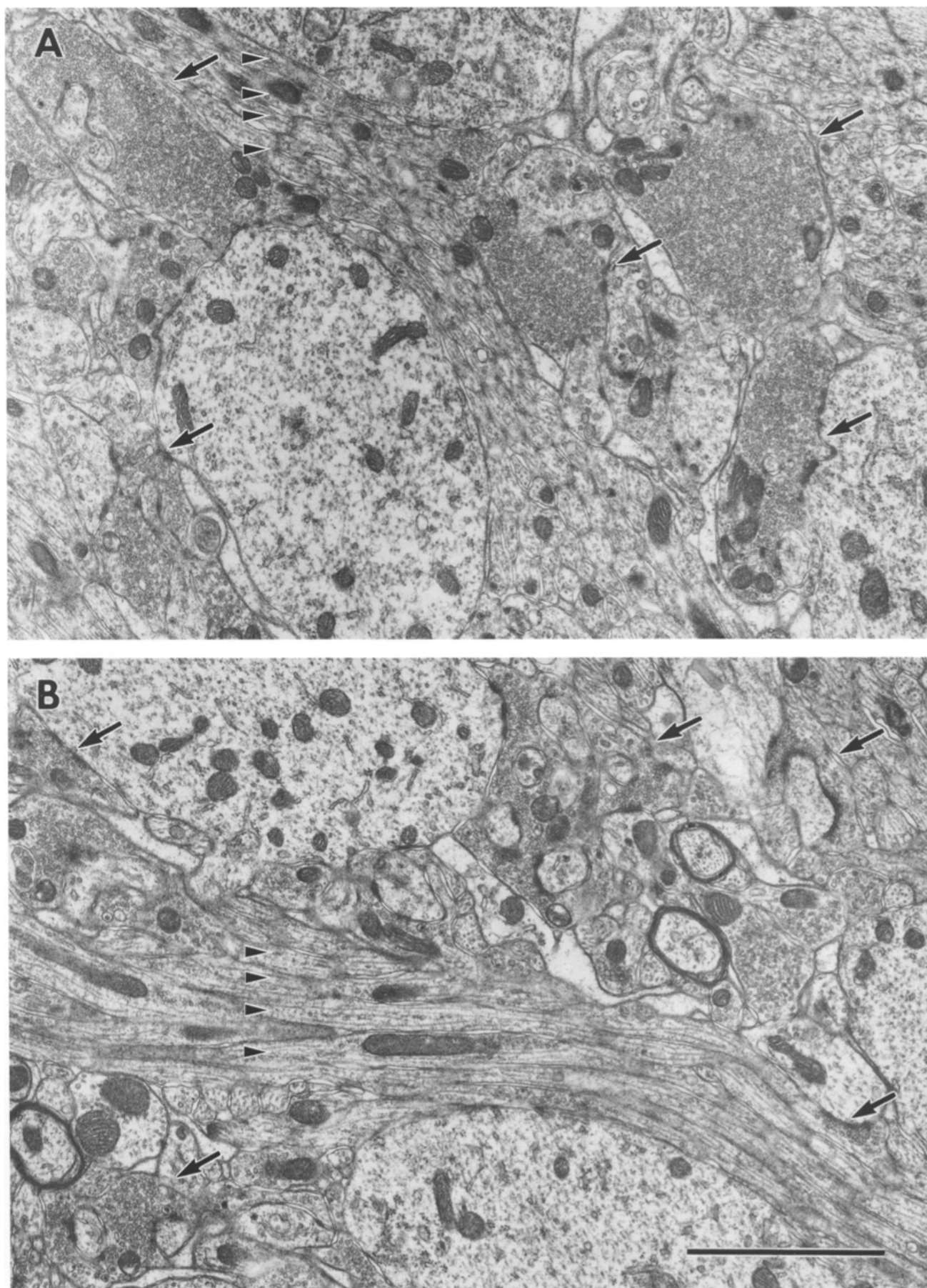


Figure 2. (A and B) Electron micrographs comparing representative areas of CA3 fields in hippocampus of wild-type (A) and synapsin I-deficient (B) mice. Many mossy fiber terminals are observed (arrows) near bundles of mossy fiber axons (arrowheads). Bar, 2 μ m.

