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Impairment of axonal development and of synaptogenesis in hippocampal neurons of synapsin I-deficient mice

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ABSTRACT **Synapsin I, the most abundant of all neuronal phosphoproteins, is enriched in synaptic vesicles. It has been hypothesized to regulate synaptogenesis and neurotransmitter release from adult nerve terminals. The evidence for such roles has been highly suggestive but not compelling. To evaluate the possible involvement of synapsin I in synaptogenesis and in the function of adult synapses, we have generated synapsin I-deficient mice by homologous recombination. We report herein that outgrowth of predendritic neurites and of axons was severely retarded in the hippocampal neurons of embryonic synapsin I mutant mice. Furthermore, synapse formation was significantly delayed in these mutant neurons. These results indicate that synapsin I plays a role in regulation of axonogenesis and synaptogenesis.**

The nerve terminal is a highly specialized structure where synaptic transmission occurs by fusion of synaptic vesicles with the plasma membrane, with consequent release of neurotransmitter into the synaptic cleft. In recent years, many synaptic vesicle proteins have been isolated, cloned, and characterized (1–5). For the most part, the molecular mechanisms by which these proteins regulate and/or mediate vesicle fusion and neurotransmitter release remain to be clarified. The synapsins are a family of proteins that are distinguishable from most other synaptic vesicle-associated proteins in two ways. (*i*) They are peripheral rather than integral membrane proteins; (*ii*) they are specific to the nervous system, as there are apparently no homologous proteins in nonneuronal tissues.

The synapsin family is composed of four homologous proteins, synapsins Ia and Ib (collectively referred to as synapsin I) and synapsins IIa and IIb (collectively referred to as synapsin II), which are products of the alternative splicing of the transcripts from two distinct (synapsin I and synapsin II) genes (6). Injection of synapsin I into *Xenopus* blastomeres accelerates the structural (7) and functional (8) development of neuromuscular synapses. In adult synapses, a variety of cell biological and electrophysiological studies are consistent with the possibility that synapsin I tethers synaptic vesicles to the actin cytoskeleton in a phosphorylation-state-dependent manner and thereby determines the proportion of vesicles in the nerve terminal that are available for release (2). In this report, and in the accompanying paper (9), we have undertaken a detailed examination of the development and function of synapses in synapsin I-deficient mice.

MATERIALS AND METHODS

Targeting Construct and Embryonic Stem (ES) Homologous Recombinants. Genomic clones containing exon 1 of the murine synapsin I gene were isolated from a 129/Sv mouse genomic library (Stratagene) by using a rat synapsin I cDNA probe (10). The targeting construct was derived by inserting a 6-kb *Sst* I–*Sma* I genomic fragment containing the 5′ flanking sequence of exon 1 and a 3-kb *Bam*HI fragment containing the 3′ flanking sequence of exon 1 into the pPNT vector, which contains the thymidine kinase gene and the neomycin-resistance gene (11). E14 ES cells (12) were transfected with 50 μg of linearized targeting construct by electroporation using a Bio-Rad Gene Pulser at 800 V and 3 μF. Selection with G418 at 150 μg/ml and 0.2 μM 1-(2-deoxy, 2-fluoro-β-D-arabinofuranosyl)-5-iodouracil (FIAU, Bristol–Meyers) was made 24 h after electroporation, and double-resistant clones were isolated after 1 week of selection. Genomic DNA from these clones was digested with *Nco* I and hybridized with a probe isolated from the 3′ flanking sequence of exon 1 (Fig. 1*A*). Positive clones were confirmed by hybridization of the same DNA with a probe isolated from the 5′ flanking sequence.

Generation of Synapsin I-Deficient Mice. ES cells from the identified homologous recombinants were injected into C57BL/6 blastocysts, which were then implanted in the uteri of pseudopregnant recipient females as described (13). Male chimeric mice were backcrossed to C57BL/6 females to screen for germ-line transmission. The genotypes of the resulting F_1 agouti mice were determined by genomic Southern blot analysis of DNA isolated from their tails. Mice heterozygous for the synapsin I mutation were bred to homozygosity.

