Suppression of Kinesin Expression in Cultured Hippocampal Neurons Using Antisense Oligonucleotides

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Abstract. Kinesin, a microtubule-based force-generating molecule, is thought to translocate organelles along microtubules. To examine the function of kinesin in neurons, we sought to suppress kinesin heavy chain (KHC) expression in cultured hippocampal neurons using antisense oligonucleotides and study the phenotype of these KHC "null" cells. Two different antisense oligonucleotides complementary to the KHC sequence reduced the protein levels of the heavy chain by greater than 95% within 24 h after application and produced identical phenotypes. After inhibition of KHC expression for 24 or 48 h, neurons extended an array of neurites often with one neurite longer than the others; however, the length of all these neurites was significantly reduced. Inhibition of KHC expression also altered the distribution of GAP-43 and synapsin I, two proteins thought to be transported in association with membranous organelles. These proteins, which are normally localized at the tips of growing neurites, were confined to the cell body in antisense-treated cells. Treatment of the cells with the corresponding sense oligonucleotides affected neither the distribution of GAP-43 and synapsin I, nor the length of neurites. A full recovery of neurite length occurred after removal of the antisense oligonucleotides from the medium. These data indicate that KHC plays a role in the anterograde translocation of vesicles containing GAP-43 and synapsin I. A deficiency in vesicle delivery may also explain the inhibition of neurite outgrowth. Despite the inhibition of KHC and the failure of GAP-43 and synapsin I to move out of the cell body, hippocampal neurons can extend processes and acquire an asymmetric morphology.

KINESIN, a microtubule-based motor protein, is a heterotetramer consisting of two 110–130 kD heavy chains and two 60–70 kD light chains (Bloom et al., 1988; Kuznetsov et al., 1988). The primary structure of the heavy chain is conserved in four widely divergent species: *Drosophila* (Yang et al., 1989), squid (Kosik et al., 1990), sea urchin (Wright et al., 1991), and human (Navone et al., in press). Purified kinesin generates ATP-dependent microtubule gliding after being adsorbed onto glass coverslips (Vale et al., 1985*a*; Scholey et al., 1985; Porter et al., 1987) or induces the transport of latex beads along microtubules after adsorption onto the bead surface (Vale et al., 1985*a*). In experiments with purified bovine chromaffin granules, the addition of kinesin and ATP was sufficient to induce translocation of the granules along microtubules (Urrutia et al., 1991). Kinesin-coated objects move unidirectionally towards the plus end of microtubules (Vale et al., 1985*b*; Porter et al., 1987). This direction corresponds to anterograde transport in neurons, since axonal microtubules have their plus ends oriented distally (Filliatreau and DiGiamberdino, 1981; Heidemann et al., 1981; Baas et al., 1988).

Several experiments also support the idea that kinesin transports organelles in vivo. Microinjection of antibodies to kinesin heavy chain (KHC)[1] inhibit tubular lysosome extension in macrophages (Hollenbeck and Swanson, 1990), pigment granule dispersion in melanophores (Rodionov et al., 1991) and organelle transport in extruded axoplasm (Brady et al., 1990). An association of kinesin with membrane-bound organelles was suggested by the immunofluorescence localization of KHC to punctate and tubular Triton X-100 soluble structures (Pfister et al., 1989; Hollenbeck, 1989; Brady et al., 1990; Wright et al., 1991; Houliston and Elinson, 1991). Higher resolution analysis by immunogold EM has shown that KHC is associated with membrane-bounded organelles (Hirokawa et al., 1991). Furthermore, after ligation of a peripheral nerve, organelles that react with KHC antibodies collect on the proximal side of the ligature, implying that kinesin is associated with anterogradely moving organelles (Hirokawa et al., 1991).

Some clues concerning the in vivo functions of kinesin

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1. *Abbreviations used in this paper*: KHC, kinesin heavy chain.

were obtained by examining the phenotype of KHC mutants. A *Drosophila* containing temperature-sensitive KHC mutant had impaired muscle activity and tactile sensitivity, most pronounced in the posterior region (Saxton et al., 1991). Mutations of the *unc*-104 locus in *C. elegans*, which encodes a kinesin-like molecule (Otsuka et al., 1991), also cause deficits in neuromuscular transmission, which appear to be secondary to a deficit in anterograde transport. These mutants exhibit a severe depletion of synaptic vesicles in presynaptic terminals and a corresponding accumulation of vesicles in nerve cell bodies (Hall and Hedgecock, 1991). Despite this defect, neurites are able to grow out and establish appropriate synaptic connections.

In this study, we examined the cellular functions of kinesin in mammalian neurons. To approach this problem, we created a pseudo-genetic null mutation of KHC in cultured rat hippocampal neurons by administering antisense oligonucleotides to suppress KHC expression. Cells treated with the KHC antisense oligonucleotides extended neurites which elongated and expressed polarity, but the neurites were significantly shorter than in untreated cells. In addition, neurons treated with KHC antisense oligonucleotides did not transport GAP-43 or synapsin I to the neurite tip, suggesting that kinesin is required for the normal anterograde transport of these molecules.

Materials and Methods

Cultures

Neuronal cultures were prepared from the hippocampi of embryonic day 18 rats as previously described (Bartlett and Banker, 1984; Goslin and Banker, 1991). In brief, cells from the dissected hippocampi were dissociated by trypsinization (0.25% for 15 min at 37°C) followed by trituration with a fire polished Pasteur pipette. For immunofluorescence, the cells were plated at a density of 100,000 cells per 60-mm petri dish on glass coverslips coated with poly-L-lysine (1 mg/ml) in MEM with 10% horse serum. In antisense experiments, cells were plated onto 12-mm-diameter coverslips that were placed with the cells facing up in 35-mm dishes with 1 ml of medium. After 2–4 h the medium was changed to MEM with N2 supplements (Bottenstein and Sato, 1979), ovalbumin (0.1%), and pyruvate (0.01 mg/ml) that had been conditioned in cultures of astroglial cells for 24 h. Long-term cultures used in immunolocalization studies were co-cultured with astroglia.

Antisense Oligonucleotides

The initial experiments were performed with oligonucleotides (−11+14 hkin) based upon the sequence of the human KHC (Navone et al., In press). Because the neuronal cultures were taken from rat brain, these experiments were subsequently repeated using oligonucleotides corresponding to the rat KHC sequence. The rat sequence in the region of the initiator codon AUG was obtained by using nondegenerate, 18-mer human primers and performing polymerase chain reaction (PCR) on reverse transcribed rat brain mRNA. An amplified band of the expected size was obtained and the DNA was cloned and sequenced. Key experiments were repeated with rat sense and antisense oligonucleotides.

