The Effect of Tau Antisense Oligonucleotides on Neurite Formation of Cultured Cerebellar Macroneurons

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Tau, a microtubule-associated protein (MAP) enriched in axons, may have a role in the generation and maintenance of an axonal morphology. Neurons from embryonic day 15 rat cerebellum in culture elaborate two morphologically distinct neurite populations—one with nontapering, elongated axonlike neurites and the other with tapered dendritelike neurites that branch frequently and are selectively stained with antibodies to MAP2. Tau antisense oligonucleotides were utilized in two ways: (1) continuous application of antisense every 24 hr for variable periods of time or (2) application of antisense that was delayed until neurite differentiation was underway. In both cases, 24 hr after the administration of the antisense, tau protein was not detected immunocytochemically. When the antisense was given continuously directly after plating, the neurites persisted as simple minor outgrowths. When antisense was added 72 hr after plating, axonlike neurites were lost, while the remaining neurites continued to grow and increase in complexity. We concluded that the initial establishment of an elongated axonlike neurite is a prerequisite for further neurite maturation; however, once the axon is established, the remaining neurites are able to grow independently and assume a tapered dendritelike appearance.

Axons are collectively defined by morphological, biochemical, and physiological criteria. A key morphological feature of an axon is its nontapering, elongated structure. Because the microtubule system represents a link to structure, it is reasonable to consider how microtubules contribute to the generation of an elongated axonlike structure. A related question is how microtubules contribute to the generation of two morphologically distinct sets of neurites—axons and dendrites. The orientation of the plus and minus ends of microtubules serves to distinguish axons and dendrites (Burton and Paige, 1981; Filliatreau and DiGiamberardino, 1981; Heidemann et al., 1981; Baas et al., 1988). In axons, the plus ends are aligned distally, whereas in dendrites, microtubules have a mixed orientation, particularly in the midportion of the dendrite. Because the two microtubule-based motor molecules, kinesin and dynein, are defined in part

by the direction that they move organelles in relation to the asymmetry of the microtubule, the contrasting orientations of microtubules within axons and dendrites could give rise to the distinct subcellular components that contribute to the ultrastructural identity of these structures (Black and Baas, 1989).

When neurons from various sites within the developing nervous system are cultured, they elaborate neurites, which may over time approximate some aspects of their *in situ* morphology. Neurites from several neuronal culture systems, including hippocampus, cerebellum, sympathetic ganglia, and cerebrocortex, develop the morphological characteristics of axons and dendrites (Bartlett and Banker, 1984; Peng et al., 1986; Kosik and Finch, 1987; Ferreira and Caceres, 1989). The axon- and dendritelike structures of cultured hippocampal neurons have been characterized in the most detail. Ultrastructural analyses of these two classes of neurites revealed the presence of ribosomes and postsynaptic elements in dendritelike processes and the absence of ribosomes and presynaptic elements in axonlike structures (Bartlett and Banker, 1984). Tritiated uridine is transported selectively into cultured hippocampal dendrites (Davis et al., 1987).

Two microtubule-associated proteins (MAPs) can also serve as markers of axons or dendrites. The MAP family is a highly diverse group of proteins that can promote microtubule polymerization and codistribute with microtubules in cells. Segregation of the somatodendritic marker MAP2 has been shown in hippocampal (Caceres et al., 1984), cerebellar (Ferreira et al., 1989), cerebrocortical (Kosik and Finch, 1987), and sympathetic cultures (Peng et al., 1986). Tau protein has been shown to segregate to the axon in cerebellar (Ferreira et al., 1989), cerebrocortical (Kosik and Finch, 1987), and sympathetic cultures (Peng et al., 1986). The principal site of synthesis of tau in the brain is in neurons, on a population of mRNAs in the neuronal cell body and within the most proximal portion of the dendrite (Kosik et al., 1989a).

