

# Unique Responses of Differentiating Neuronal Growth Cones to Inhibitory Cues Presented by Oligodendrocytes

A. Shibata,\* M.V. Wright,‡ S. David,¶ L. McKerracher,\*\* P.E. Braun,|| and S.B. Kater§

\*Department of Anatomy and Neurobiology, Colorado State University, Fort Collins, Colorado; ‡Division of Science, Truman State University, Kirksville, Missouri; §Department of Neurobiology and Anatomy, University of Utah School of Medicine, Salt Lake City, Utah; ¶Department of Biochemistry, McGill University, Montréal, Canada; ||Centre for Research in Neuroscience, Montréal General Hospital Research Institute, Montréal, Canada; and \*\*Department de Pathologie, Université de Montréal, Montréal, Canada

**Abstract.** During central nervous system development, neurons differentiate distinct axonal and dendritic processes whose outgrowth is influenced by environmental cues. Given the known intrinsic differences between axons and dendrites and that little is known about the response of dendrites to inhibitory cues, we tested the hypothesis that outgrowth of differentiating axons and dendrites of hippocampal neurons is differentially influenced by inhibitory environmental cues. A sensitive growth cone behavior assay was used to assess responses of differentiating axonal and dendritic growth cones to oligodendrocytes and oligodendrocyte-derived, myelin-associated glycoprotein (MAG). We report that >90% of axonal growth cones collapsed after contact with oligodendrocytes. None of the encounters between differentiating, MAP-2 positive dendritic growth cones and oligodendrocytes resulted in growth cone collapse. The insensitivity of differentiating den-

dritic growth cones appears to be acquired since they develop from minor processes whose growth cones are inhibited (nearly 70% collapse) by contact with oligodendrocytes. Recombinant MAG(rMAG)-coated beads caused collapse of 72% of axonal growth cones but only 29% of differentiating dendritic growth cones. Unlike their response to contact with oligodendrocytes, few growth cones of minor processes were inhibited by rMAG-coated beads (20% collapsed). These results reveal the capability of differentiating growth cones of the same neuron to partition the complex molecular terrain they navigate by generating unique responses to particular inhibitory environmental cues.

**Key words:** neuronal growth cones • inhibition • oligodendrocytes • myelin-associated glycoprotein • differentiation

**I**NITIAL development and continued restructuring of dendritic and axonal processes during periods of plasticity or recovery from injury is thought to be dependent, in large part, on activity of their growth cones. Growth cones of developing and regenerating neurites receive specific navigational instructions through recognition of particular environmental cues (for review see Kater and Mills, 1991; Schwab et al., 1993; Letourneau et al., 1994; Stirling and Dunlop, 1995). Evidence has been compiled for the existence of repulsive guidance cues that influence the outgrowth, structure, and function of developing axonal projections (Dodd and Schuchardt, 1995; for review see Keynes and Cook, 1995; Tessier-Lavigne and Good-

man, 1996). Repulsive cues are also present in the adult central nervous system (CNS)<sup>1</sup> where they present a significant barrier to regenerating axonal growth cones. Such growth inhibitory cues are concentrated in the myelin produced by oligodendrocytes (for review see Schwab et al., 1993; Filbin, 1995). Oligodendrocytes have been shown to inhibit neurite outgrowth in vitro and neurite regeneration in vivo (for review see Schwab et al., 1993; Kobayashi et al., 1995). Individual molecules expressed by oligodendrocytes such as NI-35/NI-250 (Caroni and Schwab, 1988a,b; Bandtlow et al., 1990) and more recently myelin-associated glycoprotein (MAG) (McKerracher et al., 1994; Mukhopadhyay et al., 1994; DeBellard et al., 1996; Li et al.,

Address all correspondence to Annemarie Shibata, Ph.D., The Eppley Institute for Research in Cancer and Allied Diseases, 600 South 42nd Street, Box 986805, Omaha, NE 68198-6805. Tel.: (402) 559-8271. Fax: (402) 559-4651. E-mail: ashibata@unmc.edu

1. *Abbreviations used in this paper:* CNS, central nervous system; DIV, days in vitro; Gal-C, galactocerebroside; GS, goat serum; MAP, microtubule-associated protein; MAG, myelin-associated glycoprotein; RT, room temperature.

1996), have been isolated and shown to inhibit axonal growth by their ability to cause growth cone collapse.

Despite the conspicuous inhibitory effect oligodendrocytes have on regenerating axons, little is known about the role oligodendrocyte-derived inhibitory cues play during development of postnatal neuronal architecture. Schwab and coworkers have speculated that in the CNS white matter, myelin may act as a "guardrail" to constrain long axon projections and prevent exuberant sprouting of these fiber tracts during postnatal development (for review see Schwab et al., 1993). However, oligodendrocytes are found throughout the developing and mature CNS gray matter in such areas as the basal ganglia, cerebral cortex, thalamus, and hippocampus (Brownson, 1955, 1956; Duchen, 1984; LeVine and Goldman, 1988*a,b*; Vijayan et al., 1993; Levison and Goldman, 1993). In gray matter, oligodendrocytes extend myelinating processes that are often intimately associated with neuronal cell bodies and dendrites (Kiss and Korisansky, 1975; Polak et al., 1982; Sternberger, 1984; Vijayan et al., 1993; Trapp et al., 1997). Clearly, growth cones of both differentiating dendrites and axons have opportunities to interact with oligodendrocytes during development and remodeling of the CNS. The developmental significance of this cellular organization on neurite structure and function is not known. A comparison of the effect of oligodendrocytes and oligodendrocyte-derived molecules on differentiating dendritic and axonal growth cones of the same CNS neuron might provide insight into how inhibitory cues influence the arborization of neuronal processes with which they interact.

The present study used a hippocampal pyramidal neuronal culture system to investigate how growth cones of elongating and differentiating axonal and dendritic processes react to primary cultured oligodendrocytes and to MAG, an oligodendrocyte-derived inhibitor of neurite growth (McKerracher et al., 1994; Mukhopadhyay et al., 1994; Li et al., 1996). Hippocampal pyramidal neurons extend unique processes (axons and dendrites) at well-defined developmental stages that are easily identified *in vitro* (for review see Craig and Banker, 1994). *In vivo*, processes of pyramidal neurons in the hippocampus contact oligodendrocyte membranes as early as one day after birth (Kiss and Korisansky, 1975; LeVine and Goldman 1988*a*; Vijayan et al., 1993). This culture system provided the ability to investigate how the growth cone of differentiating dendritic and axonal processes respond to inhibitory environmental cues and how responses of growth cones influence neurite structure and outgrowth.

We report here that differentiating axonal and dendritic growth cones respond differently to oligodendrocytes and MAG. These findings provide the first insight into how the outgrowth and structure of dendritic and axonal processes of the same CNS neuron are influenced by oligodendrocyte-derived inhibitory cues.