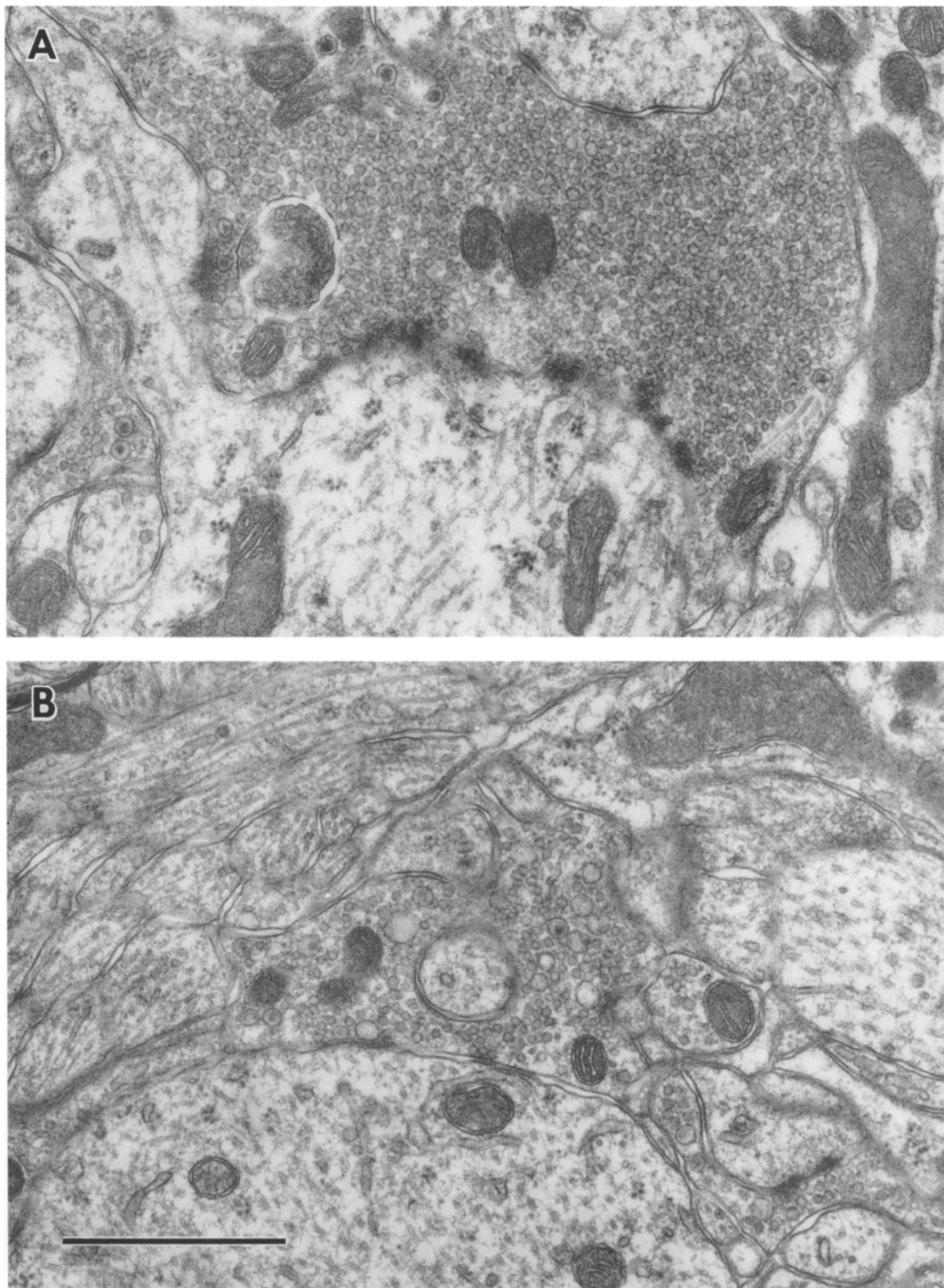


Figure 3. (*A* and *B*) Higher magnification views of hippocampal mossy fiber terminals of wild-type (*A*) and synapsin I-deficient (*B*) mice. Synaptic vesicles are reduced in number in mutants (*B*). Bar, 1 μ m.

