8. Hyperpolarization activated an inward current (I_h) that produced a depolarizing "sag" in the membrane potential (Fig. 2A). I_h deactivated slowly on release from hyperpolarization, producing a 5- to 10-mV depolarization that was sufficient to activate sodium currents and trigger action potentials in tonically firing neurons or to activate calcium currents and trigger a rebound burst in burst-firing neurons (Fig. 1B).
9. Burst amplitudes were measured several minutes after termination of the stimulation in order to assess only stable changes in neuronal properties and not those produced by fast activation or inactivation properties of individual ionic currents.
10. Average V_m was −59 ± 2 mV before and after stimulation. Average R_{in} was 60 ± 7 megohms before and 60 ± 8 megohms after stimulation.
11. The same total hyperpolarizing current delivered dc rather than in pulses produced no stable change in burst properties (n = 4). Up to 6 hours of hyperpolarizing pulses delivered to neurons that fired tonically when depolarized produced little perceptible change in activity. Depolarizing current pulses produced mixed results, leading to a small decrease in burst amplitude in some cases and a small increase in others.
12. Rebound bursts were eliminated by blocking I_h with external Cs^+, which prevented rebound bursts but left depolarization-induced burst firing intact (n = 2), or by use of neurons that did not exhibit rebound bursts (n = 4).
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Aberrant Neurites and Synaptic Vesicle Protein Deficiency in Synapsin II–Depleted Neurons

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Synapsin I and synapsin II are neuron-specific phosphoproteins that have a role in the regulation of neurotransmitter release and in the formation of nerve terminals. After depletion of synapsin II by antisense oligonucleotides, rat hippocampal neurons in culture were unable to consolidate their minor processes and did not elongate axons. These aberrant morphological changes were accompanied by an abnormal distribution of intracellular filamentous actin (F-actin). In addition, synapsin II suppression resulted in a selective decrease in the amounts of several synaptic vesicle–associated proteins. These data suggest that synapsin II participates in cytoskeletal organization during the early stages of nerve cell development.

As hippocampal neurons grow in culture, the cytoskeleton undergoes a sequential reorganization that begins with the spreading of a lamellipodial veil around the periphery of the cell (stage I) (*1*). Shortly thereafter, the veil consolidates to form an array of discrete minor neurites (stage II). The transition from stage I to stage II is associated with the establishment of a microtubule-rich domain within the shafts of the neurites and a restriction of the flattened actin-rich portion of the veil to the tips of the neurites, the site of the growth cone. Selective elongation of one of those neurites to form the axon (stage III) involves lengthening of the shaft while the growth cone is maintained in a relatively constant organizational state. This sequence occurs within 24 hours of plating (*1*). In these neurons, synapsin I (*2*) and synapsin II (*3*) are expressed before the establishment of synaptic contacts. The synapsins are associated with the cytoplasmic surface of synaptic vesicles (*4*) and have been shown to interact with actin and other cytoskeletal elements in vitro (*4*–*6*). To examine the possibility that the synapsins might function in the early stages of neuronal development, we suppressed the expression of synapsin II in hippocampal neurons.

Synapsin II became readily detectable in the cell bodies of untreated hippocampal neurons 4 hours after plating, a time that coincides with the initial neurite outgrowth stage (stage II) (*3*). Twenty-four hours after plating, nearly all hippocampal neurons had extended several minor processes and a single axon (Fig. 1A), and strong synapsin II staining was present in the cell body (Fig. 1B). Similar results were observed for synapsin I at this early stage of neuronal development (*2*).

Two nonoverlapping antisense oligonucleotides corresponding to rat synapsin II complementary DNA (cDNA) sequences from positions −13 to +10 (AS-RSII −13+10) and from positions −88 to −66 (AS-RSII −88−66) (*7*) reduced the amounts of synapsins IIa and IIb, the two isoforms of synapsin II, by about 75% after 24 hours of incubation (Table 1). On the other hand, sense oligonucleotides corresponding to the same rat synapsin II cDNA sequences, S-RSII −13+10 (Table 1) and S-RSII −88−66, did not affect synapsin II amounts in comparison with untreated control cultures.