Antisense oligonucleotide −11+14 hkin, consisting of the sequence GCCAGGTCCGCCATCTTTCTCGCAG, is the inverse complement of the human nucleotides −11 to +14. Antisense oligonucleotide −11+14 rkin, consisting of the sequence GCCGGGTCCGCCATCTTTCTGGCAG, is the inverse complement of the rat nucleotides −11 to +14. Antisense oligonucleotide −35−12 rkin, consisting of the sequence CCGGGACTGCAGGCCGGGAGCCAC, is the inverse complement of the rat nucleotides −35 to −12. Nucleotides −11+14 hkin and −11+14 rkin, which identically straddle the initiator AUG of the KHC, differ by only two nucleotides. Oligonucleotide −35−12 rkin is a nonoverlapping oligonucleotide located entirely entirely within the 5′ untranslated region of rat KHC. The antisense oligonucleotide sequences did not have significant homology with any other sequence in the database (Sequence Analysis Software Package, version 6.1; Devereux et al., 1984). The oligonucleotides were synthesized on an Applied Biosystems 380B synthesizer (Applied Biosystems, Inc., Foster City, CA), purified over a NAP5 column (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ), ethanol precipitated, and taken up in media.

The administration of the oligonucleotide was as follows: At 2–4 h after plating, when the cells were transferred to serum-free glial-conditioned medium, the oligonucleotide was added at 50 μM. The medium was supplemented with 25 μM additional oligonucleotide every 12 h from the time of plating for either 24 or 48 h. Controls were treated with the same concentration and dosage schedule of the corresponding sense strand oligonucleotide. In dose-response experiments additional antisense oligonucleotides were added every 12 h at half the initial dose.

Immunodetection

For immunostaining, the cultures were fixed for 20 min with warm 4% formaldehyde in PBS containing 0.12 M sucrose, permeabilized in 0.3% Triton X-100 for 5 min at room temperature, and rinsed in PBS. Cultures were incubated with 10% BSA for 1 h at 37°C, then exposed to primary antibody (diluted in 1% BSA/PBS) overnight at 4°C.

The following primary antibodies with their dilutions were used for this study. For kinesin staining, SUK4 (1:10), a mAb raised against the sea urchin KHC (Ingold et al., 1988), and a polyclonal antiserum raised against the squid KHC (1:500), provided by Michael Sheetz (Duke University, Durham, NC) were used. An affinity purification of the immunoglobulin fraction was prepared from the polyclonal antiserum. Both of these antibodies crossreact with mammalian brain KHC on Western blots (data not shown). Also used were a mAb raised against the heavy chain of chick brain cytoplasmic dynein taken from a mouse hybridoma tissue culture supernatant (1:2) provided by Michael Sheetz (Steuer et al., 1990); mAb DM1B (1:4,000) (Boehringer Mannheim Biochemicals, Indianapolis, IN) raised against β-tubulin; a rabbit polyclonal antibody against α- and β-tubulin (1:1,000) from Sigma Chemical Co., St. Louis, MO), an affinity-purified rabbit polyclonal antibody against synapsin I (1:250) provided by Pietro de Camilli (de Camilli et al., 1983); mAb 9-1E12(1:2,000), which reacts monospecifically with GAP-43 on immunoblots of cultured hippocampal neurons, provided by D. Schreyer and J. H. P. Skene (Goslin and Banker, 1990); and a rabbit polyclonal antibody (1:1,000–1:10,000) raised against a fusion protein in which the amino terminal 16 amino acids of rat GAP-43 were replaced by 13 amino acids from the λ gt7 capsid protein, provided by Mark Willard (Washington University, St. Louis, MO).

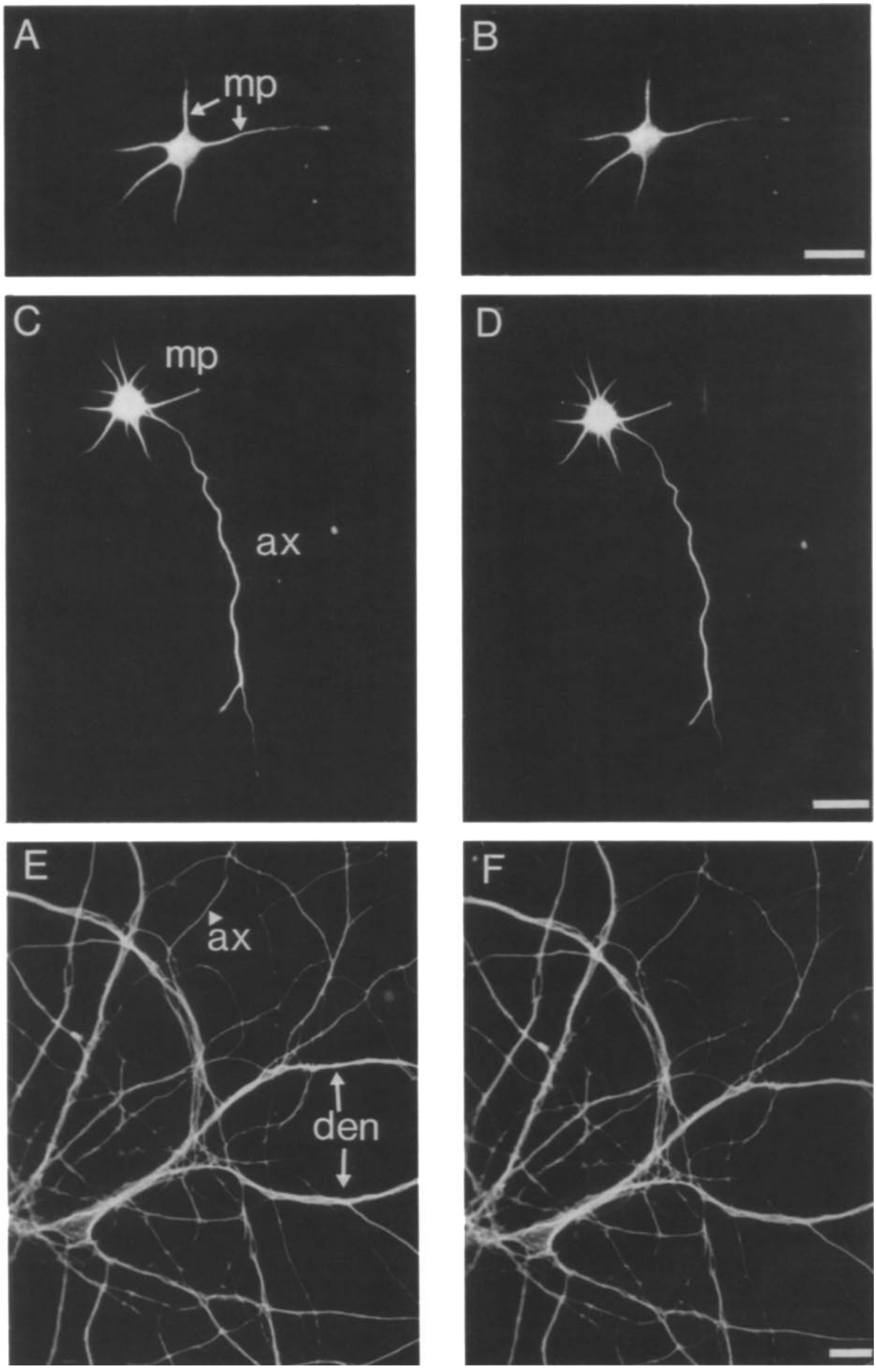
The following secondary antibodies were used. Rabbit anti-mouse IgG labeled with FITC (1:200) and goat anti-rabbit IgG labeled with rhodamine (1:200), both from Boehringer Mannheim Biochemicals. For synapsin I staining biotinylated goat anti-rabbit IgG (1:1,000) and avidin conjugated to fluorescein (1:500) (Vector Laboratories, Norwalk, CT) were used. Incubations in these antibodies were for 1 h at 37°C.