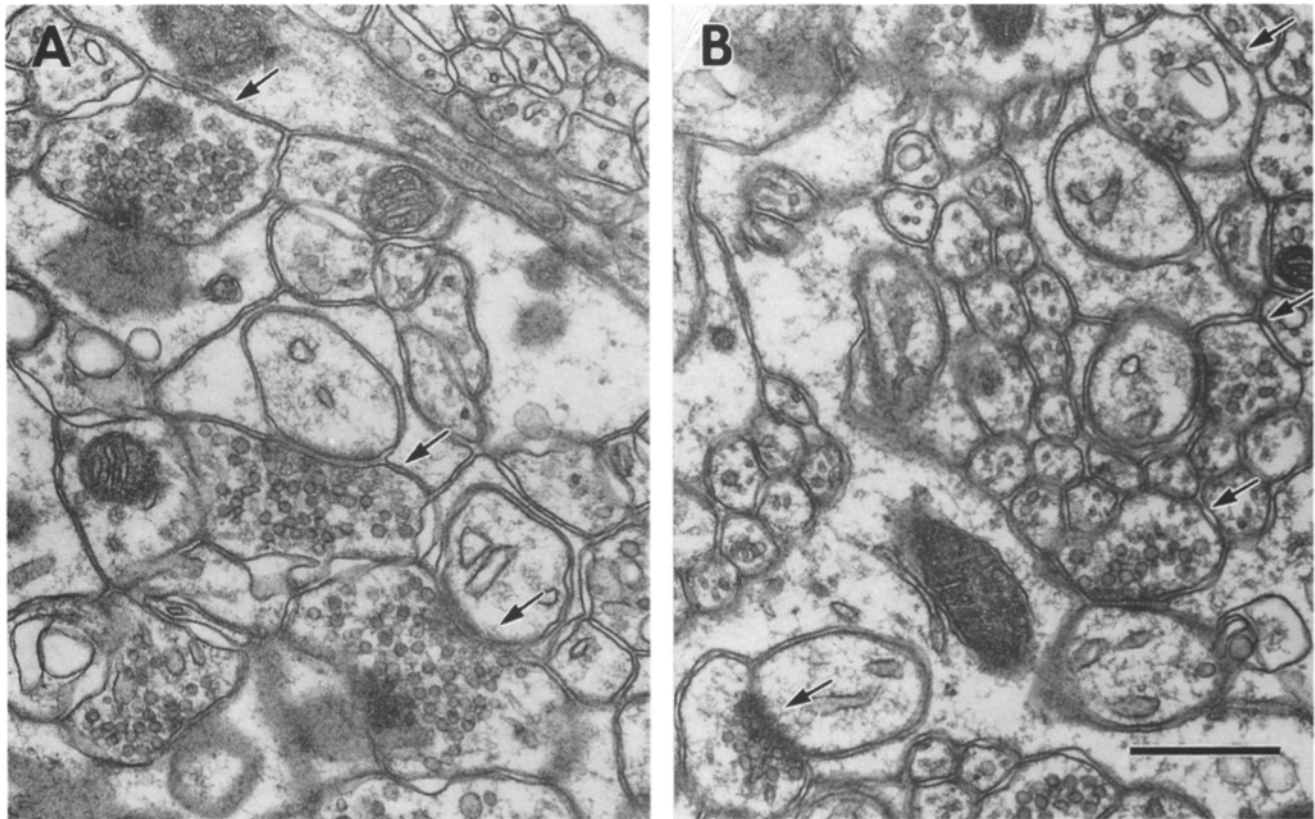


Figure 4. (A and B) Granule cell terminals (arrows) in cerebellum of wild-type (A) and synapsin I-deficient (B) mice. The number of synaptic vesicles of synapsin I-deficient animal is significantly reduced. Bar, 500 nm.

tons (Figs. 3 B and 4 B). These reductions were statistically significant in both types of synapses, and were observed in two independent mutant mouse lines (Table I, A and B). The number of dense-cored vesicles was not changed (Table I A). Occasionally we observed mossy fiber terminals containing some large vesicles with clear centers (~100–200-nm diam) in synapsin I-deficient mice.

To determine whether this reduction of the number of vesicles affects the distribution of synaptic-vesicle clusters, we subdivided cerebellar granule cell terminals into zone P (proximal region to presynaptic membrane) and zone D (distal region) as previously described (Kadota et al., 1993) (Fig. 5) and determined the number of synaptic vesicles per area in each region. In synapsin I-deficient mice, synaptic vesicles in zone D are more significantly reduced in number than in zone P (Table I C).

Size of Hippocampal Synapses Was Significantly Reduced

In addition to the reduction in synaptic vesicle densities, we observed that the mean size of mutant hippocampal mossy fiber terminals was obviously reduced in electron-microscopic sections (Fig. 2 B). Morphometric analysis showed that these differences were statistically significant (Table I A). To confirm these differences, we labeled hippocampal mossy fiber terminals of the mutant and control animals anterogradely by lipophilic tracer DiI (1-1'-diiododecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) (Honig and Hume, 1989), and examined by confocal laser

scanning microscopy (Fig. 6). The granule cells in the dentate gyrus lacking synapsin I extended mossy fiber axons and their axonal diameter did not change significantly (Fig. 6, A and B). However, the axons appeared very smooth in contour and tended to keep the constant axonal diameter (Fig. 6 B), lacking the obvious varicosity-like expansions observed in wild-type terminals (Fig. 6 A). On the other hand, we found no difference between mutant and control when examining areas of granule cell terminals in the cerebellar molecular layer by electron microscopy (Table I B).

Cytoskeletal Architecture in the Presynaptic Terminals

To further assess the structural changes in nerve terminals of the mutant mice, we examined mutant and control nerve terminals by QF-DE electron microscopy. We examined two types of synapses, hippocampal mossy fiber terminals, and cerebellar mossy fiber terminals. Reductions in the number of synaptic vesicles and the size of synapses, the findings observed by conventional electron microscope, were obvious in hippocampal mossy fiber synapses (data not shown). In hippocampal mossy fiber terminals, owing to the extremely high density of synaptic vesicles in wild-type synapses, it was difficult to identify clearly the structure associated with synaptic vesicles. However, in mutant terminals, because of the reduced synaptic vesicle density, strands associated with synaptic vesicles and the cytoskeletal structures tended to be identified easily (data not shown). Nevertheless, because of the high density of synaptic vesi-

Table 1. Morphometric Analysis of Synapsin I-deficient Mice and Wild-type Controls

