

Suppression of synapsin II inhibits the formation and maintenance of synapses in hippocampal culture

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ABSTRACT Numerous synaptic proteins, including several integral membrane proteins, have been assigned roles in synaptic vesicle fusion with or retrieval from the presynaptic plasma membrane. In contrast, the synapsins, neuron-specific phosphoproteins associated with the cytoplasmic surface of synaptic vesicles, appear to play a much broader role, being involved in the regulation of neurotransmitter release and in the organization of the nerve terminal. Here we have administered antisense synapsin II oligonucleotides to dissociated hippocampal neurons, either before the onset of synaptogenesis or 1 week after the onset of synaptogenesis. In both cases, synapsin II was no longer detectable within 24–48 h of treatment. After 5 days of treatment, cultures were analyzed for the presence of synapses by synapsin I and synaptophysin antibody labeling and by electron microscopy. Cultures in which synapsin II was suppressed after axon elongation, but before synapse formation, did not develop synapses. Cultures in which synapsin II was suppressed after the development of synapses lost most of their synapses. Remarkably, with the removal of the antisense oligonucleotides, neurons and their synaptic connections recovered. These studies lead us to conclude that synapsin II is involved in the formation and maintenance of synapses in hippocampal neurons.

Synapsins are phosphoproteins that associate with the cytoplasmic surface of synaptic vesicles and bind to the cytoskeleton. Two genes encode the synapsins, designated synapsin I and synapsin II (1). Both gene products are phosphorylated by cAMP-dependent protein kinase and Ca²⁺/calmodulin-dependent kinase I in their homologous N termini. The two genes differ in their C termini, where synapsin I, but not synapsin II, is phosphorylated by Ca²⁺/calmodulin-dependent kinase II. The function of these proteins appears to be distinct from that of the various integral membrane proteins of synaptic vesicles, some of which may be involved in the fusion of vesicles with the plasma membrane. Synapsin I may tether synaptic vesicles to actin filaments in a phosphorylation-dependent manner that controls the number of vesicles available for release at the nerve terminus (2). The synapsins have been implicated in neurite elongation (3), in the differentiation of the synaptic terminal (4), and in the clustering of synaptic vesicles into an active pool and a reserve pool (5). The synapsins also appear to play a role in the formation of synapses. The injection of synapsin I or synapsin II into *Xenopus* blastomeres accelerates synapse formation (6–8), and transfection of neuroblastoma–glioma hybrid cells with synapsin II enhances the expression of other synaptic proteins (4, 9, 10).

Hippocampal cultures provide a useful system in which to observe synapse development. Neurons taken from embryonic day 18 hippocampi and placed in dissociated culture undergo a highly predictable sequence of morphological changes as they

differentiate. Within 24 h of plating, the cells first elaborate a symmetric array of minor neurites followed by the rapid elongation of one of these neurites to form an incipient axon. The remaining minor neurites differentiate into dendrites by day 3 (11) and synaptogenesis begins around day 5 (12). Previous work showed that the suppression of synapsin II just after plating resulted in shortened processes that remained flattened and failed to consolidate into a discrete shaft (3). The aim of the present investigation was to examine the effect of synapsin II suppression on synaptic development and maintenance.

MATERIALS AND METHODS

Preparation of Hippocampal Cultures. Neuronal cultures were prepared from the hippocampi of embryonic day 18 rats as described (13). Briefly, embryos were removed and their hippocampi were dissected and freed of meninges. 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 (75,000 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 (14), 0.1% ovalbumin, and 0.1 mM sodium pyruvate.

Antisense Oligonucleotides. Two nonoverlapping synapsin II antisense oligonucleotides, As-RSII-13+10 (AGTTCAT-CATCTGGCTTGAGGGA) and As-RSII-88-66 (CGAC-CAAAGGTGGTCCGCGTCTC) (Oligos Etc., Guilford, CT), were used in this study. The oligonucleotides were S-modified (20) in the last 3 bases at the 3' end. The oligonucleotides were added at 50 μM every 12 h for 5 days, starting with 3- or 10-day-old dissociated hippocampal cultures. Control cultures were treated with the same concentration of the corresponding sense-strand oligonucleotide or without added oligonucleotide.