Dot immunobinding was performed with ^{125}I-labeled protein A as originally described by Jahn et al. (1984) and modified by Ferreira et al. (1989). The KHC polyclonal antibody was used because of the avidity of rabbit IgG for protein A. The cultures were rinsed twice in warmed PBS, scraped into Laemmli buffer (Laemmli, 1970), homogenized in a boiling water bath for 5 min, and centrifuged at 33,000 rpm. The supernatant was removed and applied to nitrocellulose paper (Ferreria et al., 1989).

Morphometric Analysis

Fixed cells were viewed from the inverted microscope by phase microscopy using a video camera and images were recorded on an optical memory disk recorder. Cells were selected at random, traced from the screen, and the lengths of their processes measured using a digitizing tablet.

Figure 1. Hippocampal neurons double labeled with polyclonal tubulin antibody (*A*, *C*, and *E*) and the kinesin mAb SUK4 (*B*, *D*, and *F*). Stage II neurons are shown in *A* and *B* with their minor processes (*mp*) indicated. Stage III neurons are shown in *C* and *D* with the axon (*ax*) indicated. Neurons at stage IV, when dendrites (*den*) become apparent, are shown in *E* and *F*. Bars, 20 μm.


A
mp
B
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Results

Immunolocalization of KHC in Cultured Rat Hippocampal Cells

Cultured rat hippocampal neurons from E18 rat brain represent a relatively homogeneous population of neurons in which pyramidal cells predominate. After plating, hippocampal neurons undergo predictable developmental changes (Dotti et al., 1988). At stage I, during the first few hours after plating, the neurons appear rounded. Stage II (Fig. 1) begins ~8 h after plating, when the neurons elaborate a symmetric array of neurites. After ~24 h in culture, one of the processes rapidly elongates (stage III); this process is the axon. During stage IV (72–96 h in culture), the remaining neurites differentiate into dendrites. Kinesin reactivity was observed at all of these stages (Fig. 1). It appeared to be homogeneously distributed, as was tubulin. Kinesin immunoreactivity was present in cell bodies and in both axonal and dendritic processes.

Antisense Oligonucleotide Affect KHC Expression

The oligonucleotides (antisense and sense) were tested for their ability to inhibit KHC expression. The sequence sites selected for the design of the oligonucleotides were centered on the initiator AUG and a nonoverlapping site located immediately upstream in the 5′ untranslated region. Cells incubated with each of the antisense oligonucleotides described in Materials and Methods showed markedly reduced reactivity to a KHC mAb (SUK4), as assessed by immunofluorescence at 24 or 48 h after plating (Fig. 2). Neurons incubated with the sense oligonucleotides, on the other hand, appeared unaffected and showed a normal degree of KHC immunoreactivity by immunofluorescence.

To verify that KHC was depleted from cells after antisense treatment, the more sensitive technique of dot immunobinding was used for detection (Fig. 3). KHC was not detectable above the background level of counts in cells grown in the presence of the antisense oligonucleotide −11+14 hkin by the dot immunobinding assay. In contrast, cells treated with sense oligonucleotides were comparable in their immunoreactivity to untreated control cells. Exposure to the antisense oligonucleotides did not affect tubulin and dynein immunoreactivity by the same assay (Fig. 3). The presence of normal levels of tubulin and dynein in the kinesin-suppressed cells suggests that the effect of the antisense oligonucleotide was specific and that the regulation of the expression of the retrograde motor, dynein, was independent of kinesin.

Effects of Antisense Oligonucleotides on Neurite Extension

Neurite extension was measured after either a 24- or 48-h exposure to the KHC oligonucleotides. Neurite elongation during the second 24-h period is considerably more affected by the antisense oligonucleotide treatment than during the first 24-h period (Fig. 4). At both time points nearly all of the neurons had two populations of neurites consisting of several short minor neurites and a single long neurite considered the axon. When the cells were exposed to the antisense oligonucleotide −11+14 hkin and fixed 24 h after plating, there was a 25% decrease in the lengths of the minor neurites and a 34% decrease in the length of the presumptive axon. The mean length of the presumptive axon was 74.5 ± 2.1 μm (compared with 111.7 ± 5.6 μm in sense-treated controls) and the summed length of the minor neurites was 79.6 ± 4.1 μm (compared with 105.2 ± 5.5 μm in sense-treated controls). Neurite growth of neurons exposed to KHC antisense oligonucleotides for 48 h is quantitated in Table I. Neurons treated with kinesin antisense oligonucleotides (50 μM) for 48 h exhibited a 50% decrease in both the lengths of the minor processes and the axon-like processes. The human antisense oligonucleotide was slightly less effective than the rat antisense oligonucleotide, but the difference was not statistically significant. Therefore, the imperfect match of the human antisense oligonucleotide, which differed in two nucleotides, did not significantly affect KHC suppression. On average the antisense-treated neurons had one less minor neurite than the sense-treated controls (Table I).

The KHC antisense oligonucleotides affected neurite outgrowth in a dose-dependent manner (Fig. 5). Increasing the concentration of the antisense oligonucleotide from 12.5 to 50 μM caused a progressive decrease in neurite outgrowth. At 12.5 μM the total neurite length was reduced 19% and at 50 μM it was reduced 50%.