In PC12 cells, neurite outgrowth correlates with the induction of tau protein (Drubin et al., 1985) and tau mRNA (Drubin et al., 1988); therefore, a suggested function of tau involves neurite elongation. When infected into the foreign host cell (Sf9 cells from the moth ovary) via a baculovirus, tau induced lengthy process outgrowths (Kosik et al., 1990). Tau may also have a role in the generation of asymmetric neuronal morphologies, because tau antisense exposure in neuronal cell culture specifically inhibits the elaboration of an elongated, nontapering axonlike structure (Caceres and Kosik, 1990).

Shortly after neurons are plated, the minor neurites begin to differentiate, the first into an axon, and then the others into

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dendrites. A minor neurite becomes defined as an axon in hippocampal neurons once it has elongated 10 μm longer than any other neurite (Goslin and Banker, 1989). Uniformly aligned microtubules are present in minor neurites (Baas et al., 1989) and therefore cannot be used to ascertain the onset of polarity. To identify those molecules important in the early definition of an axon, one strategy has been to localize molecules immunocytochemically within the neurite that is transforming to an elongated axonlike structure. The molecules reported to meet these criteria are GAP43 (Goslin et al., 1988), synapsin, and synaptophysin (Fletcher et al., 1989). However, these molecules do not provide a direct link to the microtubules, the structure required for elongation and possibly for the spatial configuration of an axonlike structure.

To study the interdependence of axonal and dendritic growth, we administered tau antisense oligonucleotides in a sustained manner from the time of plating and in a delayed manner after neurite differentiation was underway. Antisense oligonucleotides can be taken up by cells from the media in a saturable, size-dependent manner consistent with receptor-mediated endocytosis (Loke et al., 1989). We have shown that neurons with a sustained exposure to tau antisense from the time of plating remain locked as an array of minor neurites, while failing to develop the morphologically distinct neurites characteristic of axons or dendrites. On the other hand, the addition of tau antisense oligonucleotides to cultures that have established elongated axonlike structures results in the apparent regression of the most elongated neurite and the continued elaboration of the other neurites.

Materials and Methods

Cell cultures. Dissociated cultures of cerebellar macroneurons, prepared from embryonic day 15 rat embryos, were dissociated and plated onto polylysine-coated coverslips at a uniform density of 10,000–20,000/cm² (Ferreira et al., 1989). After 1 hr in serum-containing medium, cells were maintained in D-minimum essential medium/Ham's F12 supplemented with the N2 mixture of Bottenstein and Sato (1979). All the cultures were grown in a 37°C incubator with 5% CO₂. Two nonoverlapping tau antisense oligonucleotides designated RT11-14 and RT23 were used at 50 μM in all the experiments to show that the effects were specific to the tau sequence. The degree to which these oligonucleotides entered the cells and the response to varying doses were described previously (Caceres and Kosik, 1990). Antisense RT11-14 is the rat tau nucleotide GGTTCAGCCATGCTGCTCAAAGCC, which corresponds to the inverse complement of nucleotides –14 to +11 (Kosik et al., 1989b). Antisense RT23 is the nucleotide TGATAATCGACAGGAGGCGAGGACA, which corresponds to the inverse complement of nucleotides –15 to –25 (Kosik et al., 1989b). The rat tau sequences selected represent adjacent regions, one that spans the initiator AUG and the other immediately upstream. The sequences selected were not found in the data base in any other known sequence. Adjacent control wells were treated with the same concentration of the corresponding sense-strand oligonucleotide. The oligonucleotides were synthesized on an Applied Biosystems 380B synthesizer, purified over an NAP5 column (Pharmacia), ethanol precipitated, and taken up in medium.