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% (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 II (clone G316); and polyclonal anti-synapsin I (3).

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Abbreviation: DIV, day(s) *in vitro*.

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Electron Microscopy. Cells grown on coverslips were fixed in 3.5% (vol/vol) glutaraldehyde in culture medium for 30 min at 37°C, rinsed in 0.125 M sodium phosphate (pH 7.3), and stained with 1% OsO₄ in 0.125 M sodium phosphate (pH 7.3) for 1 h at 37°C in the dark. They were then rinsed, dehydrated in increasing concentrations of methanol followed by acetone, and embedded in Epon. After polymerization, the coverslips were peeled off, and the cells were punched out of the block and remounted. The cells were sectioned parallel to the glass coverslip coating substrate. Thin sections were counterstained with uranyl acetate and lead citrate and examined by using a JEOL 100 CX electron microscope.

Protein Determination, Electrophoresis, and Immunoblot Analysis. Cultures were rinsed twice in PBS prewarmed to 37°C, scraped into Laemmli buffer (15), homogenized in a boiling water bath for 5 min, and centrifuged at 33,000 rpm in a Beckman type 40.2 rotor. The supernatant was removed and stored at -80°C until use. Protein concentration was determined by the method of Lowry *et al.* (16), as modified by Bensadoun and Weinstein (17). SDS/polyacrylamide gels

were electrophoresed as described by Laemmli (15). The proteins were transferred to Immobilon membranes (Millipore) as described by Towbin *et al.* (18) and modified by Ferreira *et al.* (19). These membranes were probed with affinity-purified antibodies followed by ¹²⁵I-labeled secondary antibodies (Amersham). Autoradiograms were obtained by exposing x-ray films to immunoblots. The films were then analyzed with a PhosphorImager equipped with quantitation software (Molecular Dynamics).

RESULTS

Immunoblot analyses revealed that treatment of hippocampal cultures for 5 days with either antisense rat synapsin II oligonucleotide (AS-RSII) beginning at 3 days *in vitro* (DIV) or 10 DIV effectively reduced the amount of synapsin II by 75–90%, whereas treatment with the corresponding sense oligonucleotide (S-RSII) did not affect synapsin II levels (data not shown). The two other synaptic vesicle proteins examined were also depleted: in cultures treated with AS-RSII for 5 days