The KHC antisense oligonucleotides, while suppressing KHC expression, did not irreversibly damage the neurons (Fig. 6). When, after 48 h, the cells were released from antisense inhibition by changing the medium, neurite extension resumed at a rate that paralleled that observed under control conditions.

Distribution of GAP-43 and Synapsin in Antisense-treated Neurons

Since kinesin has been postulated to power anterograde axonal transport, we examined the effect of KHC antisense oligonucleotides on the immunocytochemical distribution of GAP-43 and synapsin, molecules that are transported to nerve termini by fast axonal transport. Under control conditions, GAP-43 was present at the tips of neurites and within the cell bodies of neurons after 48 h in culture (Fig. 7). Very little staining was detectable along the shafts of neurites. This pattern was similar to that described in Fig. 2 *H* of Goslin and Banker (1990). Likewise, synapsin I, under control conditions, was concentrated in the distal portion of the axon (Fig. 8), as described previously in hippocampal neurons (Fletcher et al., 1991). Following exposure to KHC antisense oligonucleotides, the GAP-43 and synapsin I immunoreactivities were largely restricted to the cell body; the neurites were essentially devoid of immunostaining (Fig. 7 and 8).

Discussion

Phenotype of Hippocampal Neurons After Kinesin Suppression

A powerful approach for understanding a protein's cellular function is to study the phenotype of a cell or organism deficient in that protein. Targeted gene deletion is the most widely used strategy for achieving these goals; however, such experiments are not readily possible in postmitotic neurons. Messenger RNA inactivation, either by expression of antisense RNAs or exposure to antisense oligonucleotides,

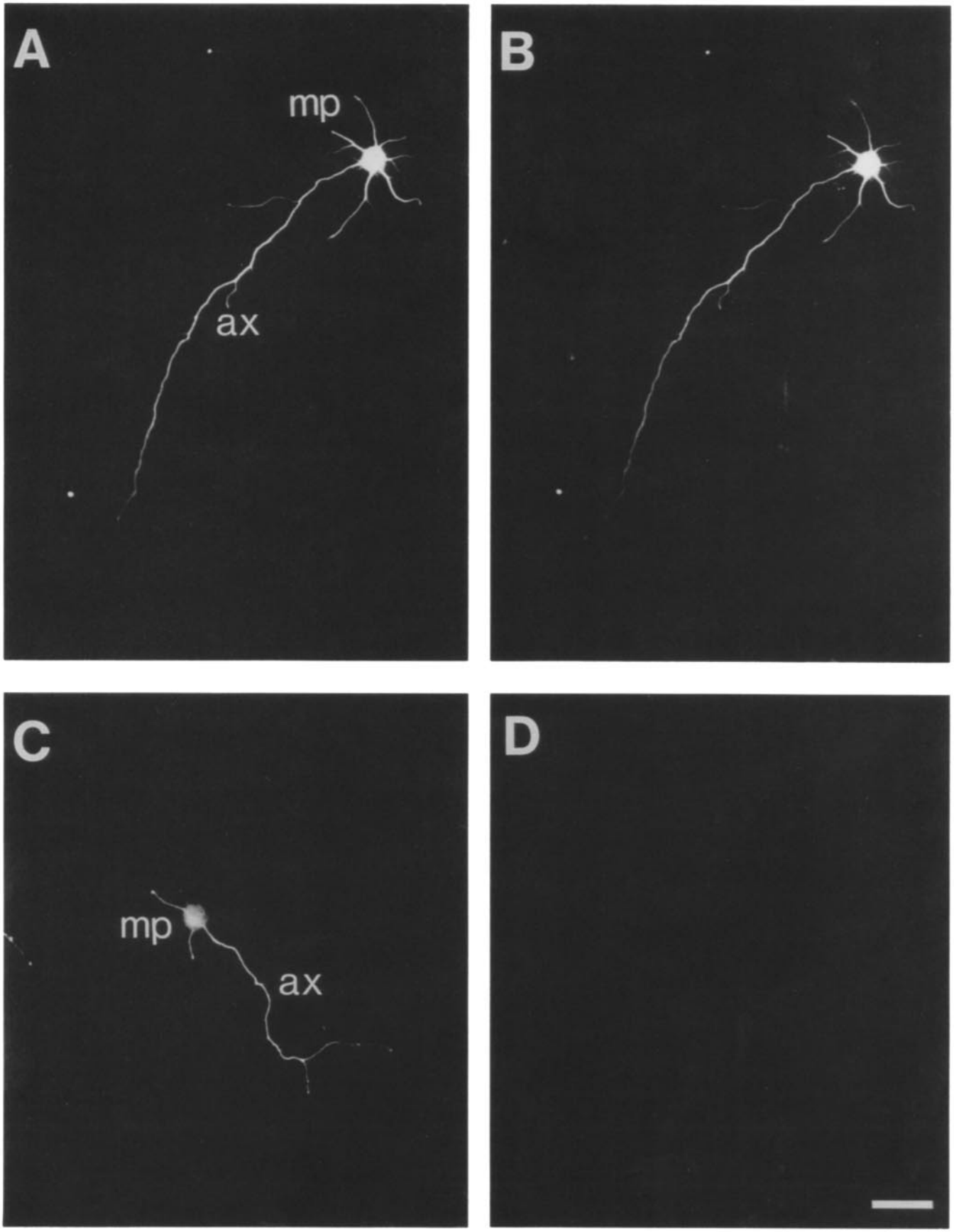

Figure 2. Hippocampal neurons fixed after 48 h in culture under sense (*A* and *B*) and antisense (*C* and *D*) conditions. The cells were double labeled with antibodies to tubulin (*A* and *C*) and kinesin SUK4 (*B* and *D*). The oligonucleotides used in this experiment were hkin −11+14 (sense) and −11+14 hkin (antisense). Identical results were observed with the other oligonucleotides. *ax*, axon; *mp*, minor process. Bar, 20 μm.

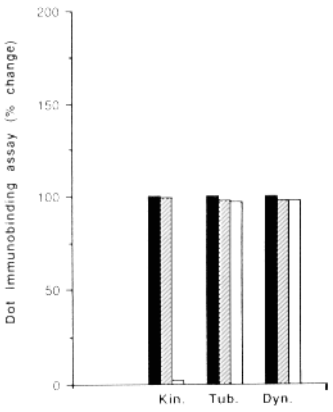

Figure 3. Dot immunobinding to quantitate rabbit kinesin (*Kin.*), tubulin (*Tub.*) (DM1B), and dynein (*Dyn.*) under sense and antisense conditions. The kinesin antibody used was the rabbit polyclonal and the tubulin antibody was DM1B. The control (■) refers to untreated cultures and the value in cpms for the control was considered 100%. The other values are expressed as a percentage of the control. ▨, sense kinesin; □, antisense kinesin.

provides an alternative means of reducing cellular concentrations of a specific protein. In this study, we have shown that exposure to antisense oligonucleotides causes a marked reduction in the levels of the KHC protein in rat hippocampal neurons. After a 24-h exposure to antisense, but not to sense oligonucleotides, the levels of KHC protein are not detectable above background by dot-immunobinding. Thus, antisense oligonucleotide treatment provides a means of creating a "null" kinesin phenotype in a mammalian neuronal cell.