Antisense oligonucleotides were either administered continuously from the time of plating or delayed until after the onset of polarity. When administered continuously, the tau oligonucleotides were added 1 hr after plating, and again at 24-hr intervals to 48 hr, and fixed for immunocytochemistry at 72 hr. When administered in a delayed fashion, a single dose of tau antisense was added after the cells were in culture for 72 hr and then fixed 24 hr later. One additional protocol was utilized in which antisense oligonucleotides were added with a delay of only 24 hr, and in some cases, a second dose was administered at 48 hr. These cultures were fixed 24 hr after the last dose of antisense. For each experiment, several plates were not fixed at the determined time point, but were allowed to recover from the effects of the antisense.

Immunocytochemistry. The tau antibodies used were 5E2 (Kosik et

al., 1988b) and tau 1 (Binder et al., 1985); both monoclonals were used at 1:20 and 1:100, respectively. The MAP2 antibodies were 5F9, a monoclonal (Kosik et al., 1988a) used at 1:100, and R4, a MAP2 polyclonal (Kosik and Finch, 1987) used at 1:500. Tubulin was labeled with TuJ1 to total tubulin (Geisert and Frankfurter, 1989). Tyrosinated tubulin and MAP1a antibodies were obtained from Sigma Chemical Co. (St. Louis, MO). The secondary antibodies used were the mouse extravidin staining kit (Sigma) with diaminobenzidine as the chromagen, rabbit anti-mouse IgG biotin conjugate (Sigma), goat anti-mouse IgG rhodamine conjugate (Calbiochem), extravidin fluorescein isothiocyanate (FITC) conjugate (Sigma), and goat anti-rabbit IgG rhodamine conjugate (Sigma).

For immunocytochemistry, cells were fixed with either cold methanol (–20°C) or 4% paraformaldehyde in PBS with 0.12 M sucrose at 37°C. Paraformaldehyde-fixed cells were permeabilized in 0.2% Triton X-100 in PBS for 5 min. For double-labeling immunofluorescence, the cells were incubated with polyclonal and monoclonal antibodies simultaneously, washed, and incubated with the secondary antibodies goat anti-rabbit TRITC conjugate, rabbit anti-mouse IgG biotin conjugate, and extravidin FITC conjugate together. In all experiments, the coverslips were preincubated with 5% bovine serum albumin. Photomicrographs were taken on an Carl Zeiss microscope equipped with epifluorescence illumination through 40 $\times$ or 63 $\times$ objectives using TMAX 100 or 400 ASA film (Eastman Kodak Co.).

Analysis of cultures. Developing cerebellar neurons were staged according to Ferreira et al. (1989) and are illustrated in Figure 1. Stage I cells are either rounded or have a lamellipodial veil. Stage II cells have a fairly symmetric array of short, unbranched neurites (Fig. 1A). The transition from stage II to stage III represents the acquisition of morphological polarity in that a single neurite begins to elongate (Fig. 1B). At stage III, one neurite has a relatively uniform diameter over most of its length and thus has the appearance of an axon (Fig. 1C). Stage IV occurs when the minor neurites develop a tapered, highly branched appearance, similar to that of dendrites (Fig. 1D,E). Analogous stages of maturation have been described for cultured hippocampal neurons (Dotti et al., 1988).

Some cultures were drawn using a 63 $\times$ ocular on the Eutectics Neuron Tracing System (Johnson and Capowski, 1983) and analyzed according to its software. Camera lucida drawings were made, including a set with a 100 $\times$ oil-immersion objective to determine neurite caliber at the tip. All drawings were made from slides stained with the tubulin or MAP2 antibody. Bright-field drawings were checked by phase-contrast microscopy to be sure all neurites were drawn.