## Materials and Methods

### Hippocampal Cell Culture

Culture methods used were modified from those established by Banker and Cowan (1977, 1979) and Mattson et al. (1988*a*). Hippocampal regions were dissected from either embryonic day 18 (E18) rat embryos or postna-

tal day 1 rat pups (P1), and then incubated at 37°C for 15 min in 2 mg/ml trypsin in Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free HBSS buffered with 10 mM Hepes, pH 7.4 (GIBCO BRL, Grand Island, NY; Sigma Chemical Co., St. Louis, MO), at 4–6 hippocampi/10 ml. Hippocampal cells were then rinsed twice in DME plus 10% FBS (GIBCO BRL), triturated through a Pasteur pipette, and then centrifuged at 400–500 *g* for 5 min. The final pellet was resuspended in 2 ml DME/10% FBS, triturated a second time with a fire-polished Pasteur pipette, and plated at 20,000 cells/mm<sup>2</sup> on 16-mm German glass-bottomed dishes (Fisher Scientific Co., Pittsburgh, PA) coated with 0.25 mg/ml poly-L-lysine hydrobromide (Sigma Chemical Co.). The following two culture conditions were established: (a) cultures were incubated in DME plus N1 supplements and biotin (DN1B; Bottenstein and Sato, 1979) (GIBCO BRL; Sigma Chemical Co.) at 37°C and 5% CO<sub>2</sub> for 1.5 d to allow for the development of one long axon-like process and several short minor processes; and (b) cultures were incubated in DN1B plus astrocyte-conditioned media at 37°C and 5% CO<sub>2</sub> for 5–6 days *in vitro* (DIV) to allow for differentiation of dendritic processes as determined by restricted staining of high molecular weight microtubule-associated proteins, MAP-2A and MAP-2B (MAP-2; Boehringer Mannheim Corp., Indianapolis, IN; see below). For all experiments presented in this study, morphological identification and MAP-2 staining was used to discern between immature processes (all processes are MAP-2 positive), axonal processes (MAP-2 negative), and differentiating dendritic (MAP-2 positive) processes. The change in the localization of high molecular weight MAPs from ubiquitous staining throughout undifferentiated hippocampal neuronal processes to the localization of MAP-2A and MAP-2B in only mature dendritic processes is commonly used as a marker for mature dendrites and axons *in vitro* (Caceres et al., 1986; Craig and Banker, 1994; Hémar et al., 1997; Perez et al., 1997; Tongiorgi et al., 1997; Cash and Yuste, 1998).

Pyramidal neurons account for 85–90% of the total neuronal population and are the most abundant cell type in the hippocampus during embryonic and early postnatal development (Schlessinger et al., 1978; Boss et al., 1987). Interneurons account for 11% of the hippocampal neuronal population with 80% of the interneurons expressing GABA (Woodson et al., 1989). The low density culture conditions used for these experiments favor the survival and differentiation of hippocampal pyramidal neurons (Banker and Cowan, 1977, 1979; Benson et al., 1994). Only 5–10% of the hippocampal cultured cells are GABAergic interneurons (Benson et al., 1994). Pyramidal neurons and GABAergic interneurons have distinct morphological characteristics *in vitro* (Benson et al., 1994; Craig and Banker, 1994). As a result of these preliminary studies, it is unlikely that hippocampal neurons other than pyramidal neurons were observed in these experiments. However to be accurate we refer to the neurons observed as pyramidal-like neurons.

### MAP-2 Staining of Hippocampal Neurons

After 1.5 DIV and 5–6 DIV, E18 and P1 hippocampal neurons were fixed with 4% PFA in PBS, pH 7.4, for 15 min. Dishes were rinsed in PBS with 5% goat serum (GS); permeabilized for 30 min in PBS with 5% GS and 0.4% Triton X-100 (Aldrich Chemical Company Inc., Milwaukee, WI); and then incubated 16–24 h at 4°C with a mouse mAb to the high molecular weight forms of MAP2, MAP-2A, and MAP-2B (1:200; Boehringer Mannheim Corp.; Cat. No. 1284959), in PBS and 5% GS. Cells were rinsed three times with PBS and 5% GS, and then incubated with a 1:400 dilution of Cy3-labeled goat anti-mouse secondary antibody (Jackson ImmunoResearch Labs Inc., West Grove, PA) in PBS and 5% GS for 30 min at room temperature (RT). Fluorescence was viewed with epifluorescent microscopy (Mercury Lamp; Nikon, Melville, NY). Nonspecific secondary antibody immunoreactivity was determined by treating the cells as described above without addition of primary antibody.

### Primary Oligodendrocyte Culture

Primary oligodendrocytes were isolated and cultured by methods adapted from McCarthy and de Vellis (1980) and Louis et al. (1992). Cerebral hemispheres from P1 rat pups were dissociated with 2.5% trypsin in HBSS for 40 min at 37°C. Cells were rinsed with DME/10% FBS (GIBCO BRL) and triturated with a wide-bore pipet. Suspended cells were centrifuged at 500 *g* for 5 min, resuspended in DME/10% FBS, and then triturated with a fire-polished Pasteur pipette. Cells were plated in polyornithine-coated 75-cm<sup>2</sup> tissue culture flasks at one brain per flask in DME + 10% FBS. Cells were allowed to adhere and grow for 10–12 d. Once oligodendrocyte progenitors reached confluency, flasks were closed tightly, sealed with parafilm, and then shaken in an orbital shaker at 250 rpm for 14–16 h at 37°C.

Medium containing detached oligodendrocyte progenitors was collected into 50-ml conical tubes and larger clumps of cells were allowed to settle. The supernatant was decanted and preplated in 10-cm petri dishes twice for 20 min each. The enriched suspension of oligodendrocyte progenitors was centrifuged, cells were resuspended in DN1B + 30% B104-conditioned medium (see below), and then seeded at 100–200 cells/mm<sup>2</sup> into 10-cm polyornithine-coated culture dishes. B104-conditioned medium maintains oligodendrocyte progenitors in a proliferate state for several cycles of cell division (Louis et al., 1992). After reaching ~500 cells/mm<sup>2</sup> cells were split 1:3 using 0.1% trypsin/EDTA, plated onto polyornithine-coated 100-mm culture dishes, and fed every other day with fresh DN1B/B104-conditioned medium until they reached confluency (Louis et al., 1992). Oligodendrocyte progenitors were then harvested as before and either cryopreserved at -70°C in DME/20% + FBS/10% DMSO or replated for differentiation into mature oligodendrocytes. To induce differentiation, either fresh or previously frozen oligodendrocyte progenitors were seeded at 100–200 cells/mm<sup>2</sup> onto poly-L-lysine- and laminin-coated culture dishes in DN1B for 3 DIV at 37°C and 5% CO<sub>2</sub>.

### **Galactocerebroside and MAG Staining of Oligodendrocytes**

To verify that our culture conditions induced differentiation of mature oligodendrocytes, oligodendrocyte progenitors were cultured for 3 DIV in DN1B at 37°C and immunostained for MAG and galactocerebroside (Gal-C). To determine whether oligodendrocytes expressed MAG, oligodendrocytes were incubated with DN1B and 5% GS for 30 min at 37°C to block nonspecific binding sites. A mouse mAb to MAG (Boehringer Mannheim Corp.) was used at 1:200 in DN1B and 5% GS, and then incubated at 37°C for 60 min. Cultures were rinsed in DN1B and 5% GS and then incubated with rhodamine-conjugated goat anti-mouse IgG (1:200; Boehringer Mannheim Corp.) for 30 min at 37°C. Cells were then fixed in 4% PFA in PBS for 15 min, rinsed in PBS and 5% GS followed by PBS alone, and then viewed with epifluorescence microscopy (Mercury Lamp; Nikon). To determine the expression of Gal-C, oligodendrocytes were fixed with 4% PFA in PBS for 15 min, rinsed in 3× PBS with 5% GS, permeabilized for 30 min in PBS with 5% GS and 0.4% Triton X-100, rinsed three times with PBS and 5% GS, and then incubated 16–24 h at 4°C with a mouse mAb against Gal-C (1:200; Boehringer Mannheim Corp.) in PBS and 5% GS. Cells were rinsed thoroughly three times with PBS and 5% GS, incubated with a 1:400 dilution of Cy3-labeled goat anti-mouse secondary antibody in PBS, and 5% GS for 30 min at RT, and then viewed for immunoreactivity (Mercury Lamp; Nikon).

### **Preparation of B104-conditioned Medium**

B104 neurogloma cells were expanded in DME + 10% FBS in 10-cm tissue culture plates. To prepare conditioned medium, B104 cells were seeded into 10-cm culture dishes at ~100 cells/cm<sup>2</sup> in DME + 10% FBS, and then incubated overnight at 37°C in 5% CO<sub>2</sub>. The next day the plates were rinsed twice with HBSS and 10 ml of DN1B was added to each dish. After 3 d the conditioned medium was collected, filter sterilized, and then frozen at -20°C for up to 3 mo (Louis, 1992).