Western Blot Analysis. Brain tissues were homogenized in 1% SDS and subjected to SDS/PAGE. The proteins were transferred onto nitrocellulose membranes and probed with affinity-purified primary antibodies, followed by incubation with ^{125}I-labeled protein A (Amersham). The blots were exposed to Kodak XAR-5 autoradiographic film or to PhosphorImager screens and quantified by using IMAGEQUANT software (Molecular Dynamics).

Preparations of Hippocampal Cell Cultures. Hippocampal cell cultures were prepared and analyzed by investigators without any knowledge of the genotype of the animal. Homozygous synapsin I-deficient and wild-type female littermates were mated with homozygous synapsin I-deficient and wild-type male littermates, respectively. The hippocampi of embryonic day 16 fetuses were dissected in parallel, freed of meninges, and cultured as described for the embryonic day 18 rat (14). The cells were dissociated by trypsinization (0.25% for 15 min at 37°C) followed by trituration with a fire-polished Pasteur pipette and plated onto poly(L-lysine)-coated coverslips (100,000 cells per 60-mm dish) in minimum essential medium (MEM) with 10% (vol/vol) horse serum. After 4 h the coverslips were transferred to dishes containing an astroglial monolayer and maintained in MEM containing N2 supplements (15), 0.1% ovalbumin, and 0.1 mM sodium pyruvate.

Immunocytochemical Procedures. Cultures were fixed for 20 min with 4% (wt/vol) paraformaldehyde in phosphate-buffered saline (PBS) containing 0.12 M sucrose. They were then permeabilized in 0.3% Triton X-100 in PBS for 5 min and rinsed twice in PBS. The cells were preincubated in 10%

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Abbreviation: ES cell, embryonic stem cell.

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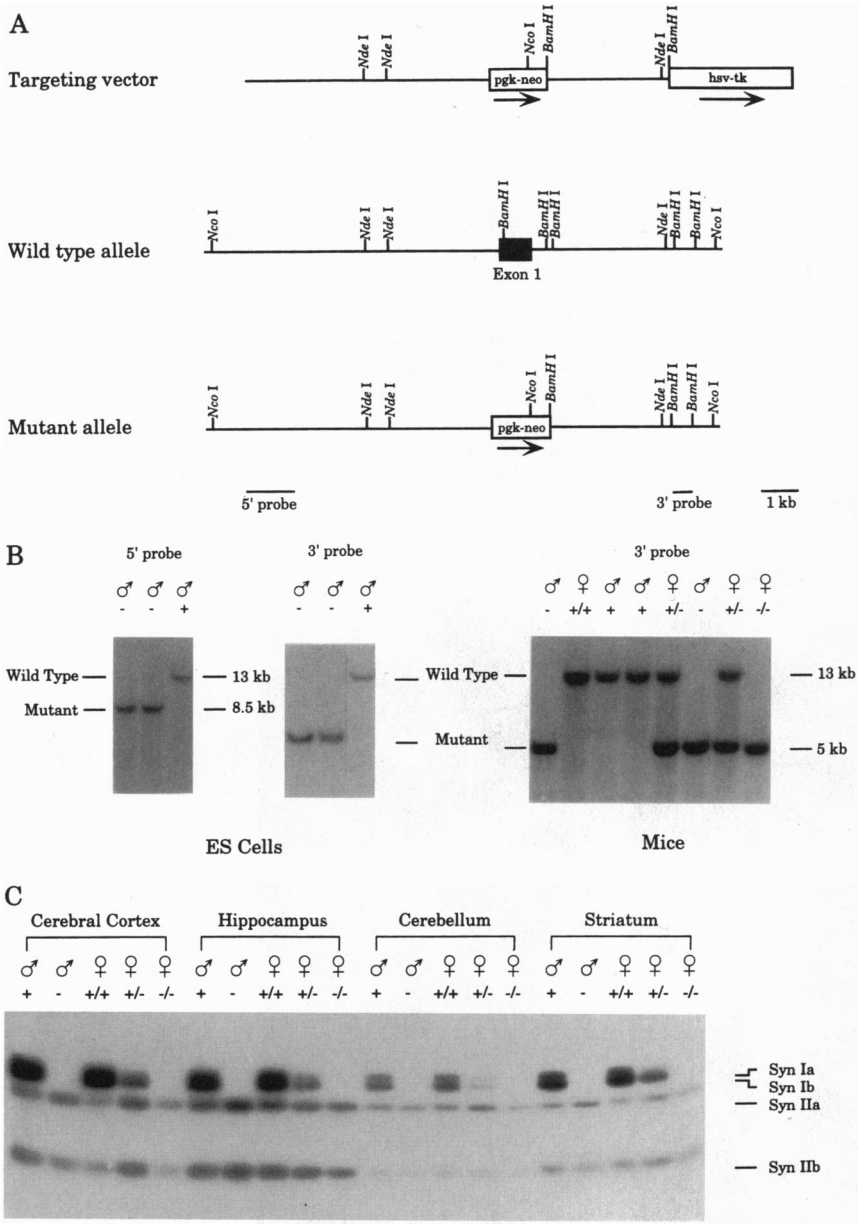