		Number of synaptic vesicles per area*	Number of dense-cored vesicles per area*	Area of nerve terminals [‡]	
A. Hippocampal mossy fiber terminals					
A-30-1 [§]	Wild type	174 ± 35 (18)	0.94 ± 0.63 (18)	2.92 ± 1.63 [¶] (61)	
	mutant	123 ± 30 (18)	1.01 ± 0.86 (18)	2.24 ± 0.96 (60)	
X-13-1 [§]	Wild type	179 ± 27 (17)	0.99 ± 0.76 (17)	3.65 ± 1.79 (33)	
	mutant	116 ± 29 (16)	1.33 ± 0.50 (16)	2.30 ± 1.18 (33)	
X-13-2 [§]	Wild type	163 ± 26 (16)	0.88 ± 0.81 (16)	2.97 ± 1.63 [¶] (44)	
	mutant	115 ± 26 (16)	0.94 ± 0.85 (16)	2.16 ± 1.08 (46)	
B. Cerebellar granule cell terminals					
A-30-2 [§]	Wild type	124 ± 36 (30)	not counted	0.31 ± 0.12 (30)	
	mutant	79 ± 15 (28)	not counted	0.28 ± 0.08 (28)	
X-13-3 [§]	Wild type	91 ± 38 [¶] (30)	not counted	0.24 ± 0.14 (30)	
	mutant	64 ± 26 (28)	not counted	0.26 ± 0.09 (28)	
C. Cerebellar granule cell terminals subdivided to zone D and zone P					
		Number of synaptic vesicles per area in zone D*	Area of zone D [‡]	Number of synaptic vesicles per area in zone P*	Area of zone P [‡]
A-30-2 [§]	Wild type	98 ± 49 (30)	0.25 ± 0.12 (30)	221 ± 57 (30)	0.06 ± 0.03 (30)
	mutant	48 ± 23 (28)	0.22 ± 0.8 (28)	189 ± 43 (28)	0.06 ± 0.02 (28)
X-13-3 [§]	Wild type	68 ± 42 [¶] (30)	0.19 ± 0.13 (30)	177 ± 66 (30)	0.05 ± 0.03 (30)
	mutant	42 ± 26 (28)	0.20 ± 0.08 (28)	162 ± 45 (28)	0.05 ± 0.03 (28)

The means ± SD (the number of synapses examined) are shown. Student's *t*-test was used to determine the significance of the changes.

*Synaptic vesicles per μm^2

[‡] μm^2

[§] Mouse lines derived from two different ES cell clones (A-30 and X-13) were examined. To analyze hippocampal mossy fiber terminal, one set of experiments (A-30-1) using A-30-derived mouse line and two sets (X-13-1 or -2) using X-13-derived mouse line were performed. In case of granule cell terminals in cerebellum, two sets (A-30-2 using A-30-derived mouse line and X-13-3 using X-13-derived mouse line) were performed. In one set of experiments, age and sex matched littermate mice were used.

^{||} Different from the values of mutants at $P < 0.001$.

[¶] Different from the values of mutants at $P < 0.01$.

cles in wild-type synapses, comparison of the synaptic vesicle-associated strands between wild-type and synapsin I-deficient synapses was difficult. Therefore to compare the nature of structures associated with synaptic vesicles between wild-type and mutant presynaptic terminals, we chose to observe cerebellar mossy fiber terminals (Fig. 7,

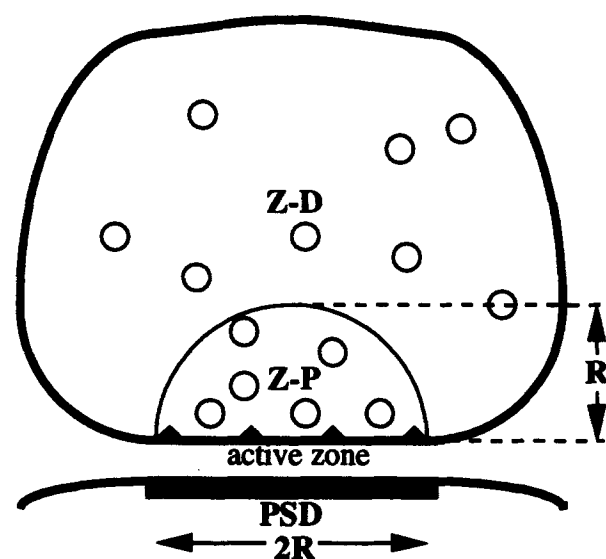


Figure 5. The areal subdivision of the cerebellar granule cell terminal. Zone P (Z-P) was the area inside the half-circle with a diameter equal to the width of postsynaptic density (PSD). Zone D (Z-D) was the remaining area excluding zone P.