Results

Effect of prolonged exposure to tau antisense

Tau antisense oligonucleotides were added to the media of cerebellar macroneurons in culture from the time of plating until 48 hr to determine the effects of sustained antisense exposure on morphological development. The abolition of detectable tau protein in antisense-treated cultures was demonstrated previously by dot immunobinding (Caceres and Kosik, 1990) and is shown here by immunocytochemistry (Figs. 2, 3). Staining by two tau monoclonal antibodies (5E2 and tau 1) revealed background levels of reaction product over the entire treated culture. Both antisense oligonucleotides (RT11-14 and RT23) were equally effective in inhibiting tau labeling. In all of the paradigms, the sense- and antisense-treated cultures synthesized other proteins as determined by their reactivity with tubulin, MAP1a, and MAP2 antibodies (Figs. 2, 3). With a sustained dose of antisense from the time of plating, cultured neurons extended initial exploratory neurites and maintained a symmetric array of minor neurites (Fig. 2). Control sense-treated cultures, on the other hand, not only extended initial neurites, but also elongated a single neurite within the first 24 hr that developed the appearance of an axon. Neurons could be kept locked at this stage by administration of tau antisense every 24 hr. At 72 hr in culture, cells treated according to the sustained

Figure 1. Development of cerebellar macroneurons labeled with tau monoclonal antibody tau 1 (*A–C*) or MAP2 (R4) antibody (*D, E*). *A*, Stage II cell with minor neurites (*arrow*) after 12 hr in culture. *B*, Transition to stage III after 24 hr in culture; the *arrow* shows an elongating neurite. *C*, Stage III cell with a single elongated neurite (*arrow*) and very-fine-caliber remaining minor neurites after 24 hr in culture. At this stage, the remaining minor neurites become thinner. *D*, Stage IV neuron after 4 d in culture, stained with MAP2 antibody, at which time the caliber of the minor neurites increases and many neurites appear tapered (*small arrows*); the *large arrows* point to a bundle of axons, which at this time point are still reactive with the MAP2 antibody. *E*, Seven days after plating, only the dendritelike neurites are labeled with the MAP2 antibody. The *arrows* point to the trajectory of an axon apparent only by phase contrast. Scale bar, 10 μ m.

exposure protocol markedly contrasted with control cells, which had elongated axonlike structures (cf. Figs. 1, 2). The effect of antisense treatment was very different from a complete interference with protein synthesis as determined by the administration of cycloheximide at comparable times. This treatment resulted in many nonviable neurons and the complete inhibition of any neurite outgrowth (data not shown).

When the daily administration of antisense was discontinued after 72 hr and the cells allowed to recover, they acquired polarity and continued to differentiate their neurites within a time frame identical to cells plated at the time antisense was discontinued (Fig. 2*D,E*). Cells fixed at 120 hr (Fig. 2*D*) and at 144 hr (Fig. 2*E*) after plating and antisense treatment first formed

a single elongated neurite (Fig. 2*D*), followed by the development of branched and tapered neurites (Fig. 2*E*). Release from the phenotypic effects of tau antisense appeared to occur within 24–48 hr of the cessation of antisense administration. This time period, which must approximate the half-life of the antisense, was the basis for selecting a 24-hr interval between doses. The cells were able to recover from the sustained exposure paradigm and resume development from the point at which they were arrested. Antisense treatment did not irreversibly impair the viability or the ability of the neurons to differentiate. However, over the period observed, neurite growth failed to catch up with the controls and maintained a lag commensurate with the time in antisense.

Figure 2. Continuous exposure to tau antisense given 1, 24, and 48 hr after plating and fixed at 72 hr. *A* and *B* are double-labeled neurons stained with MAP2 antibody R4 (*A*) and tau antibody tau 1 (*B*). Only trace tau immunoreactivity is detectable. *C* is an identically treated plate of cells stained with tyrosinated tubulin antibody. The appearance of these cells is similar to the stage II cell shown in Figure 1*A*. *D* and *E* are recovering cells stained with tau 1, 2 (*D*) and 3 (*E*) d following the cessation of antisense administration. Scale bar, 15 μ m.