When cultures were treated with S-RSII −13+10 (Fig. 1, C and D) or S-RSII −88−66, the ability of hippocampal cells

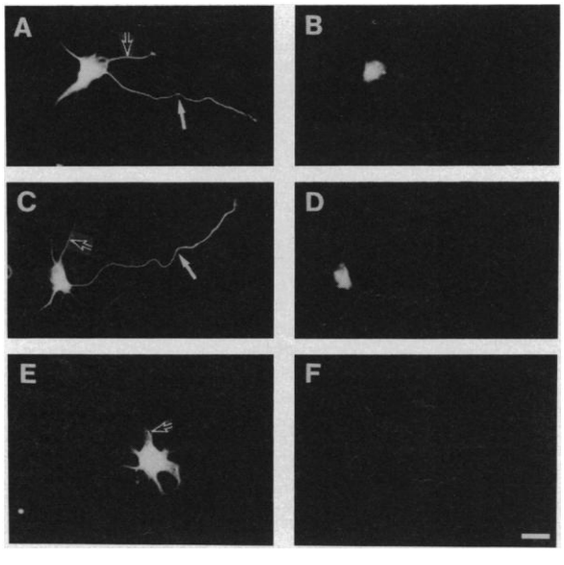

Fig. 1. Inhibition of neurite growth by synapsin II antisense oligonucleotides in cultured hippocampal neurons (*20, 21*). Control (**A** and **B**), sense-treated (**C** and **D**), and antisense-treated (**E** and **F**) hippocampal neurons were double-labeled with a monoclonal antibody against tubulin [(A), (C), and (E)] and an affinity-purified polyclonal antibody against synapsin II [(B), (D), and (F)]. No immunoreactivity for synapsin II was detected in the antisense-treated cell (F). Solid arrows, axons; outlined arrows, minor processes. Bar, 20 μm.

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to grow normal minor processes and axons and the pattern of synapsin II staining were not affected. In contrast, about 75% of hippocampal cells were found to be devoid of synapsin II immunoreactivity after 24 hours of treatment with AS-RSII −13+10 (Table 2) (Fig. 1F) or AS-RSII −88−66. Cells depleted of synapsin II showed a dramatic change in their morphology. About 20% of the synapsin II–depleted neurons failed to grow any processes and remained round (Table 2). About 80% of synapsin II–depleted neurons extended short processes with altered morphology but failed to elongate axons (Fig. 1E) (Table 2). These short processes were broad, flattened, and surrounded either totally or partially by lamellipodial veils. They were distinct from the normal minor processes, which were thin and cylindrical (compare Fig. 1E with Fig. 1, A and C). Decreasing the concentration of antisense oligonucleotides from 50 to 25 μM and 12.5 μM caused a progressive decrease in the effects observed.

Table 1. Synaptic vesicle–associated proteins and cytoskeletal proteins in sense- and antisense-treated hippocampal cultures. Embryonic day 18 hippocampal cultures were treated for 24 hours with S-RSII −13+10 (sense-treated) or AS-RSII −13+10 (antisense-treated). Similar results were obtained from cultures treated with S-RSII −88−66 or AS-RSII −88−66. Amounts of individual proteins in extracts of hippocampal neurons were determined by immunoblot analyses (*20, 23*). The values (mean ± SD) were based on the results of three or more experiments.

Protein	Protein amounts (percent of untreated control)	
	Sense-treated	Antisense-treated
Synapsin IIa	104 ± 6.1	26 ± 13*
Synapsin IIb	99 ± 8.2	25 ± 10*
Synapsin I	96 ± 5.0	49 ± 11*
Synaptotagmin	96 ± 16	51 ± 11*
Synaptophysin	94 ± 3.5	53 ± 11*
Syntaxin	104 ± 9.2	56 ± 6.1*
MAP-2	98 ± 9.6	98 ± 4.0
Tau	98 ± 15	95 ± 11
Tubulin	101 ± 12	96 ± 4.6
Actin	96 ± 5.6	99 ± 8.1

*$P < 0.001$.

In cultured neurons, outgrowth of neurites is also affected by down-regulation of the microtubule-associated proteins (MAPs) MAP-2, tau, and kinesin and of the synaptic plasma membrane protein SNAP-25 (*8–11*). However, the morphology of cells depleted of these other proteins differs markedly from that of the synapsin II–depleted neurons. Cells treated with MAP-2 antisense oligonucleotides fail to extend neurites (*8*). Tau-depleted cells elaborate normal minor processes but fail to elongate axons (*9*). Kinesin-depleted (*10*) and SNAP-25–depleted (*11*) neurons have shortened processes but maintain an asymmetric morphology that includes the elaboration of axons.