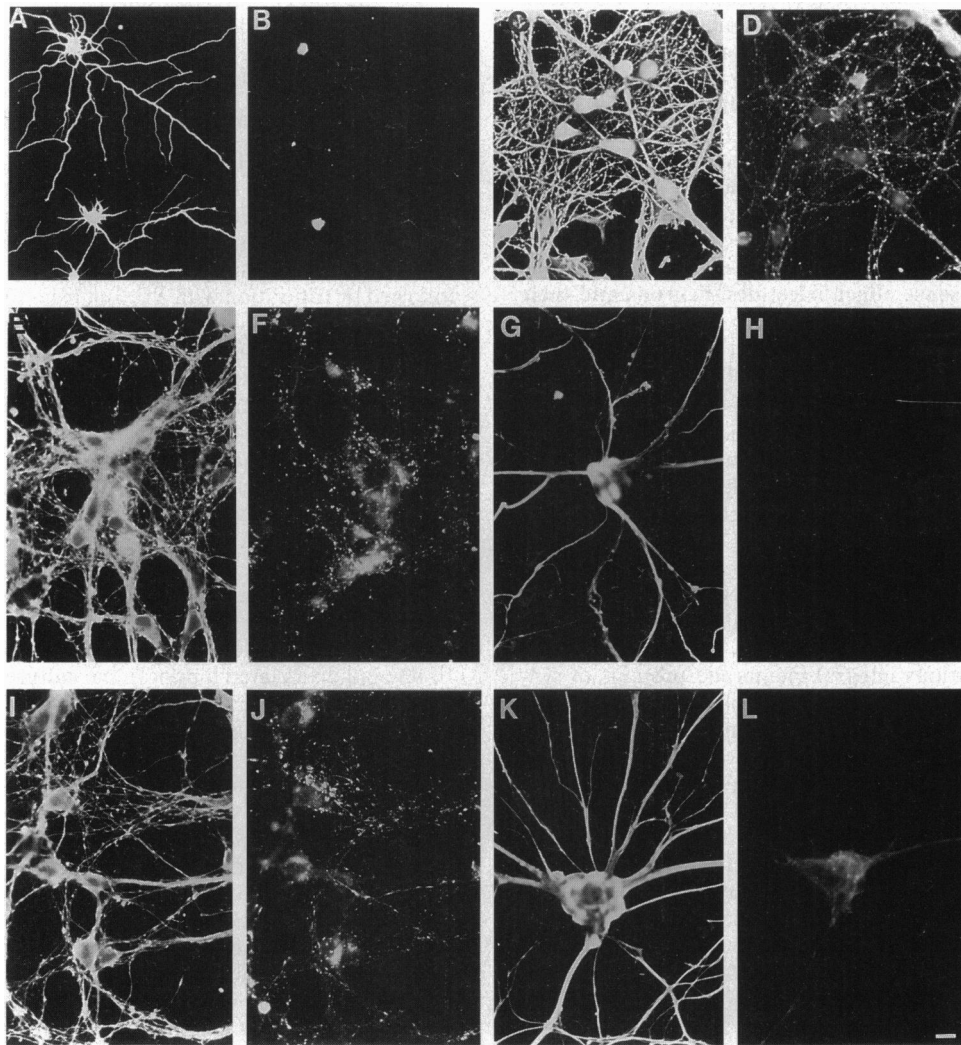


FIG. 1. Effect of synapsin II depletion on synaptogenesis in hippocampal neurons in culture. At 3 DIV, hippocampal neurons have extended an axon and several minor processes that stained with a tubulin antibody (A). At this early stage of development, synapsin II immunoreactivity is localized in the cell bodies (B). After 3 DIV, S-RSII-13+10 or AS-RSII-13+10 oligonucleotides were added to the culture medium, and the cells were allowed to grow for 5 days. At 8 DIV, nontreated control (C and D) and sense (E and F)- and antisense (G and H)-treated cultures were double-stained with tubulin (C, E, and G) and synapsin II (D, F, and H) antibodies. Numerous bright synapsin II immunoreactive spots corresponding to synapses can be seen in control (D) and sense-treated (F) neurons. Note the absence of synapsin II immunoreactivity (H) and the neuritic fasciculation (G) in antisense-treated cultures. Sense (I and J)- and antisense (K and L)-treated cultures were double-stained with tubulin (I and K) and synaptophysin (J and L) antibodies. Very few synapses were detected in antisense-treated neurons compared to sense-treated neurons. (Bar = 15 μ m.)

Table 1. Effect of synapsin II suppression on the number of synapses present in embryonic day 18 hippocampal neurons grown in culture

Days in culture prior to treatment	Synaptic marker	Number of synapses				
		Pretreatment	Treatment			Recovery
			None	Sense	Antisense	
3	Synaptophysin	0	224 ± 15	220 ± 17	33 ± 3*	139 ± 20†
3	Synapsin I	0	250 ± 18	230 ± 21	29 ± 3*	142 ± 12†
10	Synaptophysin	250 ± 20	275 ± 13	261 ± 14	78 ± 11*	320 ± 34†
10	Synapsin I	263 ± 17	289 ± 15	269 ± 15	65 ± 6*	356 ± 45†