FIG. 1. Targeted disruption of the synapsin I gene by homologous recombination. (*A*) Restriction map of the targeting vector, the 5′ part of the synapsin I gene, and the predicted structure of the disrupted synapsin I gene after homologous recombination. Horizontal arrows indicate the direction of transcription of the cassettes containing the neomycin-resistance gene under the control of the phosphoglycerate kinase 1 gene (pgk–neo) and herpes simplex virus thymidine kinase (hsv-tk). The positions of the probes used for screening of ES cell clones and mice are indicated. (*B*) Southern blot analysis of *Nco* I-digested genomic DNA from ES cell clones and from mouse tails. Hybridization was done with the 5′ probe or the 3′ probe shown in *A*. Restriction fragment lengths of the wild-type and mutant alleles are indicated. The sex and genotype of each clone and each mouse are indicated. (*C*) Western blot analysis of brain tissues from wild-type, heterozygous, and homozygous mutant mice. The sex and the genotype of each mouse are indicated. Tissue extracts were prepared from cerebral cortex, hippocampus, cerebellum, and striatum of each mouse and analyzed by using an antibody that recognizes all four synapsin isoforms.

(wt/vol) bovine serum albumin in PBS for 1 h at 37°C and exposed to the primary antibodies (diluted in 1% bovine serum albumin in PBS) overnight at 4°C. Finally, the cultures were rinsed in PBS and incubated with secondary antibodies for 1 h at 37°C. The following antibodies were used: anti-α-tubulin (clone DM1A) and polyclonal anti-tubulin (Sigma); anti-synaptophysin (clone SY38), fluorescein-conjugated anti-mouse IgG, and rhodamine-conjugated anti-rabbit IgG (Boehringer Mannheim); anti-synapsin I (clone 18.1) (6); anti-MAP2 (clone AP-14) (16).

Morphometric Analysis. Fixed cultures were viewed with an inverted phase microscope by using a video camera. Cells were selected at random and traced from the screen, and the length of their processes was measured by using a digitizing tablet.

RESULTS

Targeted Disruption of the Synapsin I Gene in ES Cells and Mice. To disrupt the synapsin I gene, a targeting vector was constructed in which exon I of synapsin I was removed and replaced with the neomycin-resistance (neo) gene under the control of the phosphoglycerate kinase 1 (*pgk*) promoter (Fig. 1*A*). The targeting vector was electroporated into E14 ES cells and selected by using G418 and 1-(2-deoxy, 2-fluoro-β-D-arabinofuranosyl)-5-iodouracil. Double-resistant clones were screened for homologous recombination by Southern blot analysis with a 3′ flanking probe and a 5′ internal probe. Since the synapsin I gene is localized on the X chromosome (17) and since the E14 ES cells were derived from male embryos, a homologous recombinant clone would be expected to contain only a mutant allele and no wild-type allele (Fig. 1*B*). Of 672 clones analyzed, 7 carried the disrupted synapsin I allele. Upon injection into C57BL/6 blastocysts, 2 of the clones gave rise to chimeric mice that transmitted the mutant allele through the germ line. Heterozygous mice were identified by Southern blot analysis and bred to obtain homozygous mice (Fig. 1*B*). The genotype distribution among total progeny fits with the Mendelian distribution predicted for an X chromosome-linked gene, suggesting that there is no appreciable short-term survival disadvantage to homozygous mutant mice compared with their wild-type or heterozygous littermates.