Effect of tau antisense administration after the onset of polarity

Tau antisense oligonucleotides were administered after the onset of polarity to determine their effect on cells that had already formed an axonlike neurite. Preliminary to determining the effects of tau antisense on neurites, we first established that two morphologically distinguishable populations of neurites were present in this culture system. Because tapering of neurites is considered a close correlate of dendritic identity (Baas et al., 1989), the degree of tapering was quantitated among primary branches and secondary branches that did not undergo further

branching in cerebellar macroneurons cultured for 7 d (Fig. 4). Neurites fell into two populations—those that tapered and those that did not. For nearly all cells, a single nontapered neurite could be identified. Measurements of the dendritic diameter just proximal to any terminal expansion gave a range of diameters from 0.8 to 1.2 μ m. The terminal diameter of axonlike neurites was 0.5–0.8 μ m. These values may also distinguish axons and dendrites (Banker and Waxman, 1988).

At 72 hr after plating, cerebellar cultures were treated with tau antisense for 24 hr and then fixed for immunocytochemistry (Fig. 3). Tau immunoreactivity was abolished; however, tubulin and MAP2 immunoreactivity were identical to the controls.

Figure 3. Delayed exposure of cerebellar macroneurons to tau oligonucleotides given as a single dose 72 hr after plating. Cells were fixed 24 hr later and double labeled with tau antibody 5E2 (1:20) and MAP2 polyclonal antibody R4 (1:500). *A* and *B* represent the tau antisense, and *C*, the sense control. Tau immunoreactivity is demonstrated in *B* and *C*, and MAP2 immunoreactivity, in *A*. Scale bar, 10 μ m.

Likewise, MAP1a immunoreactivity was identical to controls (data not shown). Double labeling of treated cells with tau and MAP2 antibodies demonstrated that cells with intense MAP2 immunoreactivity were not stained by the tau antibody under antisense conditions (Fig. 3*A,B*). The most dramatic difference between the appearance of the antisense and control cells was the nearly complete lack of neurites that resembled axons. Because tapering distinguished neurite populations, a plot of thickness versus distance from the cell body for primary and secondary neurites was used to demonstrate that the remaining population of neurites after antisense treatment had a dendrite-like morphology (Fig. 4). In untreated cultures, there was a highly significant inverse correlation for the population of dendritelike neurites; for the population of neurites arising from the cell body with an axonlike appearance, the caliber did not change as a function of distance from the cell body beyond the initial 5 μ m. Some axons arose from the proximal portion of dendritelike processes, in which there was occasionally some ta-

pering over the first 10 μ m. Thickness versus distance measurements of the neurites from cultures treated with delayed administration of antisense had a correlation coefficient identical to that of the dendritelike population of neurites from untreated cultures (Fig. 4). When these cells remained in culture beyond the 24-hr period between antisense administration and fixation, they recovered normally.

Thirty cells treated with delayed administration of oligonucleotides were labeled with the tubulin antibody, and randomly selected fields of the sense- and antisense-treated cultures were drawn with the Eutectics Neuron Tracing System (Johnson and Capowski, 1983). Representative cells are shown in Figure 5. While an axonlike neurite was identifiable in nearly all of the sense-treated neurons, only 6 of 30 antisense-treated cells had a similar axonlike neurite that could be distinguished from the other neurites on the basis of length and lack of tapering. Although these six cells appeared to have escaped the morphological effects of the antisense, the lack of tau immunoreactivity

Figure 4. Relationship of the neurite diameter to the distance from the cell body for dendritelike processes after 7 d in culture (*A*), axonlike processes after 7 d in culture (*B*), minor neurites after 1 d in culture (*C*), neurites after 4 d in culture (*D*), and neurites treated with tau antisense given at 72 hr after plating and fixed 24 hr later (*E*). Diameters are in microns, and the distance from the cell body is standardized for the variable lengths of cultures *A–E* by setting the neurite tip at 100 and measuring the diameter at three points that represent percentages of the total length. The correlation coefficients (*r*) with length are *A*, 0.92; *B*, 0.24; *C*, 0.24; *D*, 0.87; and *E*, 0.92. The analysis utilized primary neurites that did not undergo further branching. Twenty cells with an average of 2.5 neurites per cell were drawn for each graph.

was homogeneous over the entire culture. Ten antisense-treated neurons had a single neurite with unusual morphological features, such as multiple branches emanating from a common origin or unusual branch angles. The loss of a single elongated or axonlike neurite is quantitated in Figure 6, which shows that the length of the longest neurite per cell was significantly greater under the control conditions and that antisense-treated neurons had increased numbers of shorter neurites. Comparison of all the neurites from the antisense-treated population revealed a significantly shorter total summed neurite length, thicker primary and secondary neurites, and neuritic volumes equal to the sense-treated cells (Table 1).