### **NIH-3T3 Fibroblast Culture**

NIH-3T3 fibroblasts were cultured in DME + 10% FBS on plastic tissue culture plates at 37°C and 5% CO<sub>2</sub>. Cells were harvested in log phase with a non-enzymatic cell dissociation solution (CDS; Sigma Chemical Co.), centrifuged at 500 g for 5 min, and then resuspended in DME-Hepes media (GIBCO BRL). An aliquot of the cell suspension was added to the culture dish under observation on the microscope stage. As the 3T3 fibroblasts settled, attached, and spread, positions in which elongating growth cones would likely encounter the fibroblasts were stored in computer memory for repetitive observations.

### **Preparation of Recombinant MAG Isolation and Purification and Denatured rMAG Methods**

Purification of recombinant MAG (rMAG; Sf9 [*Spodoptera frugiperda*]) cells were grown in suspension in Grace's medium supplemented with 3% FBS to ~2 × 10<sup>6</sup> cells/ml, under standard conditions (Summers and Smith, 1988). Cells were infected at a multiplicity of infection of 5 with JVs-rMAG viral stock (a gift from Dr. R.J. Dunn, Centre for Research in Neurosciences, McGill University, Montréal, Canada), and infection was al-

lowed to proceed for 5 d. Infection medium containing the secreted protein, corresponding to the extracellular portion of MAG, was harvested by centrifugation and precipitation with 60% ammonium sulfate. The resulting pellet was resuspended in 25 mM Tris, pH 7.4, 50 mM KCl, and then dialyzed overnight against this buffer. To isoelectrically precipitate the rMAG, the pH of the dialysate was then lowered to 4.0 by adding dilute glacial acetic acid dropwise with stirring. This was incubated on ice for 30 min to form aggregates, and then centrifuged at 10,000 rpm for 10 min in an SS-34 rotor. Aggregated proteins were resuspended by homogenization in PBS containing 0.5% CHAPS. Any remaining insoluble aggregates were removed by a low speed centrifugation. For control experiments, rMAG was denatured by treating with 0.5% SDS and boiling at 85°C for 10 min.

### **Coupling of Proteins to Polystyrene Amino Beads**

Polystyrene beads (2.5% solid latex, 3.0 μm in diam; Polysciences Inc., Warrington, PA) were covalently coupled to rMAG using the procedure previously described by Kuhn et al. (1995). In short, glutaraldehyde-activated amino beads were incubated with either 25 μg of rMAG, denatured MAG, or no protein (for uncoated control beads) in 500 μl of PBS, pH 7.2, for 16–24 h at RT. Uncoupled sites were blocked with 0.2 methanolamine and BSA solutions for 30 min at RT. The coupled beads were stored in PBS storage buffer at 4°C until used (all solutions provided by Polysciences Inc.).

### **Image Acquisition and Behavioral Analysis**

**Image Acquisition.** An in vitro culture system was used to observe behavioral changes during interaction of growth cones with non-neuronal cell types or protein-coated beads. E18 or P1 hippocampal neurons cultured for 1.5 or 5–6 DIV were transferred into DME-Hepes media (GIBCO BRL), and then placed on a heated 37°C automated stage. Oligodendrocytes or NIH-3T3 fibroblasts previously removed from their culture dish with CDS were diluted 1:1 in 37°C DME-Hepes media and 20–50 μl of suspended cells were added to the hippocampal culture dish. Multiple positions of likely encounters between advancing growth cones and settling cells were selected and stored using a computer-controlled microscope stage (MLC-3; Marzhauser Wetzlar GmbH, Wetzlar, Germany). A 40× or 60× oil objective (Nikon) was used to view the interaction on a Nikon inverted microscope equipped with a CCD camera (CE200A; Photometrics, Tucson, AZ). Repetitive pictures were taken over time at computer-stored positions as growth cones interacted with oligodendrocytes and 3T3 fibroblasts. The same approach was used to study encounters with polystyrene beads coated with rMAG or denatured rMAG or uncoated beads. The beads were diluted 1:10 with 37°C observation media and 10–20 μl of suspended beads were added to the culture dish.

**Behavioral Analysis.** The collapse response was characterized by two criteria: (a) the permanent cessation of forward growth cone advance, and (b) the permanent loss of growth cone surface area (50% or more) resulting in a phase dark, club-shaped, immobile growth cone. Growth cones were characterized as being uninhibited after encounters with oligodendrocytes or the oligodendrocyte-derived MAG if either forward advancement or an active, flattened growth cone was maintained during the period of observation (80–120 min).

Image 1.49 (NIH) software was used to analyze encounter data. Chi square analysis was used to determine significant differences in the behavioral responses (as grouped into two groups: growth cones that collapsed, and growth cones that did not collapse) between growth cones of different processes when encountering particular stimuli and between the same type of growth cone in response to different stimuli (primary oligodendrocytes, NIH-3T3 fibroblasts, rMAG beads, denatured rMAG beads, and non-coated beads). Comparisons of mean time to collapse, growth cone surface area, and rate of elongation between types of growth cones and between stimuli were made using the one-tailed *t* test assuming unequal variances (Microsoft Excel; Microsoft Corp., Redmond, WA).

## **Results**

### **Characterization of Differentiating Hippocampal Neuronal Processes**

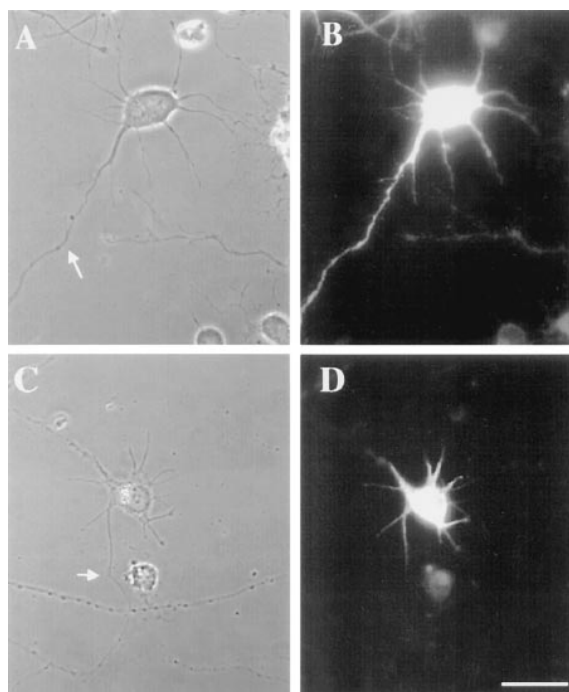
The differentiation of axons and dendrites of E18 hippocampal neurons has been well characterized. In culture,

hippocampal pyramidal neurons first extend several seemingly equivalent processes called minor processes. One of these minor processes extends at a faster rate and becomes the axon (Dotti et al., 1988). At this early stage of development the presumptive axon and minor processes express the microtubule associated protein, MAP-2. Over the next several days in vitro the remaining minor processes become mature dendrites, exhibiting a thicker, tapering morphology, and specific, localized staining of MAP-2. The axonal process continues to elongate and does not express MAP-2 (Caceres et al., 1986). Consequently, hippocampal pyramidal neurons provide a useful model system to examine the response of axonal and dendritic growth cones to inhibitory environmental cues.

To determine the development of axonal and dendritic processes of hippocampal pyramidal-like neurons of E18 and P1 rats in our culture conditions, E18 and P1 hippocampal neurons were cultured for 1.5 DIV or 5–6 DIV, and then stained with MAP-2. MAP-2 was expressed throughout the presumptive axon and minor processes of all cultured E18 hippocampal pyramidal-like neurons at 1.5 DIV ( $n = 50$ ), and was specifically localized to the soma and proximal region of the shorter, tapering processes of P1 hippocampal neurons cultured for 5–6 DIV ( $n = 90$ ; Fig. 1, C and D). These results show that E18 and P1 neurons undergo similar stages of axonal and dendritic differentiation as determined by morphological criteria and MAP-2 localization in culture and provide a useful tool to observe the behaviors of growth cones of differentiating neuronal processes to environmental inhibitory cues expressed by oligodendrocytes. Whereas MAP-2 localization is a commonly used marker for mature dendrites (for review see Craig and Banker, 1994; Hémar et al., 1997; Perez et al., 1997; Tongiorgi et al., 1997; Cash and Yuste, 1998), we refer to these processes as differentiating dendrites since they may not possess all characteristics of fully mature dendrites.