In vitro, synapsin I and synapsin II are able to bind to and bundle F-actin, as detected by light scattering and electron microscopy (*5*). Synapsin I is also able to nucleate and polymerize actin filaments (*12*). To evaluate the possibility that the effect of synapsin II depletion might be attributable in part to alterations in the actin cytoskeleton, we compared the pattern of F-actin distribution in synapsin II–depleted neurons with that in control neurons (Fig. 2). In untreated neurons or sense-treated neurons (Fig. 2B), actin filaments were concentrated in growth cones at the tips of the axons and minor processes. In contrast, in antisense-treated neurons F-actin staining was spread through the flattened lamellipodial veils (Fig. 2D). Depletion of synapsin II may lead to a failure of neurons to convert their actin organization from an isotropic network to actin bundles that are necessary to form the cortical rim along the shaft of minor neurites. Consistent with this possibility, at least some nonneuronal cells transfected with synapsin I or synapsin II show a remarkable reorganization of intracellular F-actin and a concomitant formation of highly elongated cellular processes (*13*). It is also possible that synapsin II–depleted neurons are less effective in bundling their microtubules, an organizational feature of the neurite shaft.

In NG108-15 cells, synapsin IIb overexpression was shown to increase the amounts of synaptic vesicle proteins (*14*). Therefore, using immunoblot and immunocytochemical analyses we examined various synaptic vesicle proteins in hippocampal neurons that had been treated with synapsin II antisense oligonucleotides. Concomitant with the suppression of synapsin II expression, 24 hours after incubation with either AS-RSII −13+10 (Table 1) or AS-RSII −88−66 there was a selective decrease in the amounts of the synaptic vesicle proteins synapsin I, synaptotagmin (*15*), and synaptophysin (*16*), as well as a decrease in the

Table 2. Quantitative analysis of cell morphology in sense- and antisense-treated hippocampal cultures. Embryonic day 18 hippocampal cultures were prepared as described (*20*). Twenty-four hours after treatment with S-RSII −13+10 (sense-treated) or AS-RSII −13+10 (antisense-treated), cells were fixed and double-stained for tubulin and synapsin II (*21*). Specific staining for synapsin II was determined with the nonspecific background fluorescence of glial cells as the reference point. Cells brighter than glial cells were scored as synapsin II (+), whereas cells having background fluorescence similar to that of glial cells were counted as synapsin II (−). In both control and sense-treated cultures, 98% of the total cells were synapsin II (+); 2% were synapsin II (−). In antisense-treated cultures, 75% of the total was synapsin II (−); 25% was synapsin II (+). A total of 500 cells from three experiments for each condition were counted.

Stage	Control (%)	Sense-treated (%)	Antisense-treated (%)	
			Synapsin II (−) cells (75% of total)	Synapsin II (+) cells (25% of total)
I	3	4	20	0
II	10	11	0*	5
III	87	85	0	95

*Eighty percent of the synapsin II (−) cells had an aberrant morphology resembling an incomplete transition to stage II, as exemplified by the cells shown in Fig. 1E and Fig. 2, C and D.

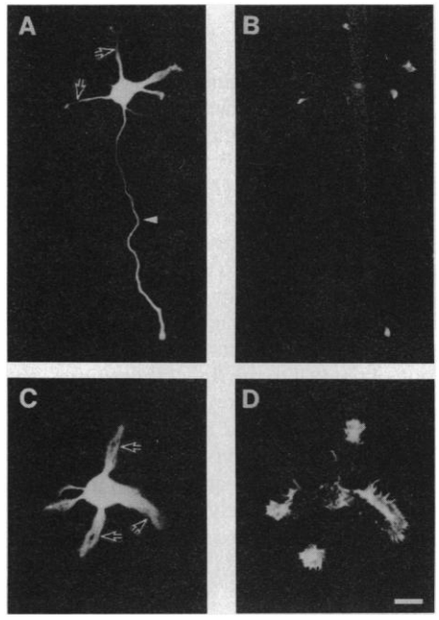

Fig. 2. Altered F-actin distribution in developing hippocampal neurons induced by synapsin II antisense oligonucleotides (*20, 22*). Sense-treated (**A** and **B**) and antisense-treated (**C** and **D**) cells were double-labeled with tubulin monoclonal antibody followed by fluorescein-labeled secondary antibody [(A) and (C)] and rhodamine-tagged phalloidin [(B) and (D)]. Arrowhead: axon; outlined arrows: minor processes. Bar, 20 μm.