The homogeneous pattern of KHC labeling in the cultured neurons is consistent with the reported large soluble pool (Hollenbeck, 1989). However other investigators have observed a punctate pattern consistent with localization to membranous organelles (Pfister et al., 1989; Wright et al., 1991). It seems likely that the punctate pattern results from some degree of extraction of KHC with the fixatives used. Brady (1991) has raised a number of plausible arguments that favor a predominant membrane localization for KHC, and has reconciled the solubility of the molecule by proposing an easily extractable relationship to membranes. The issue, however, remains problematic because recent data using extruded squid axoplasm suggests a tight association of plus-end motors with organelles that does not exchange with the soluble pool (Schnapp, B. J., T. S. Reese, and R. B. Bechtold. 1991. *J. Cell. Biol.* 115:40 Abstr.)

Table I. Effect of Kinesin Antisense Oligonucleotides (50 μM) on Neuritic Growth after 2 d in Culture

Treatment	Length			Number of Minor Processes
	Total	Axonal	Minor Processes	
	(μm)	(μm)	(μm)	
None	444 ± 16	247 ± 9	37 ± 2	4.8 ± 0.2
Sense HK11-14	420 ± 12	216 ± 11	32 ± 1	4.8 ± 0.3
Antisense HK11-14	215 ± 9	126 ± 6	20 ± 1	3.8 ± 0.3
Sense RK11-14	390 ± 12	228 ± 11	31 ± 1	4.8 ± 0.4
Antisense RK11-14	171 ± 7	116 ± 5	16 ± 1	3.3 ± 0.1
Sense RK35-12	420 ± 8	254 ± 10	29 ± 2	4.6 ± 0.2
Antisense RK35-12	180 ± 9	114 ± 7	17 ± 0.7	3.5 ± 0.6

Neurite length was determined in 100 cells for each experimental condition. Each value represents the mean ± SEM. For all of the length measurements comparisons with the controls were significant with $P < 0.005$. For the minor processes the comparison of the sense and antisense oligonucleotides HK 11-14 and RK 11-14 had a significance of $P < 0.01$, and for RK 35-12 a significance of $P < 0.05$.

Several results indicate that the phenotypic changes in hippocampal cells observed after KHC antisense oligonucleotide treatment can most likely be attributed to the reduced levels of KHC. First, the antisense oligonucleotides do not cause irreversible damage to the cell; neurons resume neurite outgrowth at normal rates following a change to medium free of the antisense oligonucleotide. Second, two different antisense oligonucleotides complementary to two different regions of the KHC mRNA produce identical morphological effects on hippocampal neurons. The finding reduces the likelihood that the observed cellular effects are because of the oligonucleotides hybridizing to an mRNA other than the KHC mRNA. Among the kinesin-like molecules sequenced to date, the most significant region of homology lies in the

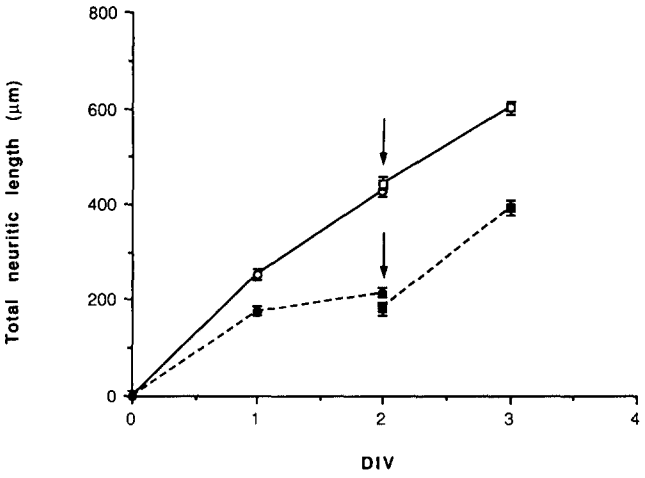

Figure 4. Total neurite length measured as a function of time in culture. The dotted line represents the antisense oligonucleotide-treated cells and the solid line represents the sense oligonucleotide-treated cells. Measurements were taken after 1 d of treatment and after 2 d of treatment. At times beyond the arrow, the medium was changed to a nucleotide-free medium. Continued neurite extension paralleled the control values.

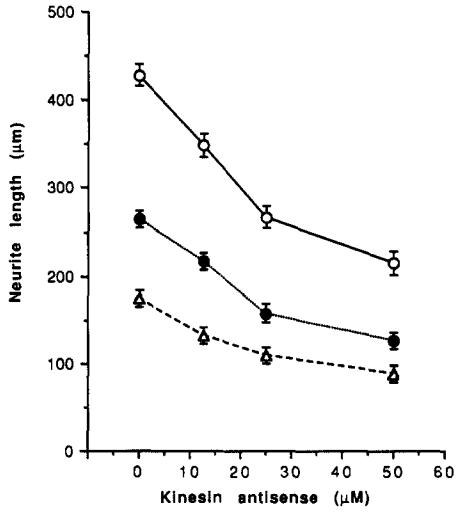

Figure 5. A dose-response curve illustrating neurite length as a function of the dose of kinesin antisense oligonucleotide. These cultures were analyzed at 48 h after plating. (—○—) Total neuritic length; (···●···) axonal length; (---△---) minor processes length.