Western blot analysis with an antibody that recognizes all four synapsin isoforms demonstrated that, in homozygous mutant mice, the targeted disruption of the synapsin I gene

specifically abolished expression of synapsins Ia and Ib in total brain (data not shown) and in different brain regions such as cerebral cortex, hippocampus, cerebellum, and striatum (Fig. 1*C*), with no apparent effect on expression of synapsins IIa and IIb. In heterozygous female mice, the expression level of synapsins Ia and Ib in brain tissue was reduced to ≈50% of the level in wild-type littermates (Fig. 1*C*). According to the Lyon hypothesis, this phenomenon can be explained by the random inactivation of one or the other X chromosome in heterozygous females (18). Results in agreement with those shown in Fig. 1*C* were also obtained by using several other antibodies directed against different epitopes of synapsin I or synapsin II (data not shown).

In most respects, homozygous synapsin I mutant mice are phenotypically indistinguishable at a gross level from their heterozygous and wild-type littermates. They appear to sniff food, explore, and mate normally. The oldest homozygous mutant mice currently have lived to 11 months of age.

Phenotype of Synapsin I-Deficient Hippocampal Neurons in Culture. Hippocampal pyramidal neurons dissociated from E16 wild-type mice differentiated in culture following a sequence of morphological events that resembled those described for the rat (19). Within 24 h after plating, the majority of neurons extended several minor neurites and a single axon (Fig. 2*A*). Axonal elongation and branching continued at a rapid rate for several days. On the other hand, little net elongation of the minor neurites was observed until day 3 in culture when dendritic development begins. By day 7 in culture, both axonal and dendritic domains were well differentiated (Fig. 3*A*).

Synapsin I-deficient hippocampal neurons also differentiated in culture; after 1 day most of them were polarized showing an axon and several minor neurites (Fig. 2*B*). However, the axon and minor neurites were considerably shorter than in the control cultures (Fig. 2, compare *A* and *B*; Table 1). Furthermore, axons in neurons lacking synapsin I were less branched than in the controls (Fig. 2 *C* vs. *D*). After 3 days in culture, the lengths of the minor neurites differed only slightly from the controls, whereas the difference in the lengths of axons between wild-type and synapsin I-deficient cultures had