A and B), where the density of synaptic vesicles is lower than that of hippocampal mossy fiber terminals. In agreement with this expectation, we could observe frequent short cross-bridges between synaptic vesicles in terminals of wild-type mice (Fig. 7 A). In mutant mice, the density of synaptic vesicles was found to be reduced (Fig. 7 B), as observed in other types of synapses, and short strands between synaptic vesicles are much less than those in wild-type synapses. From these observations, we conclude that synapsin I is a major component of the fine short strands associated with synaptic vesicles in these synapses.

Loss of Synapsin I Did Not Affect Mossy Fiber LTP

When mossy fibers are stimulated at a high frequency, the mossy fiber-CA3 synapse displays LTP, an activity dependent enhancement of synaptic transmission. It has been reported that the site for both the induction and expression of mossy fiber LTP is presynaptic (Zalutsky and Nicoll, 1990; Weisskopf and Nicoll, 1995), where cAMP-dependent phosphorylation may play a crucial role (Weisskopf et al., 1994). In our present study with synapsin I-deficient mice, we observed significant changes both in the size and structure of mossy fiber terminals. Thus, we tested the possibility that synapsin I contributes to the mossy fiber-CA3 LTP. The mossy fiber-CA3 fEPSP, was recorded in hippocampal slices from synapsin I-deficient mutant as well as wild-type mice (Fig. 8 A). Tetanic stimulation of mossy fiber inputs produced a post-tetanic potentiation followed by LTP in both mutant and wild-type mice. As shown in Fig. 8 B, no significant difference was observed in the mag-