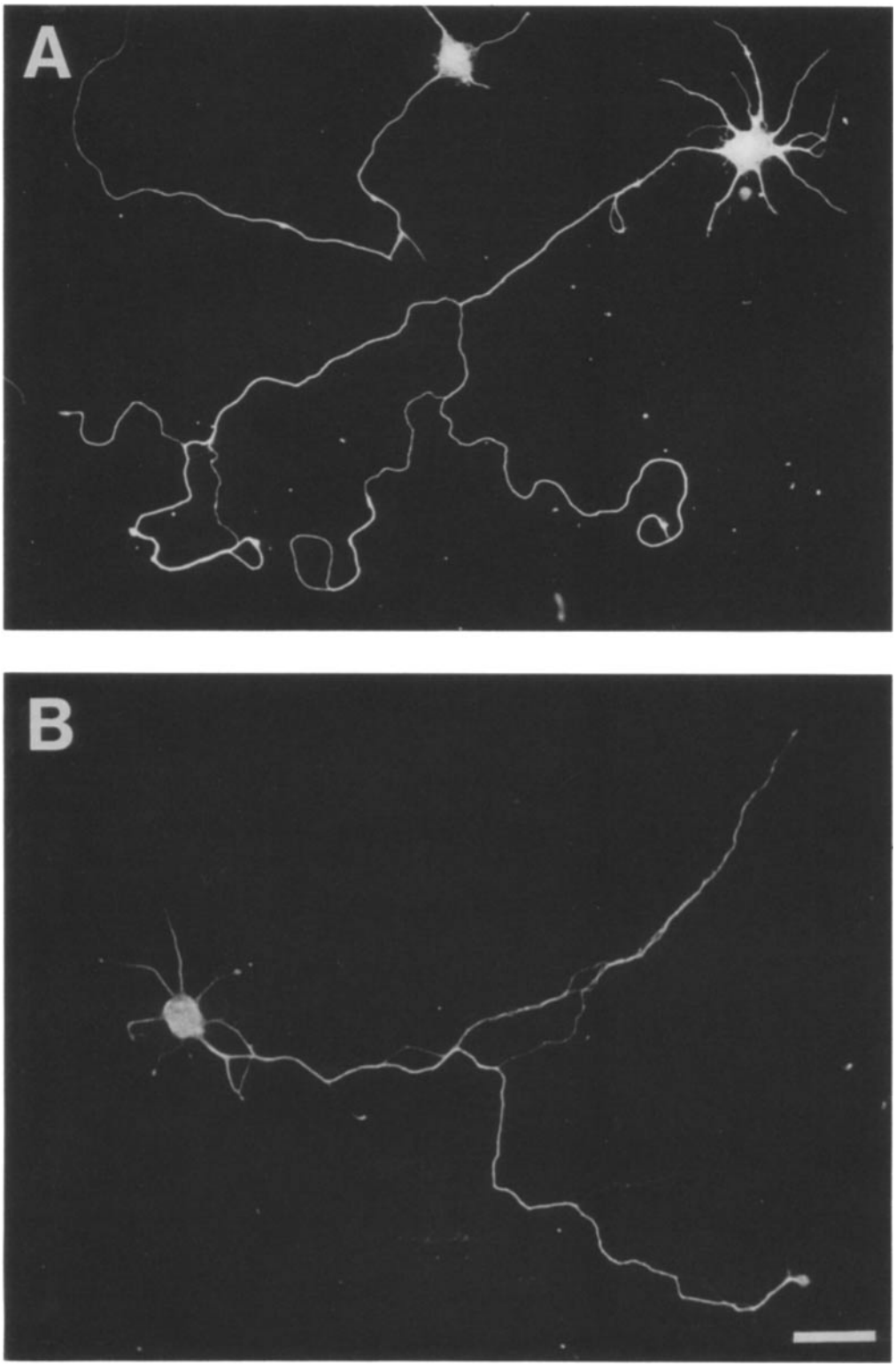

Figure 6. Recovery from antisense oligonucleotide treatment. The neurons in *A* were treated with kinesin sense oligonucleotides and in *B* with kinesin antisense oligonucleotides for 48 h. Then medium was changed and oligonucleotides were excluded. At 72 h the cells were fixed and stained with kinesin antibody (SUK4). Although the growth of the cell in *B* appears to lag behind the cells in *A*, recovery of kinesin immunoreactivity and morphological development is evident. Bar, 20 μm.

ATP- and microtubule-binding sites, not the region at the 5′ end from where we derived our antisense oligonucleotides. Third, tubulin and dynein protein levels remain normal suggesting that there is not a general reduction in protein synthetic activity.

The phenotype of KHC suppressed hippocampal neurons offers some insight into the normal physiological roles for kinesin in these cells. One striking finding is the altered distribution of synapsin I and GAP-43 in cells treated with KHC antisense oligonucleotides. In untreated cells or cells treated with sense oligonucleotides, synapsin I and GAP-43 are found in the neurites and are particularly concentrated in the termini. In antisense-treated cells, on the other hand, both proteins are confined to the cell bodies. Synapsin I and GAP-43 are both membrane-associated proteins (De Camilli et al., 1983; Valtorta et al., 1988; Gispen et al., 1985) and are