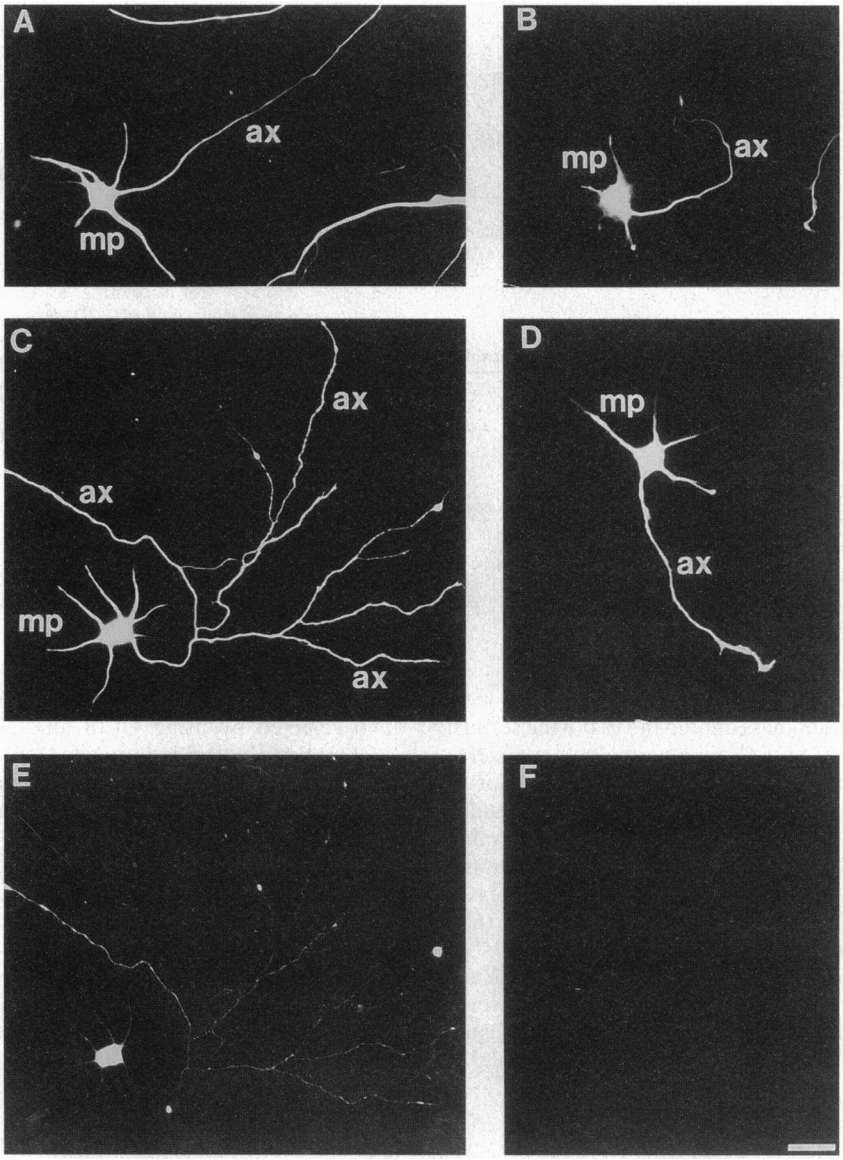

FIG. 2. Phenotype of synapsin I-deficient hippocampal neurons in culture. (*A* and *B*) Hippocampal neurons obtained from wild-type (*A*) or synapsin I-deficient (*B*) mice were cultured for 1 day and stained with a monoclonal antibody against tubulin. Both minor processes (mp) and axons (ax) are considerably shorter in synapsin I-deficient neurons. (*C–F*) Hippocampal neurons obtained from wild-type (*C* and *E*) or synapsin I-deficient (*D* and *F*) mice were cultured for 3 days and double-stained with a polyclonal antibody against tubulin (*C* and *D*) and a monoclonal antibody specific for synapsin I (*E* and *F*). Note the absence of axonal branching in synapsin I-deficient neurons. (Bar = 20 μm.)