Embryonic day 18 hippocampal neurons were grown in culture for either 3 days or 10 days, followed by treatment for 5 days with no oligonucleotide (none) or sense (S-RSII-13+10) or antisense (AS-RSII-13+10) oligonucleotides as indicated. Cells that had been treated with AS-RSII were then allowed to recover for 7 days. Morphometric analysis was carried out immediately prior to (pretreatment) or after treatment, or recovery in the absence of added oligonucleotide. Twenty fields were analyzed for each experimental condition. Each number represents the mean ± SEM. *, $P < 0.001$ compared to sense-treated cultures; † $P < 0.001$ compared to antisense-treated cultures at the time of removal of the oligonucleotide.

beginning at 3 days after plating (8 DIV), there was a 75% diminution in synapsin I and a 40% diminution in synaptophysin compared to sense-treated or untreated controls. In cultures treated for 5 days beginning at 10 days after plating (15 DIV), synapsin I and synaptophysin levels were decreased 60% and 30%, respectively (data not shown).

To determine whether synapses were formed when synapsin II was suppressed, 3-DIV hippocampal neurons were cultured in the absence or presence of S-RSII or AS-RSII for 5 days and analyzed immunocytochemically. At 8 DIV, ≈80% of the neurons were not labeled with synapsin II antibodies after treatment with either AS-RSII-13+10 (Fig. 1) or AS-RSII-