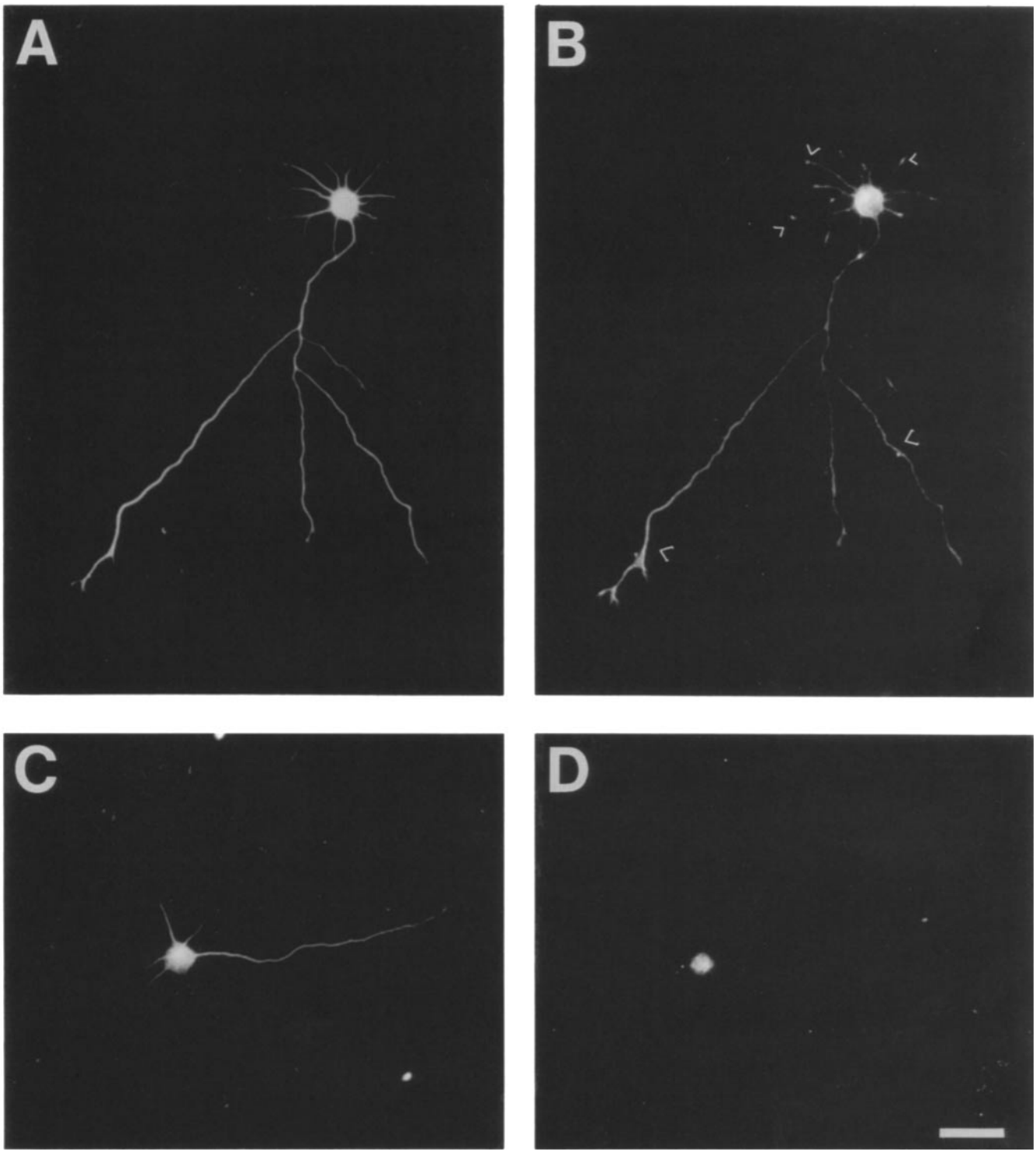

Figure 7. GAP-43 staining. *A* and *B* are kinesin sense oligonucleotide treated. *C* and *D* are kinesin antisense oligonucleotide treated. *A* and *C* are stained with the tubulin antibody and *B* and *D* are stained with the GAP-43 antibody (9-IE12). Bar, 20 μm.

delivered to neuronal termini by fast axonal transport (Baitinger and Willard, 1987; Skene and Willard, 1981; Benowitz et al., 1981). Microtubules in both minor neurites and axons are aligned with their plus ends distal to the cell body (Baas et al., 1989), so that translocation of organelles to the neurite terminus of both types of processes requires a plus-end-directed motor. The impaired delivery of synapsin I and GAP-43 to the neurite terminus under antisense conditions suggests that kinesin may serve as the plus-end motor involved in the anterograde transport of vesicles containing these proteins. This conclusion is consistent with recent findings that KHC is associated with vesicles traveling by anterograde transport that accumulate at the proximal side of a nerve ligation (Hirokawa et al., 1991). Alterna-

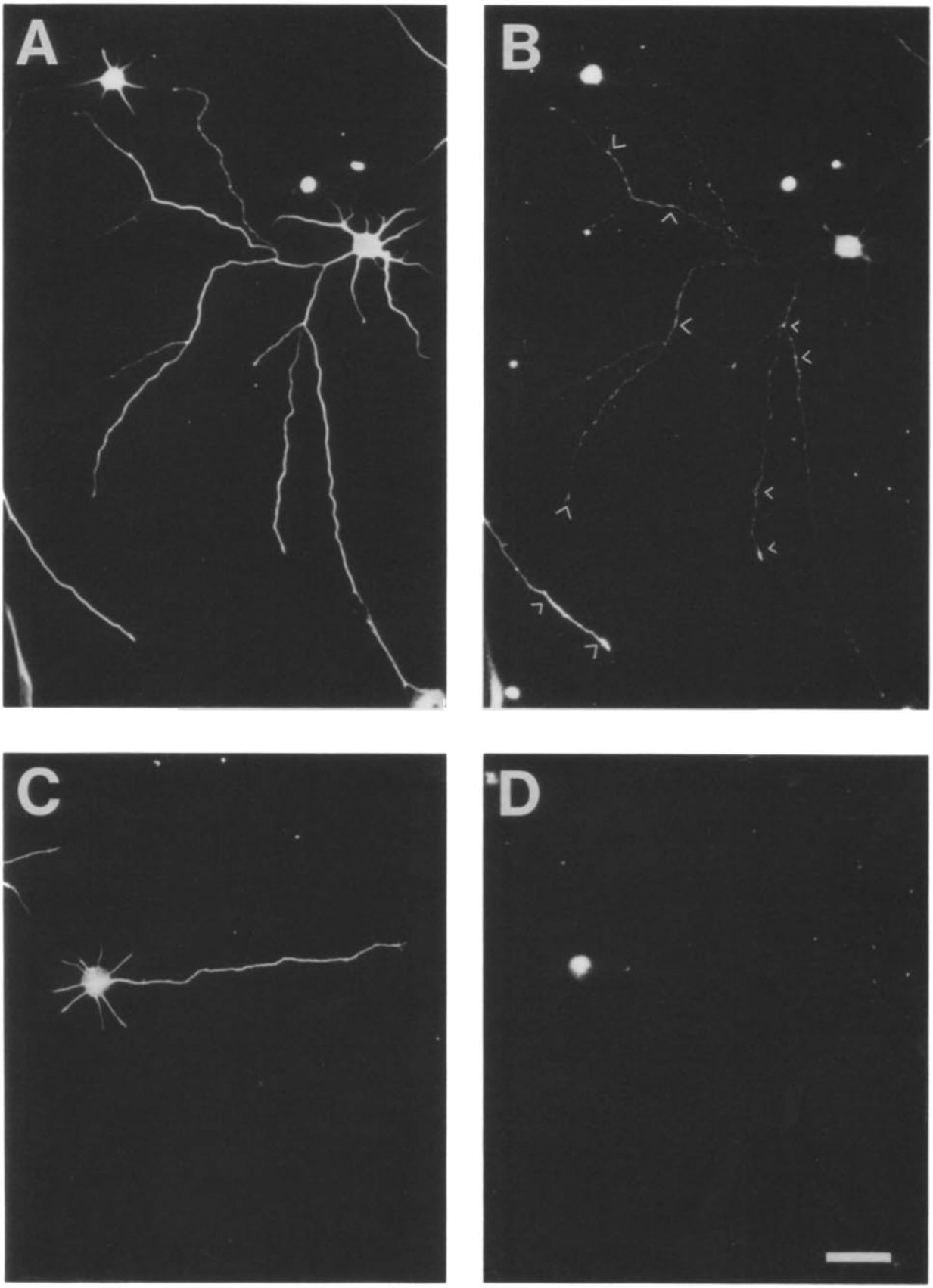

Figure 8. Synapsin I staining. *A* and *B* are kinesin sense oligonucleotide treated. *C* and *D* are kinesin antisense oligonucleotide treated. *A* and *C* are stained with the tubulin antibody (DM1B) and *B* and *D* are stained with the synapsin I rabbit antibody. Bar, 20 μm.

tively, it is formally possible that KHC is unnecessary for the transport of GAP-43 and synapsin I, but instead inhibits the transport of another protein that traps GAP-43 and synapsin I in distal neurites.