Neurite complexity was analyzed by the probability of branching from each segment (Caceres and Steward, 1983). The average number of branches per cell was greater in the antisense-treated cultures (antisense, 17.6 ± 10.8 ; sense, 11.5 ± 7.8 ; $p < 0.01$). There were 3.93 ± 0.25 second-order segments in the sense cultures and 5.86 ± 0.10 in the antisense cultures ($p < 0.001$). There were 1.93 ± 0.10 third-order branches in the sense cultures and 3.44 ± 0.16 in the antisense cultures ($p < 0.001$). The likelihood of second-order segments branching further was 0.32 for the antisense and 0.22 for the sense. The likelihood of third-order segments branching further was 0.28 for the antisense and 0.15 for the sense. The number of first-order segments arising from the soma did not differ in the two conditions (sense,

4.06 ± 1.29 ; antisense, 4.33 ± 1.83). Therefore, the increase in branch complexity is due to higher-order sprouting. Because an axon could not be definitively identified in many of the antisense-treated cells, these calculations were done in several ways: (1) inclusion of all neurites without assumptions regarding dendritic or axonal identity; (2) exclusion of the axon from the sense-treated cultures; (3) exclusion of the axon from the sense-treated cultures and the systematic exclusion of a single neurite from each of the antisense-treated cells; and (4) exclusion of any axonal-appearing structure from both groups. All methods of calculation gave significantly increased branching in the antisense cultures; the most conservative values (calculation 3) are presented.

The presence of tapered neurites that continued to increase in complexity while exposed to tau antisense suggested that this population of neurites was resistant to the effects of the antisense. Together the results from the two protocols (summarized in Fig. 7) suggested that growth of highly branched tapered neurites could occur in the absence of an elongated axonlike neurite, but only after some degree of neurite differentiation had already begun to occur. Cells with early and sustained exposure to antisense did not develop complex branched neurites. An additional intermediate time point of antisense administration was tried to demonstrate that the response to antisense at 72 hr was developmentally distinct from that observed with contin-

Figure 5. Representative cells from sense- (*A*) and antisense-treated (*B*) cultures, stained with anti-tubulin (TuJ1), and traced with the Eutectics system under 63 $\times$ oil immersion lens directly from the microscope stage. Thickness values were entered as the cells were traced. Scale bar, 15 μ m.

uous application from the time of plating. Neurons were treated with antisense oligonucleotides after 24 hr in culture, a time when the population of neurites appears to consist only of simple minor neurites and a single elongated neurite on many cells. Some cultures were given a second dose of tau antisense at 48 hr after plating. Fixation 24 hr after the last dose of antisense revealed cells identical to those treated with continuous application of antisense from the time of plating. Therefore, cerebellar neurons must develop for at least 24 hr in culture before their minor neurites are able to maintain sustained growth in the presence of tau antisense.

Finally, when the application of tau antisense was delayed until 96 hr after plating and the cells fixed 24 hr later, there was no detectable effect on tau immunoreactivity or on the morphology. Whether continuous application of tau antisense beginning at 96 hr could affect neurite growth was not tested, but might be effective if the half-life of tau has increased with neuronal maturity.