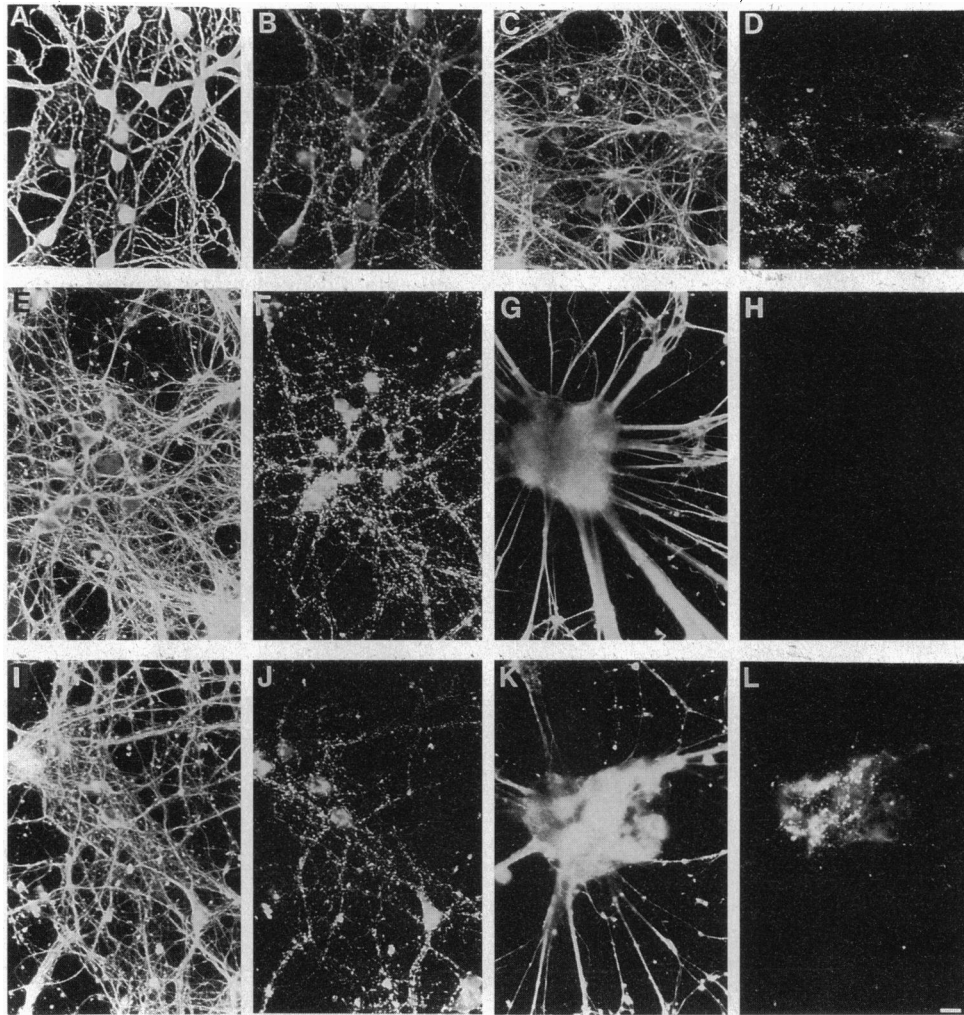


FIG. 2. Effect of synapsin II depletion on the maintenance of synapses in cultured hippocampal neurons. At 10 DIV, hippocampal neurons have a well-differentiated neuritic network that can be detected by tubulin immunofluorescence (A) and have established numerous synaptic contacts that can be detected with synapsin II antibody (B). After 10 DIV, S-RSII-13+10 or AS-RSII-13+10 oligonucleotides were added to the culture medium, and the cells were allowed to grow for 5 days. At 15 DIV, nontreated control (C and D) and sense (E and F)- and antisense (G and H)-treated cultures were double-labeled with tubulin (C, E, and G) and synapsin II (D, F, and H) antibodies. Multiple synapses could be detected in control and sense-treated neurons. No synapsin II immunofluorescence was detected in antisense-treated neurons. Sense (I and J)- and antisense (K and L)-treated cultures were double-labeled with tubulin (I and K) and synaptophysin (J and L) antibodies. Note the marked decrease in the number of synapses (L) and the neuritic fasciculation (K) in antisense-treated neurons. (Bar = 15 μ m.)

88-66 (data not shown). Staining for synaptophysin or synapsin I indicated that there was a marked reduction in the immunoreactive spots for these two synaptic markers in antisense-oligonucleotide-treated neurons compared to sense-treated or untreated controls (Fig. 1 and Table 1). Under these conditions, as analyzed by tubulin staining, the axons and dendrites tended to fasciculate, and the cell bodies tended to cluster. By 8 DIV, S-RSII-treated cultures did not show any significant changes in synapsin II immunoreactivity, axonal and dendritic development (Fig. 1), or the number of synapses (Table 1), compared to untreated controls.

To examine the possibility that synapsin II may also be required to maintain established synapses, 10-DIV hippocampal neurons were cultured in the presence of S-RSII or AS-SRII for 5 days. Treatment with either AS-SRII-13+10 (Fig. 2) or AS-RSII-88-66 (data not shown) resulted in the loss of synapsin II staining in the majority (>80%) of the cells. Synapsin I and synaptophysin staining revealed that 15-DIV AS-RSII-treated cultures had a markedly reduced number of synapses (Fig. 2 and Table 1). Synapsin II-depleted 15-DIV hippocampal cultures exhibited a morphological appearance similar to that observed in 8-DIV AS-RSII-treated cultures, including the clustering of cell bodies and the fasciculation of axons and dendrites (Fig. 2). S-RSII-treated cultures showed no changes in either cell morphology or the number of synapses compared to untreated controls (Fig. 2 and Table 1).