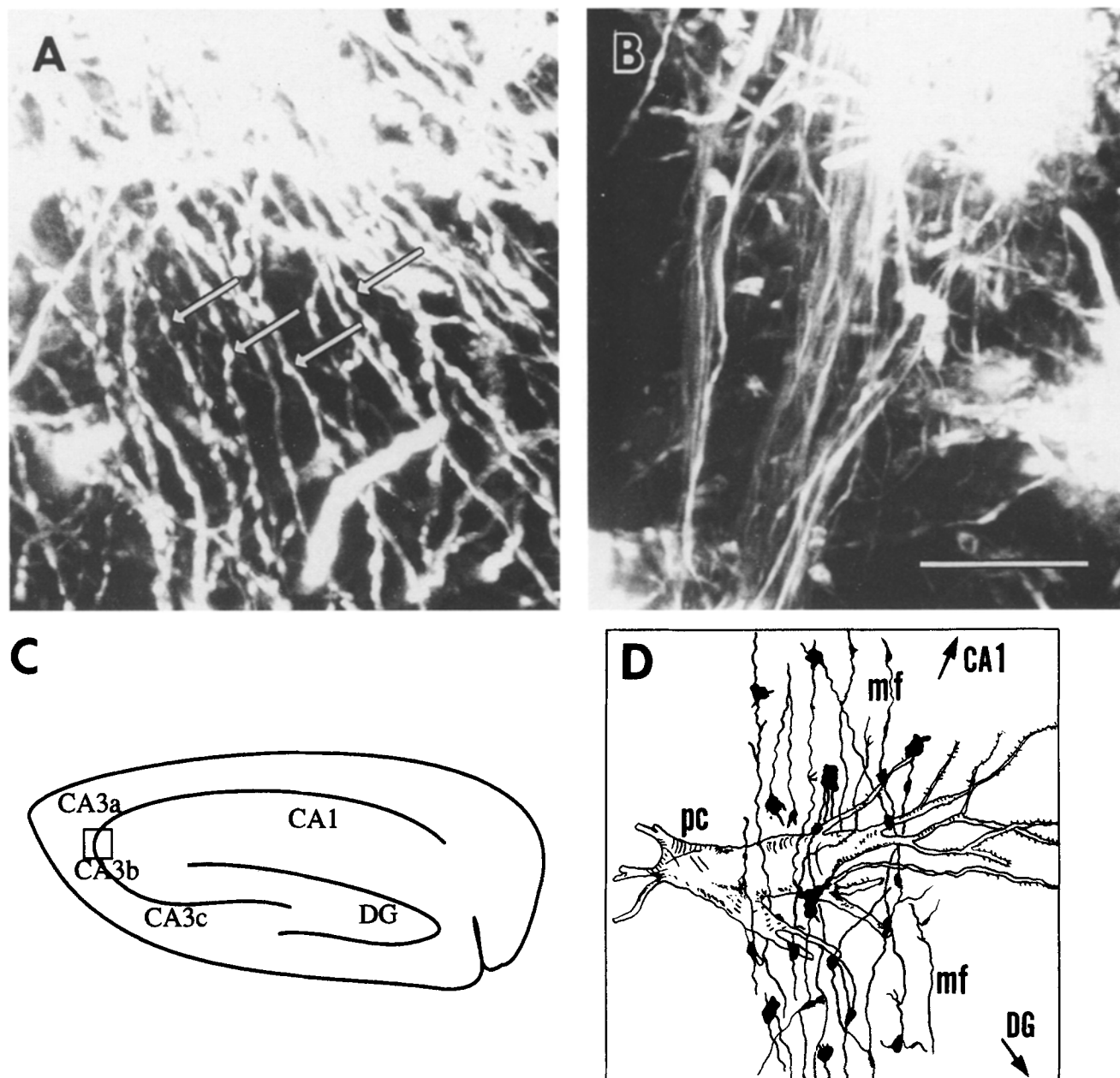


Figure 6. Anterograde labeling of hippocampal mossy fiber terminals by DiI. (*A* and *B*) Mossy fiber giant terminals in the CA3 field of wild-type (*A*) and synapsin I-deficient (*B*) mice. The mossy fibers run downward from the granule cells which were located far upside of these figures. The varicosity-like expansions (*arrows*) of mossy fibers of synapsin I-deficient mice were significantly reduced in size. (*C*) Overview of the hippocampus. Observed brain region was boxed. CA3 fields are divided into CA3a, CA3b, and CA3c subdivisions. *DG*, dentate gyrus. (*D*) Detailed drawing of the pyramidal cell (*pc*) and mossy fibers (*mf*). Bar, 25 μ m.

nitude or time course of LTP between wild-type and mutant mice.

Discussion

The temporal relationship between the peak of synapsin I expression and synapse formation (Bixby and Reichardt, 1985; DeCamilli et al., 1983a; Haas and DeGennaro, 1989; Mason, 1986) has strongly suggested that synapsins play an important role in synaptogenesis. Several recent in vitro experiments supported this idea (Ferreira et al., 1994; Han

et al., 1991; Lu et al., 1992). However, the analysis of mice lacking both synapsins showed directly that synapsins are not essential for synaptogenesis (Rosahl et al., 1995). Nevertheless, a decrease in the number of synaptic vesicles in the visual cortex and hippocampal CA1 field of double knockouts was reported (Rosahl et al., 1995), and the injection of antibodies directed against synapsin I to lamprey reticulospinal axons resulted in a loss of synaptic vesicles (Pieribone et al., 1995). These experiments suggested that the absence of synapsins strongly influences the turnover of synaptic vesicles. We examined hippocampal mossy fiber