Discussion

The analysis of 10-d-old cerebellar neurons revealed two populations of neurites—those that tapered and branched at relatively short distances, and those that did not taper and had an elongated appearance. As neurites are elaborated from cerebellar neurons, they follow a highly reproducible pattern of maturation (Fig. 1). Within the first 12 hr of plating, the neurons elaborate a symmetric array of minor neurites that are not tapered (Fig. 1*A*). By 24 hr, most of the cells have elaborated an axonlike structure, while the remaining minor neurites have not appre-

Figure 6. The left shift to shorter neurites is shown in the antisense-treated population. The mean value for the longest sense-treated neurite is 302.0 μ m, with a range of 45.7–551.3 μ m. The mean value for the longest antisense-treated neurite is 130.5, with a range of 27.1–298.3 μ m. This difference was highly significant ($p < 0.0003$) using the Mann-Whitney U test for the nonparametric distribution of the histogram.

ciably changed. At 4 d in culture, the minor neurites show tapering and the complex branching pattern typical of dendrites (Fig. 1*D*).

The continuous exposure to tau antisense permitted the hypothesis that the prior development of an elongated axonlike neurite was necessary for the conversion of minor neurites into dendrites. Once the cell passed the stage of axonal formation, a distinct population of neurites emerged that resembled dendrites. With application of the antisense delayed until 72 hr, these dendritelike neurites were capable of continued growth in the absence of an identifiable axon. Therefore, the transition from minor neurites to dendritelike processes requires a chronologically prior stage of axonal initiation. Because this observa-

Table 1. Effects of tau antisense on neuronal shape parameters

	Total length (μ m)	Total volume (μ m ³)	Thickness/ 1° segment	Thickness/ 2° segment	Soma area (μ m ²)
Sense	420.7 (75.6–691.8)	167.9 (27.5–482.3)	1.1 $\pm$ 0.07	0.5 $\pm$ 0.05	105.2 $\pm$ 4.11
Antisense	252.6 (63.8–439.2)	188.5 (16.7–737.8)	1.4 $\pm$ 0.11	0.7 $\pm$ 0.05	106.8 $\pm$ 4.87
Significance	$p < 0.005$	$p > 0.1$	$p < 0.05$	$p < 0.01$	$p > 0.2$

The parameters listed were compared for cultures treated with either sense or antisense oligonucleotides added at 72 hr after plating and fixed 24 hr later. For the total length and total volume, the significance was determined by the Mann-Whitney U test because the distribution of values was skewed. For the remaining determinations, a two-tailed Student's t test was used. All values were generated with $n = 30$, and errors refer to SEM. The numbers in parentheses are ranges.

Figure 7. Camera lucida drawings of anti-tubulin-labeled neurons treated with either continuous or delayed exposure to tau antisense oligonucleotides for the number of days in culture indicated along the top. Row A cells were continuously exposed to tau antisense from the time of plating (see Materials and Methods). Row B cells received a single dose of tau antisense 72 hr after plating and were fixed 24 hr later. At 3 d with daily exposure to antisense since the time of plating, only short minor neurites are evident, whereas untreated cells (row B) at 3 d show an elongated process. With the cessation of daily antisense treatment, the cells begin to show recovery at 4 d, whereas delayed antisense administration results in the loss of the axonlike neurite and continued growth of the other neurites. By 5 d, cells that were initially exposed to daily antisense administration continue to recover and look similar to untreated cells at day 3. With a single dose of antisense at day 3, recovery of the axon by day 5 is apparent. Arrows correspond to neurites that extend beyond the figure.

tion suggested a developmental threshold, an additional intermediate time point of antisense application was studied. Oligonucleotides given at 24 hr in culture, a time when polarity is just becoming evident, resulted in morphologies indistinguishable from continuous antisense application from the time of plating. Thus, at 24 hr, neurites still require tau synthesis for any differentiation beyond minor neurites. At 72 hr, only the elongation of the axonlike neurite is dependent upon continued tau synthesis.