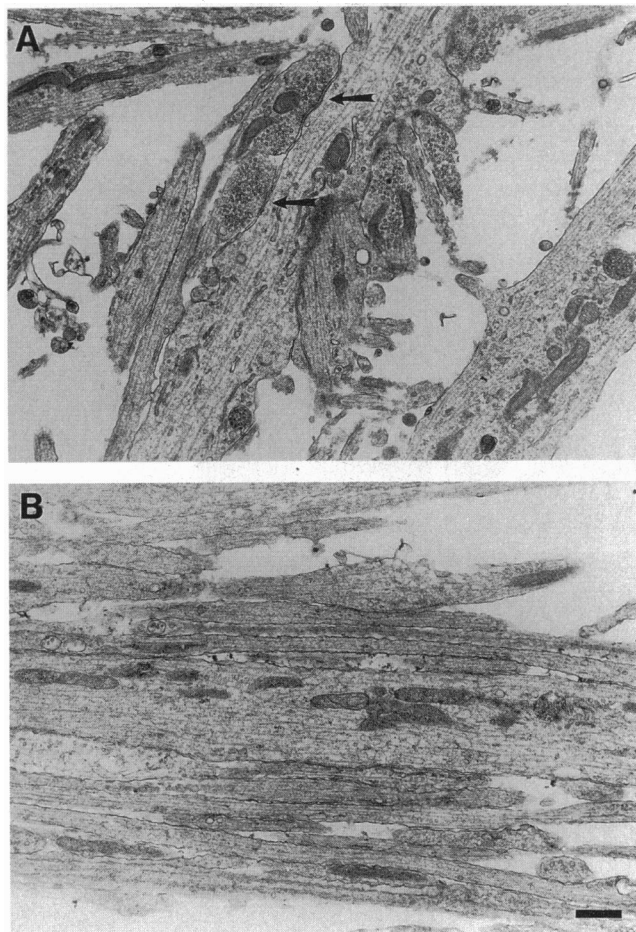


FIG. 3. Ultrastructural features of 15-DIV hippocampal cultures treated with sense or antisense synapsin II oligonucleotides. Representative electron micrographs were taken from 15-DIV cultures that had been treated for 5 days with S-RSII-13+10 (A) or AS-RSII-13+10 (B). Arrows in A point to synapses. Note the apparent lack of synaptic vesicle clusters and synaptic membrane specializations in B. (Bar = 500 nm.)

Electron microscopy was used to determine directly whether synaptic ultrastructural features were present after these various treatments of hippocampal cultures. In 8-DIV (data not shown) and 15-DIV (Fig. 3) S-SRII-treated cultures, numerous synapses were found with typical ultrastructural features (i.e., the clustering of synaptic vesicles in the vicinity of specialized synaptic membranes). In contrast, AS-SRII-treated cultures at 8 DIV (data not shown) and 15 DIV (Fig. 3) had very few ultrastructural synapses.

To analyze whether the morphological changes induced by AS-SRII treatment might have been due to the functional impairment of synapses, generation of action potentials in hippocampal neurons was blocked with tetrodotoxin. Treatment of the hippocampal cultures with tetrodotoxin at 1 μ M for 15 days failed to elicit any noticeable changes in cell morphology (data not shown).

Finally, we examined whether the removal of the synapsin II antisense oligonucleotides would reverse the altered cell morphology and whether synapses might reform. Cultures at 8 DIV and 15 DIV that had been treated with either AS-RSII-13+10 or AS-RSII-88-66 for 5 days were transferred to medium containing S-RSII or no oligonucleotides. Under these conditions, synapsin II immunoreactivity became detectable 2 days after the removal of the AS-SRII. After the reexpression of synapsin II, there was a progressive increase in the number of synapses as shown by the reappearance of an increasingly higher number of punctate immunoreactive spots staining for synapsin II, synaptophysin, and synapsin I (Fig. 4 and Table 1). Remarkably, the fasciculated neuritic bundles gradually spread into an elaborate network. Neurites in cultures maintained in the presence of AS-RSII remained fasciculated.

DISCUSSION

It was found (3) that the suppression of synapsin II by antisense oligonucleotides resulted in the inhibition of neu-