### Characterization of Oligodendrocytes

To confirm the differentiation of our primary cultured oligodendrocytes, we immunostained isolated primary cells for the expression of Gal-C and MAG. Most cells were Gal-C positive (87.3%;  $n = 156$ ) and 91.7% ( $n = 277$ ) of the isolated cell culture showed positive immunoreactivity for MAG. These results confirmed the enrichment of mature oligodendrocytes in our cultures. Detachment and addition of oligodendrocytes to the hippocampal cultures did not alter the differentiated phenotype. After detachment and addition to neuronal cultures, mature oligodendrocytes were identified by a complex lace-like network of processes surrounding the round cell body. We determined that 94.6% ( $n = 211$ ) of the oligodendrocytes added to neuronal cultures and identified morphologically as mature oligodendrocytes exhibited positive immunoreactivity for MAG (Fig. 2, A and B) and 85.9% ( $n = 141$ ) showed Gal-C immunoreactivity. These results indicated morpho-



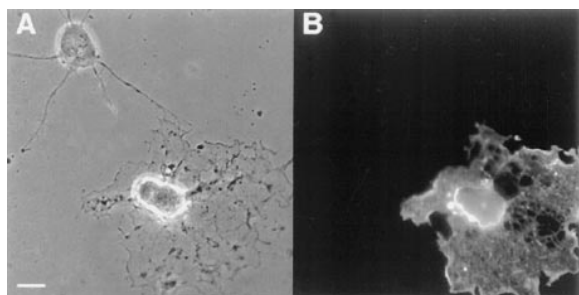
**Figure 1.** Postnatal hippocampal pyramidal-like neurons differentiate mature axons and dendrites. (A) Hippocampal pyramidal-like neuron cultured for 1.5 DIV. One neurite, the presumptive axon, has extended at a greater rate than the other minor processes. (B) MAP-2 stains all neurites (the presumptive axon [arrow] and all minor processes) at this early developmental stage. (C) After 5–6 DIV, postnatal hippocampal neurons extend a long, thin axon and several shorter tapering presumptive dendrites. (D) The differentiation of mature dendrites was exhibited by the specific localization of MAP-2 to the dendro-somatic region of all cultured hippocampal pyramidal-like neurons observed after 5–6 DIV. The mature axon, indicated by the arrow, does not express MAP-2. Bar, 25  $\mu\text{m}$ .

logical criteria could be used to identify mature oligodendrocytes for these experiments.

### Differentiating Hippocampal Axonal Growth Cones Collapse After Contacting Oligodendrocytes

To determine whether MAP-2–negative axonal growth cones of hippocampal pyramidal-like neurons are inhibited by oligodendrocytes, a coculture system was devised to stage encounters between axonal growth cones and primary cultured oligodendrocytes. Non-inhibitory 3T3 fibroblasts were used for control encounters. Differentiated primary oligodendrocytes or 3T3 fibroblasts were added to hippocampal cultures and allowed to adhere on the culture dish. This in vitro approach allowed for high resolution monitoring of growth cone–oligodendrocyte or growth cone–fibroblast interactions.

Nearly all MAP-2–negative axonal growth cones of postnatal day 1 (P1) hippocampal pyramidal-like neurons cultured for 5–6 DIV were inhibited by contact with primary cultured oligodendrocytes (90.3%, 28/31; Table I). Fig. 3, A–A'' shows a typical collapse response of an axonal growth cone after contact with the membrane of an oligodendrocyte. In all 28 encounters, growth cones un-



**Figure 2.** Primary cultured oligodendrocytes express MAG in culture. **A** dropped, differentiated oligodendrocyte used for coculture experiments was stained with anti-MAG antibody. Phase images of the cocultured hippocampal neuron and oligodendrocyte are shown in **A**. The corresponding fluorescent image is shown in **B**. The spread oligodendrocyte shows positive immunoreactivity for MAG, as do 94.6% of the cultured oligodendrocytes used in our experiments. Neuronal processes were not immunopositive for MAG. Bar, 10  $\mu$ m.

derwent sudden paralysis after contact, resulting in a significant decrease in the average rate of outgrowth compared with the average velocity before contact ( $P < 0.001$ ; Table II). Many of the axonal growth cones that collapsed also retracted from the site of contact (78.6%, 22/28). Collapsed growth cones lost on average 69.5% of their original growth cone surface area within 10.2 min of contacting oligodendrocytes (Table II). In all cases, axonal growth cones that collapsed did not recover active motile growth cones during the remainder of the experiment (80–120 min). Only 9.7% (3/31) of axonal growth cones did not collapse after contact with an oligodendrocyte. One uninhibited axonal growth cone remained in contact with the oligodendrocyte while maintaining a motile, flattened lamellipodium but did not elongate past the oligodendrocyte. The remaining two growth cones grew around the oligodendrocyte following contact (Table I).

**Table I.** P1 Hippocampal Growth Cone Response to Oligodendrocytes and Fibroblasts

	<i>n</i>	Collapse (%)
Oligodendrocytes		
Axon	31	90.3*‡
Dendrites	29	0.0§
Minor processes	37	67.6*
NIH-3T3 fibroblasts		
Axon	25	4
Dendrites	16	6.3
Minor processes	16	6.3

Encounters between cultured growth cones of P1 hippocampal pyramidal neurons were experimentally staged with primary cultured oligodendrocytes and 3T3 fibroblasts (see Materials and Methods). Chi squared analysis was used to determine significant differences between the number of growth cones that did or did not exhibit the collapse response after encounters (see Results).

\* $P < 0.001$  compared to the percent collapse after contact with 3T3 fibroblasts.

‡ $P < 0.001$  compared to the percent collapse of dendritic growth cones after oligodendrocyte-encounters.

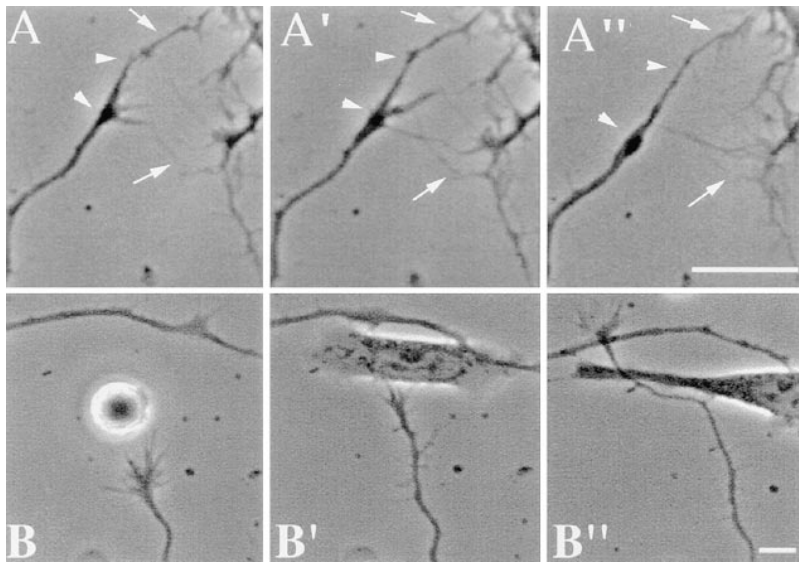
§ $P < 0.001$  compared to the percent collapse of minor process growth cones after oligodendrocyte-encounters.