Another consistent change in the antisense-treated cells was a reduction in neurite length that affected both the axon and the minor processes which will become dendrites. Both classes of neurites were reduced in length by ~50% after 48 h in antisense-containing medium, but resumed a normal growth rate upon removal of the oligonucleotides. The greater effect on the minor neurites after 48 h compared to just 24 h in the antisense-containing media suggested that the longer the processes grew, the more dependent they became on kinesin for further elongation (Fig. 4). It is generally thought that the addition of new surface membrane necessary for neurite elongation occurs predominantly at growth cones (Pfenninger and Maylie-Pfenninger, 1981). Thus impaired anterograde transport of vesicles in KHC null cells may be the underlying cause for the reduced rate of process outgrowth.

If this explanation is correct, why is neurite elongation not entirely blocked? It may be that neurite outgrowth can occur in the absence of anterograde vesicle transport to growth cones. Membrane addition within the cell body could be sufficient to support a reduced rate of process outgrowth, at least for short distances (Small et al., 1984) and in fish epidermal keratocytes there is a rapid forward transport of concanavalin A–coated gold particles on the dorsal surfaces of lamellipodia that is blocked by treatment with cytochalasin D (Kucik et al., 1989). An alternative possibility is that a different anterograde motor drives the transport of other classes of organelles that can, in part, support neurite elongation. In *Drosophila*, for example a dozen or more different kinesin homologs have been identified (Endow and Hatsumi, 1991).

The particular class of organelles translocated by specific motors remains problematic, particularly in comparing diverse systems. Some degree of organelle specificity was suggested for the putative motor gene *unc*-104 in *C. elegans* because neurons from the mutant phenotype accumulated synaptic vesicles in nerve cell bodies and were depleted of such vesicles in presynaptic terminals (Hall and Hedgecock, 1991). The protein encoded by the *unc*-104 gene bears homology to KHC only in the force-generating head domain (Otsuka et al., 1991). *Unc*-116 in *C. elegans*, is a distinct locus, which encodes a kinesin that is 55% identical over its entire length to the *Drosophila* and squid KHC (de Feo, G., N. Patel, and J. R. Mancillas. 1991. *Soc. Neurosci. Abstr.* 17:58). One allele at this locus has defects restricted to the nervous system (de Feo, G., N. Patel, and J. R. Mancillas. 1991. *Soc. Neurosci. Abstr.* 17:58), and this mutant may represent a closer analogy to the defect in the oligonucleotide-induced KHC null neurons.

The ultimate loss of ribosomes during the transformation of a minor neurite into an axon, and thus the loss of a basis for local protein synthesis, places a high priority on axonal transport to support this rapidly elongating structure. However, some degree of process outgrowth might be a consequences primarily of the polymerization and/or sliding of microtubules. Microtubule polymerization can produce work in vitro (Hotani and Miyamoto, 1990) and appears to drive process formation in Sf9 cells that express high levels of τ protein (Knopps et al., 1991). Furthermore, recent studies (Tanaka and Kirschner, 1991) indicate that intact microtubules are pushed anterograde in neurons; such sliding forces may also contribute to process extension. It will be important to examine the growing tips of processes grown in KHC antisense conditions for the possibility that they are enriched in cytoskeletal structures but devoid of organelles.

Development of Neurite Asymmetry in Null Cells

During their initial growth, hippocampal neurons extend several processes; one of these ultimately differentiates into a long and slender axon while the remainder become dendrites. The commitment of a neurite to an axonal identity occurs when that process becomes significantly longer than the others (Goslin and Banker, 1989). In this study we find that hippocampal neurons that lack detectable KHC nevertheless become polarized, at least as assessed by morphologic criteria. Although process length is reduced, the cells nonetheless exhibit a distinctly asymmetric form, with one neurite far longer than any of the others. Appropriate markers will be necessary to establish the identity of the long process as an axon. Because treatment with KHC antisense oligonucleotides prevents the transport of GAP-43 and synapsin I into neurites, these commonly used axonal markers cannot be utilized. Other markers, like MAP-2 and τ become segregated relatively late in the course of neuronal development in culture (Caceres et al., 1984; Kosik and Finch, 1987), too late for use in the present work.

Appropriate sorting of molecules must occur to create and maintain the molecular differences between axons and dendrites. The selective elongation of a single neurite is an early step in the development of neuronal polarity and represents the formation of an incipient axon. The results here suggest that the selective localization of proteins whose transport requires kinesin cannot be essential for the elaboration of an asymmetric array of neurites. GAP-43 appears to be among this class of proteins. The function of GAP-43, and its possible role in neurite outgrowth, remain controversial. Although it has been reported that GAP-43 antibodies delivered into neuroblastoma cells prevent neuritogenesis (Shea et al., 1991) and overexpression of GAP-43 accelerates neurite outgrowth in PC12 cells (Yankner et al., 1990), neurite outgrowth does occur normally in a PC12 line deficient in GAP-43 (Baetge and Hammang, 1991). GAP-43 normally accumulates in the axonal growth cones of cultured hippocampal neurons coincident with the emergence of the axon, suggesting that it could play a role in the establishment of polarity (Goslin and Banker, 1990). Since the inhibition of KHC expression resulted in the restriction of GAP-43 to the cell body, it is reasonable to suggest that GAP-43 need not be present in axons and their growth cones for axonal outgrowth and for the development of neurite asymmetry. There are, however, certain caveats to this interpretation. First, if the half-life of KHC is sufficiently long, then this motor protein may be present for some portion of the initial 24-h period. Some GAP-43 may have been transported into neurites during the first day in culture, before KHC levels had been completely reduced, but may have disappeared by 24 h, when we determined its localization. It is also possible that a small amount of GAP-43 was present in axons, but was below the level of detection by immunofluorescence.

The specific suppression of KHC expression by antisense

techniques permits the assessment of the role of this motor and the transport of specific molecules in neuronal development. Retained neuronal function in the absence of the motor suggests that additional motors or other mechanisms may be operative in neurite elongation and the development of polarity. Which classes of organelles are transported by kinesin and exactly what degree of cellular integrity is retained in a KHC-null state are compelling topics of further investigation.

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