The presence of neurites with only minimal axonal features when antisense was administered at 72 hr suggested neurite regression and a role for tau in sustaining an axon. Dendritic branching complexity has been described as a function of plating density (Banker and Waxman, 1988); however, in this study, under conditions of decreased cell–cell contact due to the loss of the long axon, branching complexity was increased in comparison to controls. In many systems, particularly *in vivo* systems, when a neuron is deprived of its axon, sprouting occurs. This response usually occurs from the axon stump, but can occur ectopically from dendritic tips after a close axotomy (Hall et al., 1989). It is possible that the enhanced branching complexity after delayed tau antisense administration represented a reactive response to the loss of an axonlike process.

How neurites gain distinct identities as axons and dendrites must entail a cascade of molecular events that lead to a mature morphology. One aspect of axonal identity is the distinct morphology attained by a single elongated, nontapering structure among a population of neurites. While tau protein probably has a role in the determination of the axon's unique morphology (Caceres and Kosik, 1990), neuronal polarity does not arise from the selective segregation of tau protein to a single neurite. Tau is present in all neurites, including minor neurites at the time of axonal formation. Therefore, a mechanism must exist to

inhibit other minor neurites from becoming axons. In hippocampal neurons, the transection of an axon close to the cell body at early time points releases the inhibition on elongation and permits the conversion of one of the other early neurites to an axon (Goslin and Banker, 1989). To perform a unique role within only one of the neurites, either tau is distinct within a single neurite undergoing elongation, or, as an effector molecule, it acts differentially upon a substrate that is distinct within the incipient axon. Although tau does undergo complex developmentally regulated splicing events (Kosik et al., 1989b), there is no change in the electrophoretic migration of the tau isoforms during the time course of these experiments (Ferreira et al., 1989). A more likely neurite-specific alteration of tau is in its relationship to the microtubule. The binding of tau to microtubules selectively within a single neurite may occur stochastically and serve as a critical step in the formation of the axon. Once tau binds, it may engender cooperative phenomena that enhance additional binding and lead to neurite elongation. Dendritic differentiation can proceed in the absence of tau, once neurons have entered stage III. The MAP2 molecule, with its ability to displace tau from microtubules (Sandoval and Vandekerckhove, 1981), represents a good candidate for a complementary role in dendritic differentiation.

When the 72-hr cultures were fixed after only 16 hr in antisense, considerable tau immunoreactivity remained. Because a 24-hr exposure to the antisense resulted in clear effects on morphology and tau immunoreactivity, the briefer 16-hr interval may represent insufficient time for the turnover of the tau protein pool synthesized before the administration of the antisense. Fixation after 48 hr in the antisense revealed the reappearance of tau immunoreactivity, probably due to exhaustion of the antisense oligonucleotide. When tau antisense was added after 96 hr in culture and analyzed 24 hr later, there was only minimal

loss of the axonlike neurite. The failure to see a decrement in tau protein after 24 hr, when the antisense was added at 96 hr rather than 72 hr in culture, could be explained by a lengthening of the tau protein half-life between these time points. The observed decrement in tau mRNA levels with maturity (Mangin et al., 1989) is consistent with a concomitant lengthening of its half-life. Tau could become less vulnerable to proteolysis by binding to microtubules and proteolytically eliminated when free of microtubules (Okabe and Hirokawa, 1989). Thus, binding of tau to microtubules may represent a critical step in the transformation of an initial exploratory neurite to an axon. Indirect support for the importance of microtubule stability in the elaboration of an axonlike structure stems from the finding that, in cultured, cerebellar neurons, microtubules are first acetylated in the axon (Ferreira and Caceres, 1989). Furthermore, the addition of tau antisense just after plating results in the failure to detect acetylated microtubules, despite the persistence of tubulin reactivity (A. Caceres and K. S. Kosik, unpublished observations). Because the phosphorylation state of tau is known to affect its binding to microtubules (Lindwall and Cole, 1984), a balance between cellular kinases and phosphatases may regulate tau binding to microtubules and thus operate in the generation of polarity.

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