To determine whether collapse of axonal growth cones in response to contacting oligodendrocytes was a cell-specific response, we staged encounters between elongating axonal growth cones and 3T3 fibroblasts. 3T3 fibroblasts clearly did not inhibit axonal growth cones since only 4% of the encounters (1/25) resulted in collapse (Table I; Fig. 3, *B–B'*). Axonal growth cones did not show significant changes in growth cone surface area or velocity after encounters with 3T3 fibroblasts ( $P > 0.05$ ; Table II). These results indicate that MAP-2–negative axonal growth cones of hippocampal neurons are specifically inhibited by isolated primary oligodendrocytes.

### Differentiating Hippocampal Dendritic Growth Cones Are Not Inhibited by Oligodendrocytes

To determine whether MAP-2–positive, differentiating dendritic growth cones of hippocampal pyramidal-like neurons (P1, 5–6 DIV; Fig. 1, *C* and *D*) are inhibited by oligodendrocyte-derived inhibitory molecules, encounters were staged between MAP-2–positive growth cones and oligodendrocytes. Differentiating dendritic growth cones were clearly not inhibited after encountering oligodendrocytes. In all 29 encounters, MAP-2–positive growth cones that contacted oligodendrocytes did not collapse. Five MAP-2–positive growth cones led their extending process over the encountered oligodendrocytes (17.2%, 5/29; Table I). In six encounters, uninhibited differentiating dendritic growth cones remained in contact with oligodendrocytes, and maintained active lamellipodial structure throughout the experiment (80–120 min). An example of this behavior is shown in Fig. 4. The remaining observed differentiating dendrites exhibited periods of extension and retraction after initial contact. These growth cones encountered the oligodendrocyte several times while maintaining nearly all of their original growth cone surface area (18/29; Table II). The average time between encounters was 26.4 min. It is important to note that periods of extension and retraction during outgrowth resulted in differentiating dendritic processes of varying lengths. Encounters of MAP-2–positive growth cones with the membrane of randomly dropped oligodendrocytes were likely the result of short periods of rapid neurite extension. After contact with oligodendrocytes, the average velocity of MAP-2–positive growth cones decreased but was not significantly different from the average velocity when these growth cones advanced without encountering environmental cues ( $2 \pm 2.1 \mu\text{m/h SEM}$ ,  $n = 12$ ,  $P > 0.05$ ; Table II). These data show that oligodendrocytes do not cause paralysis of differentiating dendritic growth cone of CNS neurons.

3T3 fibroblasts were used for control experiments to determine whether the response of MAP-2–positive growth cones to oligodendrocytes was cell type specific. As shown in Table I, the majority of MAP-2–positive growth cones were not inhibited by contact with fibroblasts. The rate of dendritic outgrowth after contact with 3T3 fibroblasts was highly variable but was not significantly different from the rate of outgrowth after encounters with inhibitory oligodendrocytes ( $P > 0.05$ ; Table II) or the rate of dendritic outgrowth when not encountering environmental cues ( $2 \pm 2.1 \mu\text{m/h SEM}$ ,  $n = 12$ ; Table II). The variable elongation rate of dendritic growth cones after encounter with 3T3 fi-



**Figure 3.** Phase images of hippocampal axonal growth cones encountering a primary cultured oligodendrocyte and 3T3 fibroblast. An axonal growth cone (arrowheads) contacted the extended membrane of an oligodendrocyte (A, upper right corner, arrows) and sampled the membrane with motile filopodia and lamellipodium (3.2 min after initial contact, A'). After 16.6 min of contact the growth cone collapsed. The lamellipodium folded in on itself becoming club shaped and phase dark and the growth cone retracted except for the thin filopodial thread that remained (A''). The collapse was manifested as the loss of 60% of the original growth cone surface area. A 3T3 fibroblast was dropped into the path of an axonal growth cone (B). Filopodia of an axonal growth cone contacted the spread fibroblast membrane (B'). After 10.4 min the axonal growth cone had traversed over the 3T3 fibroblast as well as a distal neurite (B') and had increased its growth cone surface area by 35%. The axonal growth cone continued to elongate for the duration of the experiment (~80 min). Fibroblasts were often stimulated to move away from the site of contact with neuronal growth cones (B''). Bar, 10  $\mu$ m.

broblasts was most likely due to movement of 3T3 fibroblasts away from the site of encounter with dendritic growth cones. In some cases, the moving 3T3 fibroblast “pulled” and elongated the adhered dendritic process. Taken together, the data show that differentiating dendritic growth cones exhibit significantly different behaviors than axonal growth cones in response to contact with the inhibitory cues expressed by oligodendrocytes ( $P < 0.001$ ).

### Hippocampal Minor Processes Collapse After Contacting Oligodendrocytes

Our results revealed that the growth cones of differentiating dendrites are insensitive to the inhibitory cues ex-

pressed by oligodendrocytes. We were interested in investigating how the growth cones of minor processes, the earliest formed, undifferentiated, neuronal processes of P1 hippocampal pyramidal (1.5 DIV), respond to these inhibitory environmental cues. Unlike the response of growth cones of specifically MAP-2-positive differentiating dendrites, 67.6% of the growth cones of minor processes (25/37) collapsed within an average of 12.2 min after contact with oligodendrocytes (Table I). Growth cones of minor processes exhibited a significant decrease in velocity and growth cone surface area after contact with the oligodendrocyte ( $P < 0.01$ ; Table II). An example of a minor process-oligodendrocyte encounter is shown in Fig. 5. Minor processes were not inhibited by control 3T3 fibroblasts,

**Table II.** Effect of Oligodendrocytes and Fibroblasts on Growth Cone/Neurite Characteristics

	Velocity before contact (mean)	Velocity after contact (mean)	Change in neurite length after contact (mean)	Surface area lost or gained after contact (mean)
	$\mu$ m/h	$\mu$ m/h	$\mu$ m	%
<b>Oligodendrocytes</b>				
Axons (31)	26.1 $\pm$ 5.4	-10.9 $\pm$ 3*‡	-6.5 $\pm$ 2.7	-69.5 $\pm$ 2.3
Dendrites (29)	11.4 $\pm$ 6.4	1.4 $\pm$ 2.4§	0.5 $\pm$ 3.4	0.43 $\pm$ 3.4
Minor processes (37)	21.3 $\pm$ 3.7	-13.4 $\pm$ 2.8*‡	-11 $\pm$ 2.6	-50.5 $\pm$ 8
<b>NIH-3T3 fibroblasts</b>				
Axons (25)	26.3 $\pm$ 8.4	24.7 $\pm$ 8	25.3 $\pm$ 6.5	16.8 $\pm$ 33.8
Dendrites (16)	10.6 $\pm$ 3	7.9 $\pm$ 6.5	8.4 $\pm$ 4.8	1.1 $\pm$ 0.95
Minor processes (16)	25.7 $\pm$ 8.5	14.5 $\pm$ 4.4	12.7 $\pm$ 3	-19 $\pm$ 13.3

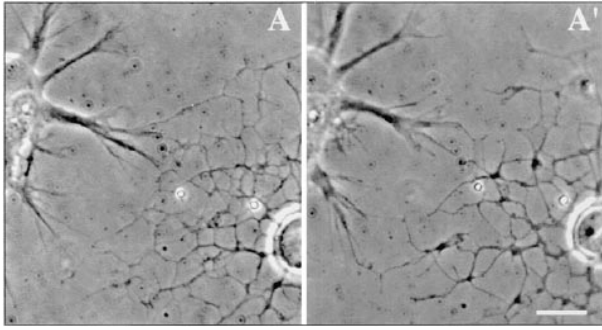
Numbers of P1 hippocampal pyramidal growth cone interactions are given in parentheses. Neurite length, growth cone velocity, and surface area were determined using NIH Image software. Negative values indicate a velocity of retraction, decrease in neurite length, or decrease in growth cone surface area;  $\pm$  values are SEM ( $t$  test assuming unequal variances). Measurements represent the growth cone advance, neurite length and growth cone surface area during the 80–120 min of experimental observation. Velocity of growth cone advance and neurite elongation are relative to the time of observation. Chi square analysis was used to determine significant differences between the velocity before and after contact with oligodendrocytes or 3T3 fibroblasts.