amount of the presynaptic membrane protein syntaxin (*17*). In contrast, these synaptic proteins were not affected by treatment with sense oligonucleotides S-RSII −13+10 (Table 1) or S-RSII −88−66. To rule out the possibility of a nonspecific effect of antisense synapsin II oligonucleotides on protein synthesis, we examined several cytoskeletal proteins in control, sense-, and antisense-treated hippocampal cultures. No changes were observed in the amounts of MAP-2, tau, tubulin, or actin (Table 1). Consistent with the immunoblot analyses, immunofluorescence staining of synapsin II antisense-treated cells was much weaker for synapsin I and synaptotagmin but appeared equally strong for MAP-2 and tau, as compared to that of untreated or sense-treated cells.

The observations that antisense suppression of synapsin II expression disrupts early neuronal development and selectively reduces the amounts of synaptic vesicle proteins indicate that synapsin II is involved in the maturation of synaptic pathways before the formation of synaptic contacts. Complementing the present findings, other studies have demonstrated that introduction of synapsin I or synapsin II into neuronal cells accelerates the rate of functional and structural synaptogenesis (*14, 18*). It has been reported that mice without detectable synapsin I manifest no apparent gross changes in function or morphology of the adult nervous system (*19*). However, other proteins, by serving a compensatory role, may account for the absence of a markedly altered phenotype in the nervous systems of those mice.

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7. The sequences of the two nonoverlapping synapsin II antisense oligonucleotides used here are 5′-AGTTCATCATCTGGCTTGAGGGA-3′ (AS-RSII −13+10) and 5′-CGACCAAAGGTGGTCCGCGTCTC-3′ (AS-RSII −88−66). These sequences are based on rat synapsin II cDNA in the antisense orientation. Two antisense oligonucleotides corresponding to rat synapsin I cDNA sequences were ineffective in reducing synapsin I levels in hippocampal cultures. The numbers in the abbreviations correspond to the positions in the nucleotide sequence, with negative numbers referring to the 5′ untranslated regions.
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20. Sparse neuronal cultures were prepared from embryonic day 18 rat hippocampus as described (*1, 10*). Sense or antisense oligonucleotides were added at a final concentration of 50 μM at 0 and 12 hours after plating.
21. Twenty-four hours after plating, control, sense-, and antisense-treated cultures were fixed in warm 4% paraformaldehyde in 0.12 M sucrose and phosphate-buffered saline (PBS; pH 7.4) for 20 min. After fixation, the cultures were rinsed in PBS and blocked in 10% bovine serum albumin in PBS for 30 min. After incubation in 0.3% Triton X-100 in PBS for 5 min to permeabilize the cell membrane, the cultures were incubated in primary antibody solution containing a mixture of monoclonal antibodies to tubulin (clone DMIA) and affinity-purified polyclonal antibodies to synapsin II (G316) overnight at 4°C. The cultures were rinsed in PBS and incubated in secondary antibody solution containing fluorescein-labeled goat antibody to mouse immunoglobulin G (IgG) and rhodamine-conjugated goat antibody to rabbit IgG for 1 hour at 37°C. The cultures were rinsed thoroughly in PBS and examined under a Zeiss fluorescence microscope.
22. Twenty-four hours after plating, control, sense-, and antisense-treated cultures were fixed. After permeabilization of the cell membrane, the cultures were stained for tubulin with monoclonal antibodies to tubulin (clone DMIA) followed by fluorescein-labeled secondary antibody. To visualize F-actin, rhodamine-labeled phalloidin (Molecular Probes) was included in the secondary antibody solution.
23. After incubation for 24 hours in the absence or presence of synapsin II sense or antisense oligonucleotides, whole-cell extracts were prepared from the cultures. After determining the total protein concentration of the extracts, we separated equal amounts of protein by SDS–polyacrylamide gel electrophoresis. The proteins were transferred onto nitrocellulose membranes that were then probed with affinity-purified primary antibodies, followed by probing with ^{125}I-labeled secondary antibodies (Amersham). The immunoblots were then analyzed with a phosphorimager equipped with quantitation software (Molecular Dynamics).

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