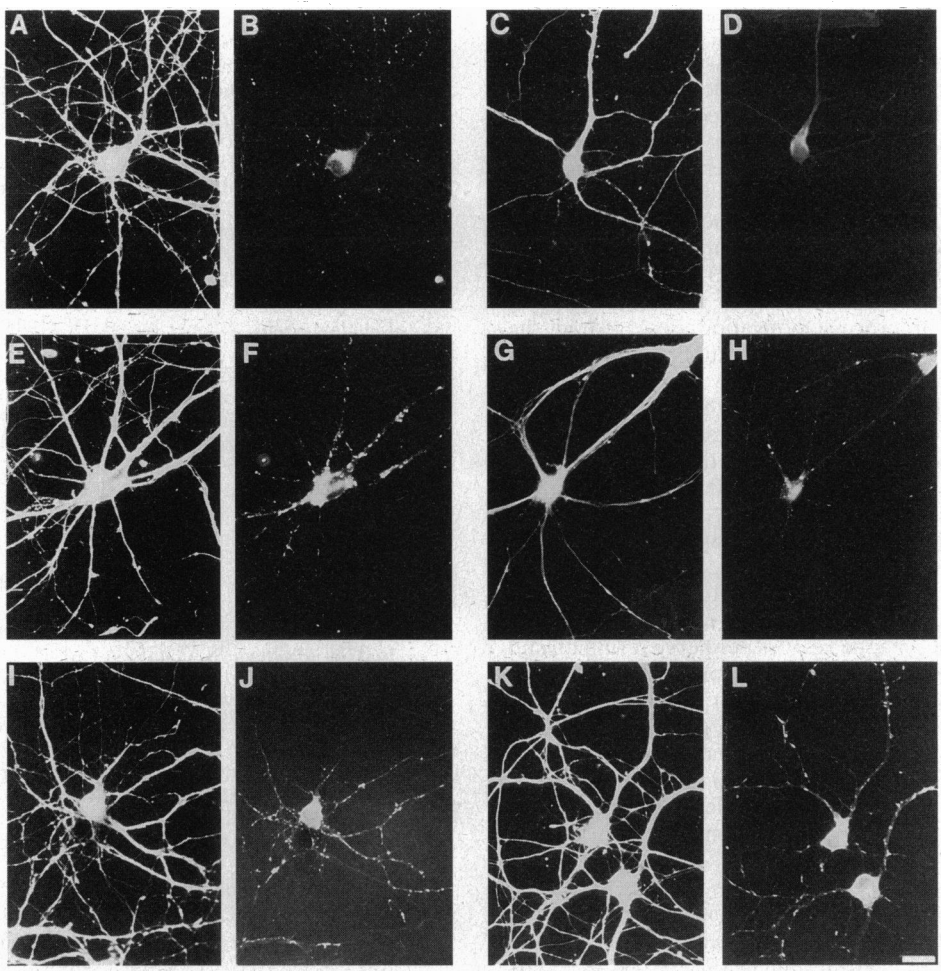

FIG. 3. Immunocytochemical detection of synapses in wild-type and synapsin I-deficient hippocampal neurons. Hippocampal neurons obtained from wild-type (*A, B, E, F, I,* and *J*) or synapsin I-deficient (*C, D, G, H, K,* and *L*) mice were cultured for 7 (*A–D*), 10 (*E–H*), or 14 (*I–L*) days and double-stained with a polyclonal antibody against tubulin (*A, C, E, G, I,* and *K*) and a monoclonal antibody against synaptophysin (*B, D, F, H, J,* and *L*). Numerous synaptophysin immunofluorescence spots could be seen at 7 days in wild-type neurons (*B*). Synaptophysin immunofluorescence spots were not detected until 10 days in synapsin I-deficient neurons (*H*). (Bar = 20 μm.)

increased. With the onset of dendritic differentiation at day 3, the growth of these predendritic processes approached that of the controls. In contrast, axonal elongation remained retarded at day 3 (Table 1). Therefore, the absence of synapsin I had a greater effect on the growth of processes destined to become presynaptic. The restoration of normal dendritic growth before that of axons temporally parallels the loss of synapsin I from early dendrites and its consequent segregation to axons (20).

Synapse Formation Is Delayed in Synapsin I-Deficient Hippocampal Neurons. To determine whether synaptogenesis was altered by inactivation of synapsin I expression, we immunolabeled synapses in hippocampal cultures 3, 7, 10, and 14 days after plating by using synaptophysin antibodies as a synaptic marker (20). Soon after the onset of dendritic differentiation at day 3, axo-dendritic and axo-somatic synaptic contacts appeared in wild-type hippocampal neurons (data not shown). By day 7, bright punctate staining with synaptophysin antibodies was observed in the presynaptic areas (Fig. 3). The number of synapses increased with time of development in culture.

In synapsin I-deficient neurons, the formation of synaptic contacts was delayed and did not appear until day 10 in culture (Fig. 3 and Table 2). Once synapses formed in these synapsin I-deficient neurons, they continued to increase in number until by day 14 the number of synapses in cultures of these neurons was similar to that observed in cultures of wild-type controls.