\* $P < 0.001$  compared to the velocity before contact.

‡ $P < 0.001$  compared to the velocity after contact with 3T3 fibroblasts.

§ $P > 0.05$  compared to the velocity of dendrites not encountering cellular cues (2.0  $\pm$  2.1  $\mu$ m/h,  $n = 12$ ).

|| $P > 0.05$  compared to the velocity of after contact with 3T3 fibroblasts.



**Figure 4.** Hippocampal dendritic growth cone encountering a primary cultured oligodendrocyte. (A) Dendritic growth cone encounters the web-like membrane of an oligodendrocyte with its extended filopodia. (A') After 17.2 min of contact and throughout the experiment (120 min) the dendritic growth cone retains active filopodia and has increased its growth cone lamellipodium surface area by 24%. Bar, 10  $\mu$ m.

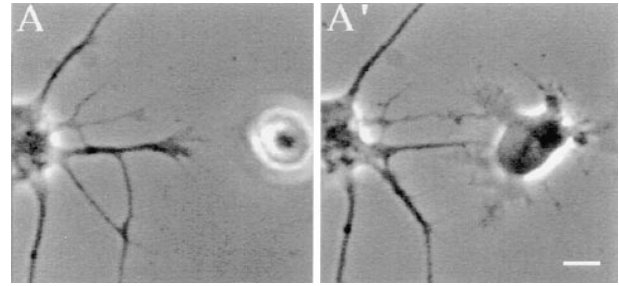
with only 6.3% collapsing after fibroblast encounter (Table I). These results indicate that growth cones of undifferentiated minor processes of P1 pyramidal-like neurons are sensitive to the inhibitory cues expressed by oligodendrocytes.

#### **Response of Embryonic Growth Cones After Contacting Oligodendrocytes**

To determine if responses of differentiating axonal and dendritic growth cones and undifferentiated growth cones of minor process to inhibitory cues are present at an age when these growth cones are less likely to encounter oligodendrocytes *in vivo*, we cultured E18 hippocampal neurons and observed growth cone behavior after oligodendrocyte encounter. Behaviors of E18 differentiating axonal or dendritic growth cones were not significantly different from those of P1 axonal and dendritic growth cones after encounters with oligodendrocytes and control fibroblasts ( $P > 0.05$  for all growth cone types). The majority of MAP-2-negative axonal growth cones collapsed after contact with oligodendrocytes. These axonal growth cones did not collapse after fibroblast-encounters (Table III). Most MAP-2-positive differentiating dendritic growth cones (5–6 DIV) did not collapse after contact with either oligodendrocytes or fibroblasts (Table III). The majority of undifferentiated growth cones of minor processes (1.5 DIV) were inhibited by oligodendrocyte encounter. Encounters with 3T3 fibroblasts did not inhibit the advance of growth cones of minor processes (Table III). Our results suggest that differentiating axons and dendrites of hippocampal pyramidal-like neurons are pre-programmed, even at early stages of development, to respond in specific ways to inhibitory cues expressed by oligodendrocytes.

#### **Postnatal Hippocampal Growth Cone Responses After Contacting Recombinant MAG**

MAG has been shown to inhibit neurite initiation and axonal elongation *in vitro* (McKerracher et al., 1994; Mukhopadhyay et al., 1994; Bartsch et al., 1995; Li et al., 1996; Schafer et al., 1996). Additionally, we found that oligodendrocytes in our culture system expressed MAG (Fig. 2, A



**Figure 5.** Growth cone of a hippocampal minor process encounters a primary cultured oligodendrocyte. (A) The growth cone of a minor process extended as a dropped oligodendrocyte settled to the dish and began to spread its processes. (A') After 18.9 min of contact the growth cone has collapsed. The encounter results in a 98% loss of the original growth cone surface area. Bar, 10  $\mu$ m.

and B). The second objective of this study was to compare the responses of differentiating axons and dendrites of hippocampal processes to MAG and examine whether growth cone responses to MAG were similar to those after oligodendrocyte encounter. To test the inhibitory effects of MAG, rMAG was covalently linked to polystyrene beads and encounters were staged between rMAG-coated beads and hippocampal growth cones. rMAG-coated beads from the same batch were used in all experiments.

**MAP-2-negative Axonal Growth Cones.** In most (18/25) of the encounters, P1 axonal growth cones cultured for 5–6 d collapsed upon contact with rMAG coated beads (Table IV). The average time for collapse to occur was 20.8 min after contact with an average reduction of 79.3% of the growth cone surface area. Also, 50% (9/18) of the collapsed growth cones retracted (average distance 19.2  $\mu$ m) after paralysis of the growth cone. An example of an axonal growth cone encountering a rMAG-coated bead is shown in Fig. 6, A–A'. Out of 25 encounters, 72% of axonal growth cones collapsed after contact with rMAG beads, while 12% of the axonal growth cones did not elon-

**Table III. Embryonic Hippocampal Growth Cone Response to Oligodendrocytes and Fibroblasts**

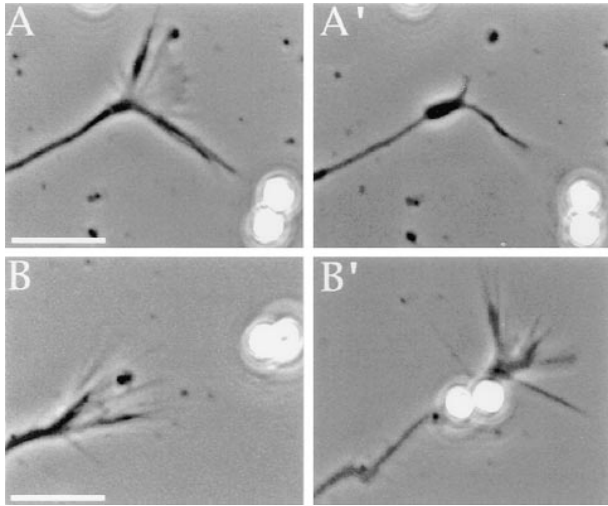
	<i>n</i>	Collapse (%)
Oligodendrocytes		
Axons (5–6 DIV)	15	93.3*‡
Dendrites (5–6 DIV)	23	17.4§
Minor processes (1.5 DIV)	26	65.4*
Immature axons (1.5 DIV)	26	88.5*‡
NIH-3T3 fibroblasts		
Axons (5–6 DIV)	15	6.7
Dendrites (5–6 DIV)	16	6.3
Minor processes (1.5 DIV)	23	0
Immature axons (1.5 DIV)	20	10

Encounters between cultured growth cones of E18 hippocampal pyramidal neurons were experimentally staged with primary cultured oligodendrocytes and 3T3 fibroblasts (see Materials and Methods). Chi squared analysis was used to determine significant differences between the number of growth cones that did or did not exhibit the collapse response after encounters (see Results).

\*  $P < 0.001$  compared to the percent collapse after contact with 3T3 fibroblasts.

‡  $P < 0.001$  compared to the percent of collapse of dendritic growth cones after oligodendrocyte encounters.

§  $P < 0.01$  compared to the percent collapse of minor process growth cones after oligodendrocyte encounters.