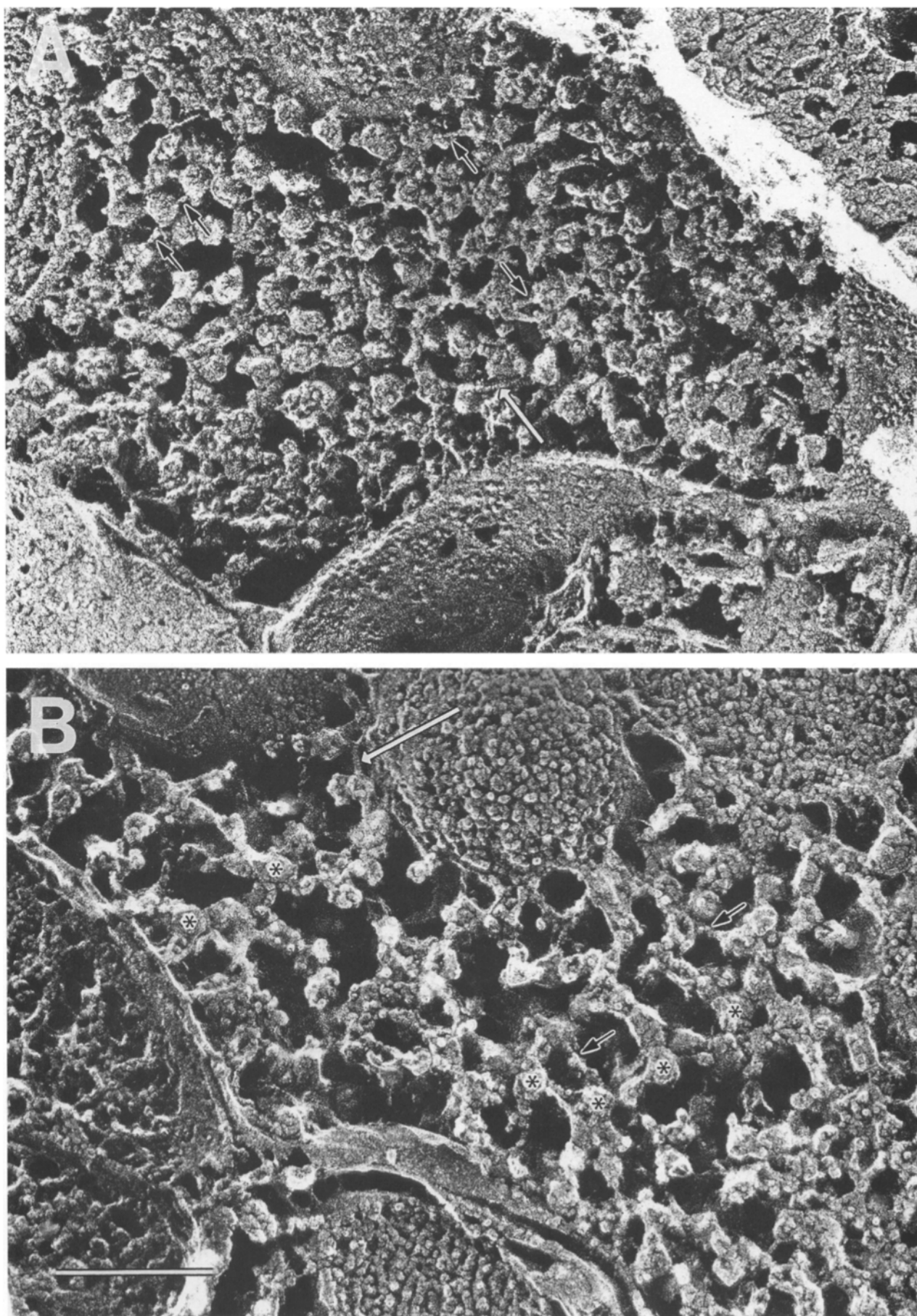


Figure 7. Quick-frozen, deep-etched cerebellar mossy fiber terminals of wild-type (*A*) and synapsin I-deficient (*B*) mice. Short cross-bridges (arrows) between synaptic vesicles (asterisks) were relatively sparse in nerve terminals lacking synapsin I. Occasionally, actin filaments were observed in the presynaptic terminals (open arrows). Bar, 200 nm.

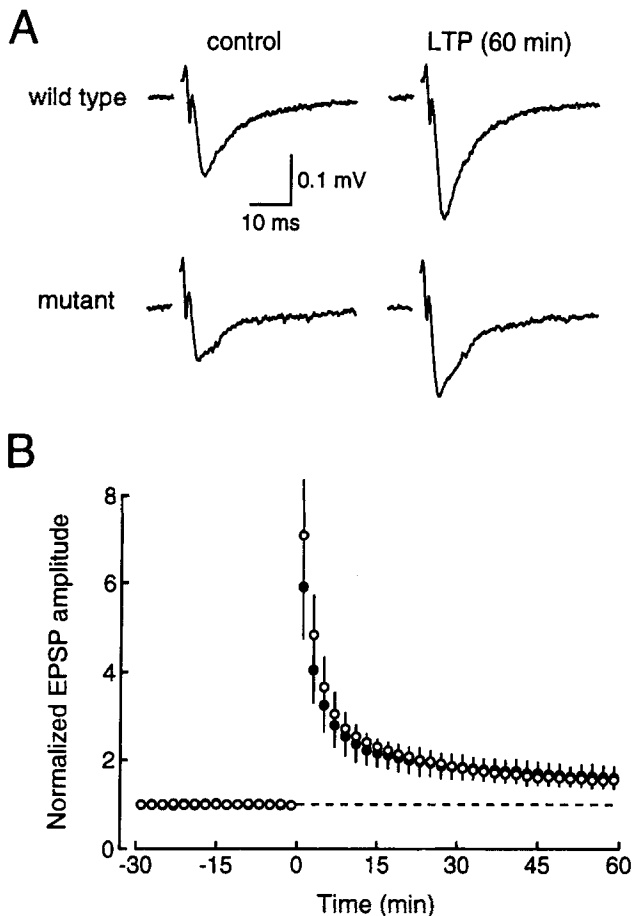


Figure 8. Mossy fiber LTP in area CA3 of hippocampal slices. (A) Typical recordings of mossy fiber fEPSP in the wild-type (upper traces) and mutant (lower traces) mouse before (left) and 60 min after (right) the induction of LTP. Each trace shows an average of 10 consecutive recordings. Stimulus artifacts are truncated. (B) Time course of mossy fiber LTP in wild-type (○; $n = 10$ slices) and mutant (●; $n = 9$) mice. Tetanic stimulation (see Materials and Methods) was applied at time 0. To exclude the possible contribution of inputs other than mossy fibers to LTP, 40 μ M D-AP5 (D[-]-2-amino-5-phosphonopentanoic acid) was superfused for at least 7 min prior to the tetanic stimulation. Each point represents averaged fEPSP amplitude (mean \pm SD) normalized to base-line amplitude. All experiments were carried out without pre-knowledge of genotype.

giant terminals in CA3 fields and cerebellar granule cell terminals of synapsin I-deficient mice by conventional electron microscopy and by QF-DE method and observed a significant reduction in the number of synaptic vesicles. Moreover, we found that synaptic vesicles distal from, rather than proximal to, the presynaptic membrane were more severely reduced in number in cerebellar granule cell terminals. This result, together with our previous immunocytochemical study showing that synapsin I tends to be located distally from the presynaptic membrane (Hirokawa et al., 1989) and a recent report that presynaptic injection of synapsin antibodies resulted in the selective loss of vesicles distal from the presynaptic membrane (Pieribone et al., 1995), are compatible with the idea that synapsin I forms reserve pool of synaptic vesicles by forming cross-bridges

among synaptic vesicles and the cytoskeleton. The down-regulation of p38 expression in synapsin I-deficient mice is considered to be a consequence of this reduction of synaptic vesicles. As expected, the number of large dense-cored vesicles, not associated with synapsin I, was not affected. The reduction in synaptic vesicles was not so severe as previously reported in double knockouts (Rosahl et al., 1995), suggesting that synapsin I and II function cooperatively for the maintenance of synaptic vesicles, and that synapsin I and synapsin II have a shared function with regard to the turnover and stabilization of synaptic vesicles.

