Cortical Neurite Outgrowth and Growth Cone Behaviors Reveal Developmentally Regulated Cues in Spinal Cord Membranes

Masabumi Nagashima,1* Erik W. Dent, 2 Xiu-Zhen Shi,1** Katherine Kalil1,2

1 Department of Anatomy, University of Wisconsin, 1300 University Avenue, Madison, Wisconsin 53706

2 Neuroscience Training Program, University of Wisconsin, 1300 University Avenue, Madison, Wisconsin 53706

ABSTRACT: Corticospinal axon outgrowth in vivo and the ability to sprout or regenerate after injury decline with age. This developmental decline in growth potential has been correlated with an increase in inhibitory myelin-associated proteins in older spinal cord. However, previous results have shown that sprouting of corticospinal fibers after contralateral lesions begins to diminish prior to myelination, suggesting that a decrease in growth promoting and/or an increase in inhibitory molecules in spinal gray matter may also regulate corticospinal axon outgrowth. To address this possibility, we carried out in vitro experiments to measure neurite outgrowth from explants of 1-day-old hamster forelimb sensorimotor cortex that were plated onto membrane carpets or membrane stripe assays prepared from white or gray matter of 1- to 22-day-old cervical spinal cord. On uniform carpets and in the stripe assays cortical neurites grew robustly on young but not older membranes from both white and gray matter. Mixtures of membranes from 1- and 15-day spinal cord inhibited neurite outgrowth, suggesting that the presence of inhibitory molecules in the 15-day cord overwhelmed permissive or growth promoting molecules in membranes from 1-day cord. Video microscopic observations of growth cone behaviors on membrane stripe assays transferred to glass coverslips supported this view. Cortical growth cones repeatedly collapsed at borders between permissive substrates (laminin or young membrane stripes) and nonpermissive substrates (older membrane stripes). Growth cones either turned away from the older membranes or reduced their growth rates. These results suggest that molecules in both the gray and white matter of the developing spinal cord can inhibit cortical neurite outgrowth.

Developing corticospinal axons from the sensorimotor cortex extend over long distances to innervate specific targets in the spinal cord. In rodents, this outgrowth occurs during the first several postnatal weeks (Reh and Kalil, 1981; Schreyer and Jones, 1982; Stanfield et al., 1982; Stanfield and O’Leary, 1985; Kuang and Kalil, 1994). The cessation of axon growth in the more mature spinal cord and the failure of regeneration after adult injury have been attributed in part to the down-regulation of growth promoting factors as well as the increase in inhibitory components in the cellular environment (Caroni and Schwab, 1988; Savio and Schwab, 1990; Schnell and...
Schwab, 1990; Schwab et al., 1993; Bahr and Bonhoeffer, 1994; Schwegler et al., 1995; Wanner et al., 1995; Filbin, 1995; Li et al., 1996; Brosamle and Schwab, 1997). Since development of myelin in the corticospinal tract coincides with cessation of axon growth, myelination could possibly contribute to inhibition of axon outgrowth. This view is supported by results showing that prevention of myelin formation in the spinal cord (Vanek et al., 1998) or treatment of the adult rat corticospinal pathway with antibodies against inhibitory myelin-associated proteins promotes increased axon regeneration and recovery of function (Schnell and Schwab, 1990; Bregman et al., 1995; Z’Graggen et al., 1998).

Nevertheless, previous results (Kuang and Kalil, 1990) have also shown that sprouting of intact corticospinal fibers in response to lesions of the contralateral corticospinal tract is robust in young animals but declines after the first postnatal week and ceases altogether by 3 weeks postnatal. This developmental decline in sprouting occurs prior to extensive myelination of the corticospinal pathway. One possibility is that the developmental program for cortical axon outgrowth is turned off during postnatal development. Another possibility is that an increase in other non-myelin-associated inhibitory molecules and/or a decrease in growth-promoting properties of gray matter in the spinal cord may also contribute to diminished axon outgrowth. To address the latter issue, in the present study we carried out in vitro experiments to compare cortical neurite outgrowth on spinal cord membranes prepared from white or gray matter at various postnatal ages. Previous studies (Kuang et al., 1994) have shown that extensive neurite outgrowth occurs from explants of newborn hamster sensorimotor cortex. We therefore used these explants to assess cortical neurite outgrowth on either uniform membrane carpets or on membrane stripe assays, which consisted of alternating lanes of spinal cord membranes obtained from spinal cord white or gray matter of various postnatal ages. Similar membrane stripe assays have been used to test the growth preferences of retinal axons for different tectal targets (Walter et al., 1987; Wizenmann et al., 1993; von Boxberg et al., 1993; Bahr and Bonhoeffer, 1994; Bahr and Wizenmann, 1996) and preferences of thalamic and cortical axons for various cortical targets (Hubener et al., 1995; Castellani and Bolz, 1997). In addition, we carried out time-lapse video microscopy on growth cones at substrate borders, suggesting a guidance mechanism based on repulsion by inhibitory membrane cues.