**Figure 6.** Axonal growth cones of rat hippocampal neuron encountering rMAG-coated and control beads. (A) Several filopodia of the large active axonal growth cone contact rMAG-coated beads. At contact the growth cone exhibits a spread lamellipodial veil. (A') After 17.3 min of contact the growth cone lamellipodium has collapsed leaving only 22% behind as a club-shaped, phase-dark growth cone with one or more thin threads remaining in contact with the bead as the neurite retracts. Similar to oligodendrocyte-induced collapse of axonal growth cones, this growth cone does not recover from rMAG-induced collapse during the period of observation (85 min). (B) An axonal growth cone elongated toward a control, denatured rMAG-coated bead. (B') The growth cone contacted the beads and elongated underneath them without effecting the overall activity or morphology of the growth cone. The growth cone did not show significant changes in growth cone surface area. Bar, 10  $\mu$ m.

gate past the rMAG bead but remained in contact without collapsing. Few axonal growth cones grew past the rMAG-coated beads (16.0%; Table IV). In control experiments, axonal growth cones did not collapse when encountering beads coated with denatured rMAG. Of these, 72.7% grew past the bead whereas only 13.6% (3/22) of axonal growth cones collapsed after encountering drMAG beads. Fig. 6, B–B', shows an axonal growth cone encountering a denatured rMAG bead. Similarly, uncoated beads were not inhibitory since only 7.1% (1/14) of encounters resulted in growth cone collapse (Table IV). The percent change in the average axonal growth cone surface area after contact with control beads was not significantly different from non-contacting axonal growth cones ( $P > 0.05$ , data not shown).

These results demonstrate that rMAG inhibits the advance of MAP-2-negative, hippocampal axonal growth cones in our growth cone collapse assay. Chi square analysis showed that rMAG-coated beads had statistically significant collapse-inducing effects compared with denatured rMAG-coated and -uncoated beads ( $P < 0.001$ ). Also rMAG-coated beads elicited 58.4% more growth cone collapse than a non-inhibitory control bead (Table IV).

**MAP-2-positive Differentiating Dendritic Growth Cones.** To evaluate more fully the inhibitory role of rMAG, the behaviors of differentiating, MAP-2-positive, dendritic growth cones (P1, 5–6 DIV) were observed during encounters with rMAG-coated beads. Similar to their re-

**Table IV.** P1 Hippocampal Growth Cone Response to Recombinant MAG and Control Beads

	<i>n</i>	Collapse (%)
rMAG-coated beads		
Axons	25	72* <sup>§§</sup>
Dendrites	21	28.6
Minor processes	20	20
Denatured rMAG beads		
Axons	22	13.6
Dendrites	32	12.5
Minor processes	12	16.7
Uncoated beads		
Axons	14	7.1
Dendrites	13	15.4
Minor processes	10	0

Encounters between cultured growth cones of P1 hippocampal pyramidal neurons were experimentally staged with polystyrene beads coated with rMAG. Denatured rMAG or uncoated beads were used for control encounters. Chi square analysis was used to determine significant differences between the number of growth cones that did or did not exhibit the collapse response after encounters (see Results).

\* $P < 0.001$  compared to the percent collapse after contact with denatured rMAG-coated and uncoated beads.

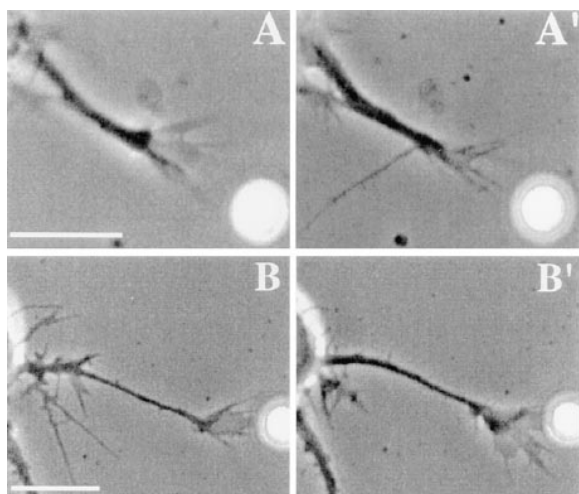
<sup>§</sup> $P < 0.001$  compared to dendritic growth cone response to rMAG-coated beads.

<sup>§§</sup> $P < 0.001$  compared to minor process growth cone response to rMAG-coated beads.

sponse after oligodendrocyte encounter, the majority of MAP-2-positive growth cones contacting rMAG-coated beads did not collapse (71.4%, 15/21; Table IV). Nine of these growth cones made contact without collapsing but did not elongate; three growth cones repetitively encountered rMAG without collapsing; and three growth cones contacted and grew past rMAG-coated beads (Table IV). An example of MAP-2-positive, growth cone behavior during contact with a rMAG-coated bead is shown in Fig. 7, A–A'. During this encounter the surface area of the growth cone remained unchanged while contacting the rMAG-coated bead. Similarly, denatured rMAG-coated beads and uncoated beads did not significantly change the growth cone surface area or motility of specifically MAP-2-positive dendritic growth cones. Few differentiating dendritic growth cones collapsed after contact with denatured rMAG-coated beads and uncoated beads (Table IV). Chi squared analysis determined that the treatments (rMAG, denatured rMAG, and uncoated beads) did not elicit statistically different behaviors from MAP-2-positive growth cones ( $P > 0.05$ ). The percent change of growth cone surface area after encounters with rMAG-coated beads was not significantly different from that of growth cones encountering control beads or of non-contacting growth cones ( $P > 0.05$ ; data not shown). These results reveal a clear difference in the response of differentiating axonal and dendritic growth cones to contact with substrate-bound rMAG, similar to that seen with primary cultured oligodendrocytes.

**Growth Cones of Minor Processes.** To determine whether growth cones of undifferentiated minor processes are sensitive to rMAG, P1 hippocampal neurons were cultured for 1.5 DIV, and encounters with rMAG-coated and control beads were staged. Interestingly, few minor processes were inhibited by rMAG. Only 20% (4/20) of the growth cones collapsed upon encounter with rMAG-coated beads (Table IV). This result was significantly different from the 67.6% of growth cones of minor processes that collapsed





**Figure 7.** Growth cones of dendritic and minor processes encountering rMAG-coated and control beads. (A) A dendritic growth cone extends thin filopodia to contact a dropped rMAG-coated bead. (A') After 17.9 min of contact the dendritic growth cone remains active with motile filopodia and a spread lamellipodium. (B) Growth cone of a minor process encounters a rMAG-coated bead and is unaffected by the encounter. The growth cone contacts the bead with filopodia and the distal edge of its lamellipodium. (B') 19.2 min after initial contact the growth cone has nearly doubled its original growth cone surface (increase of 82%). For the remainder of the experiment (~87 min) the growth cone continued motile activity but did not elongate. Bar, 10  $\mu\text{m}$ .

after encountering primary oligodendrocytes ( $P < 0.01$ ). Fig. 7, B–B' shows a growth cone of a minor process encountering a rMAG-coated bead. Control beads, denatured rMAG, and uncoated beads did not cause these growth cones to collapse (Table IV). The behavioral responses of undifferentiated growth cones of minor processes after contact with rMAG and control beads were not significantly different ( $P > 0.05$ ). These data show that rMAG-coated beads did not mimic the oligodendrocyte-induced inhibition of growth cones of minor processes although rMAG does have similar effects on mature axonal and dendritic growth cones. These results suggest that oligodendrocytes may express additional inhibitory molecules to which growth cones of minor processes are sensitive. It should be noted that while these experiments reveal qualitative information about the inhibitory nature of rMAG, we have not ruled out a quantitative, concentration-dependent effect.

## Discussion

The architecture of mammalian CNS neurons is a consequence of precise differentiation and outgrowth of axons and dendrites. Outgrowth of axonal and dendritic processes is thought to be directed by dynamic behavior of their growth cones. Individual growth cone behavior is influenced by the intrinsic ability of growth cones to respond to environmental cues that they encounter. Inhibitory guidance cues have been shown to be important for proper axonal guidance during development of vertebrate ner-

vous systems (Cox et al., 1990; Davies et al., 1990; Faissner and Kruse, 1990; Snow et al., 1990; Stahl et al., 1990; Walter et al., 1990; Lou et al., 1993; Pini, 1993; Taylor et al., 1993; Colamarino and Tessier-Lavigne, 1995; Fan and Raper, 1995; Messersmith et al., 1995; Püschel et al., 1995). In CNS white matter, oligodendrocytes are potent inhibitory cues sensed by regenerating axons after injury and may provide restrictive boundaries for the guidance of later growing axonal tracts and collateral branching during development (Schwab and Schnell, 1991; Kapfhammer et al., 1992; Müller, 1993; Steindler, 1993; Schwegler et al., 1995). In CNS gray matter, oligodendrocytes are often closely associated with neuronal cell bodies and dendrites but the functional significance of these cellular interactions is not clearly understood. Oligodendrocytes in gray matter express the same proteins as mature oligodendrocytes in white matter and recently have been shown to myelinate (Ludwin, 1979; Leveille et al., 1980; Sternberger, 1984; Trapp et al., 1997). It is possible that in later developing areas of CNS gray matter inhibitory cues expressed by oligodendrocytes play an active role in shaping and maintaining neuronal architecture and synaptic organization by influencing intrinsic outgrowth patterns of axonal and dendritic processes.