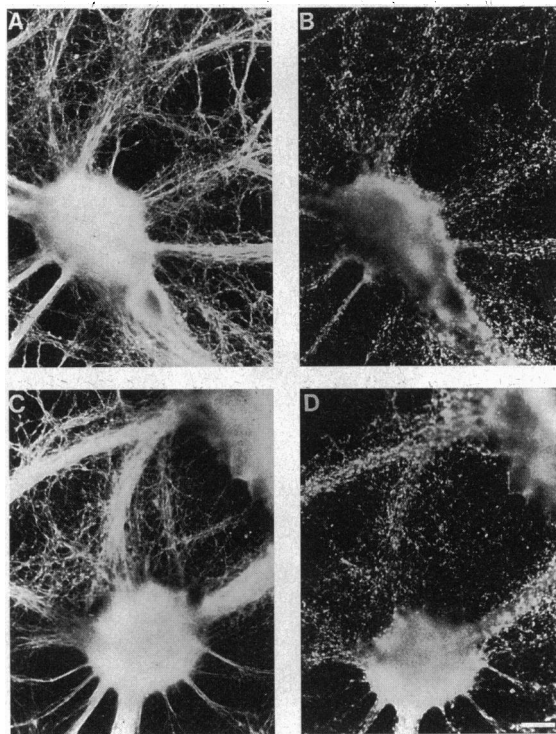


FIG. 4. Recovery from antisense oligonucleotide treatment. Cultures at 15 DIV that had been treated with AS-RSII-13+10 from days 10 to 15 were allowed to grow in the absence of oligonucleotides for 10 days. Cultures were then double-labeled with tubulin (A and C) and synapsin II (B) or synaptophysin (D) antibodies. The reexpression of synapsin II is accompanied by the formation of numerous synapses and the spreading of the processes. (Bar = 20 μ m.)

rite elongation in cultured hippocampal neurons. We report herein that the suppression of synapsin II by antisense oligonucleotides after axons have formed prevents synapse formation. We also provide evidence indicating that synapsin II is required for the maintenance of synapses in the central nervous system. Antisense oligonucleotide suppression of synapsin II resulted in the partial suppression of both synapsin I and synaptophysin. A similar treatment shortly after plating also caused the suppression of synaptotagmin (3). Each of the two nonoverlapping antisense oligonucleotides suppressed these other synaptic components. Sense oligonucleotides did not have this effect. There are several possible explanations for these results. One is that the synapse, including synaptic vesicles, relies on synapsin II for the integrity of the entire structure and without this molecule the other components of the synapse degrade. Another possibility is that synapsin II may have a regulator function in the expression of synaptic proteins. In support of the latter possibility, when synapsin II was overexpressed in neuroblastoma-glioma hybrid cells, the expression of synapsin I and synaptophysin was also induced (4). A third possibility is that the antisense oligonucleotides suppress, but are not specific for, synapsin II. However, if this were the case, it would not be expected that two different antisense oligonucleotides would give identical phenotypes and various control oligonucleotides would leave the cultures unaffected. Nevertheless, it is possible that oligonucleotides can work in unexpected ways, including an inhibitory action directly on proteins (20).

Data from two different knockout mice demonstrate that synapses can form in the absence of synapsin I (21, 22) but their formation in culture is delayed (22). It will be important to determine whether synapse formation is also delayed *in situ* in the synapsin I knockout mice. Although synapses are present in the adult mutant mice, their ultrastructure and function are impaired (23).

The suppression of synapsin II is accompanied by the fasciculation of axons and dendrites. The formation of a spread network of processes in culture could be considered the result of a balance between molecules that promote adhesion to the substratum and those that induce adhesion among neurites. Concomitant with the loss of synapsin II, axons and dendrites have reduced ability to attach tightly to the polylysine-coated surface, and under these conditions the neurites tangle together. The loss of adhesiveness to the surface may be due to a decrease of extracellular matrix proteins. Recently, it has been shown that synapsin transfection of neuroblastoma cells resulted in a 3-fold increase in laminin levels (9).

A remarkable finding of the present study was the ability of the neurons to recover after the removal of the antisense oligonucleotides. It has been the impression from many studies of cultured neurons that, once bundled, neurites do not reverse

to a more spread pattern. In the balance between neurite adhesion and repulsion, there may be additional molecules that can modulate the formation of neurite patterning. Molecules associated with synapse formation and possibly coregulated with synapsin II may enhance a more spread pattern to increase the opportunity for diverse fiber types to come into contact with each other.

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