Table 1. Effect of synapsin I null mutation on neurite outgrowth in hippocampal neurons

Days in culture	Neurite outgrowth	Length, μm	
		Wild type	Mutant
1	Axon	217.1 ± 13.6	161.8 ± 8.2*
	Minor processes	37.1 ± 1.3	28.9 ± 1.2*
	Total	356.4 ± 20.7	259.2 ± 13.5*
3	Axon	767.3 ± 79.4	253.7 ± 15.3*
	Minor processes	57.9 ± 4.3	45.7 ± 3.1†
	Total	1061.5 ± 49.4	467.8 ± 24.4*

Sixty cells from three cultures were analyzed for each time point. Results are expressed as the mean ± SEM. Total neurite length is the total length of axons plus axonal branches plus minor neurites. *, Differs from wild type, $P < 0.001$; †, differs from wild type, $P < 0.05$.

Table 2. Effect of synapsin I null mutation on synapse formation in hippocampal neurons

Days in culture	Number of synapses per cell	
	Wild type	Mutant
7	40.8 ± 3.9	0.5 ± 0.2*
10	64.2 ± 3.9	37.5 ± 4.9*
14	75.5 ± 6.5	70.6 ± 6.4

Sixty cells from three cultures were analyzed for each time point. Results are expressed as the mean ± SEM. *, Differs from wild type, $P < 0.001$.

DISCUSSION

The dramatic effect of deletion of the synapsin I gene on neurite outgrowth and synaptogenesis demonstrated in the present investigation provides strong support for the concept that this protein is involved in regulation of synapse formation. The ability of synapsin I-deficient nerve cells to form synapses, but after a delay, reconciles earlier studies suggesting a role for synapsin I in synaptogenesis (7, 8) with the "normal" appearance of the synaptic architecture of adult mutant mice observed at the light microscope level (9, 21).

The delay in synapse formation may be attributable in part to the retardation in process elongation observed in the synapsin I-deficient cultures. Control cultures showed greater process length and, therefore, had a greater likelihood of making contacts with other neurons. On the other hand, even dense cultures, in which differentiation is accelerated, showed a delay in synapse formation, indicating that limited contact alone was probably not a sufficient explanation for delayed synapse formation.

Other evidence has suggested that synapsin I and synapsin II may be involved in neuronal development and synaptogenesis (7, 8, 22–25). Transfection of synapsin II into NG108 cells resulted in an increase in the number of nerve terminals per cell, in the number of synaptic vesicles per nerve terminal, and in the amount of several synaptic vesicle proteins (22). Moreover, injection of synapsin I or synapsin II into early blastomeres of *Xenopus laevis* resulted in a greatly accelerated structural (7) and functional (8, 23) maturation of synapses. In addition, treatment of embryonic day 18 hippocampal neurons with synapsin II antisense oligonucleotides impaired neurite elongation (24) and the formation and maintenance of synapses (25).

In addition to its ability to regulate various stages of neuronal development, synapsin I has been shown to regulate clustering of synthetic phospholipid vesicles (26) and of synaptic vesicles in mature nerve terminals (9, 27). These results suggest a role for the synapsins in organizing the structure of the nerve terminal.

It will be of importance to determine whether the effects of synapsin I observed at different stages of neuronal development reported here are attributable to the same or different mechanisms of action. Similar considerations apply to the results obtained with synapsin II antisense oligonucleotides (24, 25). During the initial phases of neurite outgrowth, antisense suppression of synapsin II resulted in shortened broad neurites. When synapsin II was suppressed later in development, there was a failure to develop synapses. When synapsin II was suppressed after formation of synapses, there was a loss of synaptic contacts. Finally, the impairment of neuronal development observed when either synapsin I or synapsin II is depleted may be attributable to a gene dosage effect. Alternatively, the results raise the possibility that the two types of synapsins use distinct complementary mechanisms to achieve the same common goal of organizing the nerve terminal and the synapse.

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