In the hippocampus, oligodendrocytes have been identified in areas CA1, CA2, CA3, fascia dentata, and dentate gyrus with an antibody (Rip) that had been previously shown to stain mature, myelin basic protein-positive oligodendrocytes (Friedman et al., 1989; Berger and Frotscher, 1994). The presence of oligodendrocytes in the deep pyramidal layer of CA3 is particularly interesting because mossy fiber axons form synaptic connections with dendrites of CA3 pyramidal neurons as late as two weeks after birth, well after most oligodendrocytes have migrated, differentiated, and begun myelination (Angevine, 1965, 1975; Schlesinger et al., 1975; LeVine and Goldman 1988a,b; Hardy and Reynolds, 1993; Levison and Goldman, 1993). Dendritic differentiation and maturation of dendritic fields in the hippocampus and other regions of the CNS are primarily postnatal events (Pokorny and Yamamoto, 1981; Jacobson, 1991). Consequently, both axons and dendrites of hippocampal pyramidal neurons are likely to contact oligodendrocytes during development. To gain a better understanding of the developmental significance of interactions between oligodendrocytes and neuronal processes, we have investigated how oligodendrocytes and one of its inhibitory components, MAG, influence the outgrowth of differentiating axonal and dendritic growth cones of hippocampal neurons in culture. To our knowledge, this is the first study to investigate how developing dendritic processes respond to inhibitory cues expressed by oligodendrocytes.

The present study reveals a striking difference in the behavior of differentiating axonal and dendritic growth cones of hippocampal pyramidal-like neurons (postnatal and embryonic) after encountering oligodendrocytes in culture. Nearly all MAP-2-negative axonal growth cones are inhibited by contact with oligodendrocytes (Fig. 3; Table I). These results confirm previous work showing that axonal growth cones of several other neuronal cell types are inhibited by contact with oligodendrocytes in culture (for review see Schwab et al., 1993) although not all CNS

neurons have been shown to collapse (Kobayashi et al., 1995). In these experiments, the few axonal growth cones that did not collapse may have encountered oligodendrocytes that were not fully mature or whose expression of surface glycoproteins was altered by the plating procedure. In contrast, none of the encounters between differentiating dendritic growth cones and oligodendrocytes resulted in growth cone collapse (Fig. 4; Table I). This insensitivity to inhibitory oligodendrocytes appears to be developmentally regulated since the majority of growth cones of minor processes collapsed upon oligodendrocyte encounter (Fig. 5; Table I). The small percentage of growth cones of minor processes that did not collapse after oligodendrocyte encounter may represent growth cones that were at or near the final stages of differentiation and therefore, displayed the behavioral characteristic of maturing dendritic growth cones.

MAG is first expressed by mature oligodendrocytes before myelination (Quarles, 1984). Recently, this protein was shown to inhibit neurite initiation and growth cone motility of axons (McKerracher et al., 1994; Mukhopadhyay et al., 1994; Li et al., 1996; DeBellard et al., 1996). We used the hippocampal growth cone assay to compare the inhibitory activity of substrate-bound rMAG to that of oligodendrocytes. Similar to oligodendrocyte encounters, contact with rMAG-coated beads caused collapse of axonal but not specifically MAP-2-positive dendritic growth cones. Unlike their collapse response to oligodendrocyte encounter, the majority of minor processes did not collapse after encounters with rMAG-coated beads (Table IV). These results suggest that individual growth cones of neuronal processes may possess a specific receptor, or combination of receptors, to the inhibitory cues expressed by oligodendrocytes. Minor processes and differentiating dendritic growth cones may not possess the specific putative neuronal sialoglycoprotein receptor necessary for binding MAG and/or the distinct neurite inhibition site required for MAG-induced inhibition (Collins et al., 1997; Tang et al., 1997). In addition to MAG, another oligodendrocyte derived protein called NI-35/NI-250, has been identified and may contribute to the oligodendrocyte-induced collapse of neuronal growth cones (for review see Schwab et al., 1993).

Whereas previous experiments showed that soluble MAG did not cause growth cone collapse of P1 or adult rat DRG neurons (Bartsch et al., 1995), substrate-bound MAG appears to inhibit axonal outgrowth (Mukhopadhyay et al., 1994; McKerracher et al., 1994; Li et al., 1996). Other cell surface and extracellular matrix molecules have specific effects when bound to a substrate. Astrocyte-derived extracellular matrix proteins affect axonal outgrowth differently depending on whether they are soluble or substrate-bound (Chamak and Prochiantz, 1989; Lochter et al., 1991). More recently it was shown that neuronal Eph-related receptor tyrosine kinases require that their ligands be in membrane-bound form to induce clustering activity (Davis et al., 1994). The activity of MAG appears to be dependent upon its full-length structure that includes a specific sialic acid binding site and a distinct inhibitory activity site (Collins et al., 1997; Tang et al., 1997). Identification and characterization of the neuronal MAG sialoglycoprotein receptor will be necessary to fully understand the role

of this protein in the non-permissive growth characteristics of oligodendrocytes.

The results of this study and previous experiments provide strong evidence for selective sensitivity and behavioral responses of differentiating axonal and dendritic processes to particular environmental cues. For instance, axonal growth cones, as a general feature, do not seem to be hypersensitive to just any cellular cue(s). They do not, for example, collapse after contact with 3T3 fibroblasts (see Results). In addition, Mattson et al. (1988a,b) directly compared the response of axons and dendrites of hippocampal neurons to the neurotransmitter glutamate and showed that dendritic growth cones of hippocampal neurons are more sensitive than axonal growth cones to the application of glutamate. Additionally, axons and dendrites seem to have specific growth requirements and respond to trophic factors expressed by astrocytes differently. Dendrites of mesencephalic and striatal neurons exhibit extensive growth in homotypic astrocytic cultures while axonal outgrowth is favored in heterotypic astrocytic cultures (Chamak et al., 1987; Autillo-Touati et al., 1988; Bruckenstein and Higgins, 1988a,b; Rousset et al., 1988). Recently, it has also been suggested that the support of axonal but not dendritic outgrowth by astrocytes is dependent upon the developmental state of the cocultured astrocytes (LeRoux and Reh, 1994). Many intrinsic differences found so far between axons and dendrites (Craig and Banker, 1994; Prochiantz, 1995) closely reflect the specific roles axons and dendrites serve in the CNS. The different behaviors we have described for differentiating axonal and dendritic growth cones upon encountering oligodendrocyte-derived inhibitory cues may be another important adaptation enabling both processes to react differently to surrounding inhibitory molecules during development and periods of plasticity or regeneration.

In conclusion, the present investigation has revealed that differentiating dendritic and axonal growth cones of the same CNS neurons are differentially influenced by oligodendrocytes and one of their inhibitory components, MAG. These data suggest that during development the acquisition of distinct, intrinsic sensory components by axons and dendrites allows for the differential modulation of axonal and dendritic architecture even while encountering the same inhibitory environmental cue. Inhibitory proteins expressed by oligodendrocytes in CNS gray matter areas, such as the hippocampus, may direct or constrain the growth and pathfinding of axonal growth cones while concomitantly allowing a high degree of plasticity in differentiating dendritic growth cones and resultant, developing dendritic arbors.

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