However, in addition to this finding, we observed that the sizes of mossy fiber terminals were significantly reduced. Three distinct experiments, using conventional electron microscopy, QF-DE electron microscopy, and confocal laser scanning microscopy with anterograde labeling by DiI, supported this finding. On the contrary, the mean size of cerebellar granule cell terminals of synapsin I-deficient mice was similar to controls. In double knockout mice, the sizes of the presynaptic areas in hippocampal CA1 fields and the visual cortex were reported to be unchanged (Rosahl et al., 1995). This discrepancy could be explained by some particular characteristics of mossy fiber terminals. First, mossy fiber terminals have a larger size with a complex shape and higher densities of synaptic vesicles (Gaarskjaer, 1986). Because of their large volume and high synaptic vesicle density, they might be unable to maintain giant presynaptic expansions when synaptic vesicles are destabilized and reduced in number in synapsin I-deficient mice. Second, the relatively high expression of synapsin I in mossy fiber terminals (Melloni et al., 1993) might result in a more severe effect of synapsin I deficiency in this type of presynaptic terminal than in other types. In other words because the size of cerebellar granule cell terminals and the total numbers of synaptic vesicles in these terminals are not large compared to the mossy fiber terminals in CA3 fields the difference between synapsin I-deficient and wild-type mice may be difficult to be detected. Possibly, in synapsin I-deficient mice there might be some alteration in synthesis, transport, and turnover of membranes composing presynaptic terminals and synaptic vesicles.

To investigate the synaptic vesicle associated structures in the nerve terminals of synapsin I-deficient mice, we performed the QF-DE method, by which the reduction of synaptic vesicles could be visualized more distinctly. We showed that short cross-bridges between synaptic vesicles were greatly reduced in number in cerebellar mossy fiber terminals. In previous reports we have suggested that fine short strands existing between actin filaments and synaptic vesicles, and between synaptic vesicles both in vivo and in vitro, are mainly synapsin I (Harada et al., 1990; Hirokawa et al., 1989; Hirokawa, 1990), and this was strongly supported by the present finding. The strands associated with synaptic vesicles in terminals lacking synapsin I could be synapsin II or other synaptic vesicle-associated molecules. These observations led us to conclude that synapsin I is a main element of short linkages between synaptic vesicles in nerve terminals.

To test whether these structural alterations affect synaptic function, we investigated mossy fiber LTP as a model of synaptic plasticity. LTP at the Schaffer collateral-commissural fiber-CA1 pyramidal synapse, NMDA receptor-depen-

dent LTP, was not severely affected by the inactivation of synapsin I gene (Rosahl et al., 1993). However the hippocampal mossy fiber LTP has been reported to have different mechanisms from this type of LTP. The hippocampal mossy fiber LTP does not require postsynaptic NMDA receptor activation, membrane depolarization, or a rise in calcium. It is associated with reduced PPF and is therefore suggested to be induced and expressed presynaptically (Zalutsky and Nicoll, 1990). A recent report showed an increase in the probability of transmitter release using the open-channel NMDA receptor antagonist MK-801 (Weisskopf and Nicoll, 1995). However we could find no difference in the time course and magnitude of mossy fiber LTP between synapsin I-deficient mice and controls. We conclude that synapsin I is not involved in the presynaptic processes responsible for mossy fiber LTP.

In conclusion, synapsin I is not essential for transmitter release, neurite outgrowth, or the development of neuronal cytoarchitecture. However, the absence of synapsin I resulted in a decreased number of synaptic vesicles and a reduction of fine short strands associated with synaptic vesicles. Furthermore, hippocampal mossy fibers of synapsin I-deficient mice showed a decrease in the size of nerve terminals. The relationship between the decreased number of synaptic vesicles and the reduced size of terminals is unclear, but the size of relatively large nerve terminals with extremely high densities of synaptic vesicles might be comparatively prone to intra-terminal structural change. The reduction of synaptic vesicles in synapsin I-deficient mice was not so severe as reported in mice lacking both synapsin I and II (Rosahl et al., 1993), suggesting that the two synapsins share common mechanisms in synaptic vesicle turnover. The altered synaptic vesicle-associated structures revealed by QF-DE method confirmed that the observed short fine cross-bridges associated with synaptic vesicles are mainly synapsin I. All these results strongly suggested that synapsin I contributes significantly to the formation and maintenance of the presynaptic structure. We are currently analyzing the alteration in the dynamics of synaptic vesicles caused by the absence of synapsin I electrophysiologically, which should additionally elucidate the function of synapsin I.

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