**MATERIALS AND METHODS**

**Preparation of Membranes**

Preparation of membranes from hamster spinal cord ranging in age from 1 to 22 days postnatal was carried out with methods adapted from Walter et al. (1987) and Vielmetter and Stuermer (1989). Animals were sacrificed with an overdose of Nembutal and the cervical spinal cord was exposed and removed. White and gray matter were separated by first bisecting the spinal cord into two transverse slabs and then carefully dissecting away the outer white matter with fine tungsten needles. Spinal cord tissues were then transferred to ice-cold L-15 Medium (Gibco) and dounce homogenized in buffer containing 10 mM Tris-HCl (pH 7.4), 1.5 mM CaCl₂, 1 mM Spermidine, 15 μg/mL glucosidase inhibitor (2,3-dehydro-2-deoxy-N-acetyllactosaminic acid), 25 μg/mL aprotinin, 25 μg/mL leupeptin, and 5 μg/mL pepstatin. The homogenate was centrifuged (20 min, 50,000 × g, 21,000 rpm; Beckman SW-50.1 rotor, 4°C) into a 5%-50% sucrose step gradient (sucrose in homogenization buffer plus inhibitors listed above). Membranes at the 5%-50% interface were twice washed in 1 mL of phosphate-buffered saline (PBS) plus inhibitors and centrifuged in an Eppendorf microfuge (16,000 × g, 10 min, 4°C). The final pellet was resuspended in 1 mL PBS without inhibitors and membrane concentration was determined by reading the optical density (220 nm) of a 50-μL sample dissolved in 950 μL PBS with 2% sodium dodecyl sulfate (SDS). The final volume of the total membrane suspension was adjusted to give an optical density of 0.76. To visualize cell membrane borders in the stripe assays after filtration into the Nucleopore filter culture support, fluorescent rhodamine or fluorescein beads (Polysciences) were added to the membrane suspension at 1:50,000. In some experiments, spinal cord membranes were subjected to heat at 56°C for 8 min to determine effects of inactivating membrane-associated proteins.

**Preparation of Membrane Carpets and Stripe Assays**

Uniform membrane carpets were created on Millipore CM filter supports (0.4 μm pore, 30 mm diameter). Approximately 1.8 mL of the membrane suspension was vacuum filtered through the Millipore filter supported upon a fritted glass surface (240 μm² accessible to vacuum). After filtration was complete, the filter support was transferred to 35-mm petri dishes containing tissue culture medium, taking care not to allow the membranes to dry. Stripes of spinal cord membranes were prepared as described in Walter et al. (1987). A polycarbonate filter (Nucleopore; pore diameter 0.1 μm) was placed on a silicone matrix (a kind gift from Dr. F. Bonhoeffer) with 90-μm-wide channels, which was
laid on top of a porous glass frit. A small volume of the membrane suspension (about 100 μL) was pipetted onto the filter and a vacuum was applied to the frit. Excess membrane suspension was washed away with PBS and the filter was transferred to a fine nylon mesh onto the frit. For two stripe assays, the same volume of a different membrane suspension was pipetted onto the filter and a vacuum was applied. The filter, now covered with alternating membrane stripes, was rinsed again with PBS and stored on wet agar. To obtain clear phase-contrast images, the membrane stripes were visualized under fluorescence optics. Images of fluorescently labeled explants and neurites were captured under low light levels with a camera system and stored on an optical memory disc recorder (Panasonic). Neurite outgrowth on membrane carpets was measured by levels of brightness from the fluorescence images. Several low-magnification images were obtained of the entire perimeter of the explant. Then, by means of software programs in the Image I system, brightness thresholds were set to include all fluorescently labeled neurites, whose extent was manually traced. Measurements of areas above threshold, excluding the explant itself, gave the measure of total neurite outgrowth from each explant. To assess differential neurite outgrowth on the membrane stripe assays, single phase images were obtained from either fixed or live cultures. Adopting the scale of Bahr and Wizenmann (1996), we rated random neurite outgrowth as no preference (0), slight preference (1), or clear-cut preference (2). Each case consisted of a single explant. The borders of the membrane stripes were visualized under fluorescence optics.

Preparation of Cortical Explants

Under sterile conditions, cortical explants approximately 900 μm wide and 300 μm thick were obtained from the forelimb region of sensorimotor cortex of 1-day-old postnatal hamsters. Cortex was dissected from fresh brains and sectioned on a Vibratome into chilled sodium-free artificial cerebral spinal fluid (10 mM d-glucose, 2 mM MgSO4, 2 mM CaCl2, 1.25 mM Na2HPO4, 5 mM KCl, 25 mM Hepes, and 252 mM sucrose), after which wedge-shaped explants spanning the full radial thickness of the forelimb sensorimotor cortex were cut from the sections (Kuang et al., 1994). Explants were allowed to equilibrate at 4°C for 30 min, and then eight to 10 of the explants per dish were positioned on the filters containing uniform membrane carpets or on the membrane stripe assays that had been transferred to laminin-coated coverslips. Cultures were placed in 35-mm petri dishes and sufficient culture medium (MEM supplemented with 10% fetal bovine serum, 26 mM NaCO3, 0.3% glucose, and 0.5 mM L-glutamine) was added such that the explants were just barely covered. The cultures were kept humidified at 37°C in a 5% CO2 incubator for 48 h before assessment of neurite outgrowth.

Video Microscopy

To carry out video microscopy of behaviors of living cortical neurites and their growth cones responding to membrane stripes, the petri dish containing the explants was filled with culture medium and sealed with silicon grease. Temperature was maintained during observation at 37°C using an Air Stream Incubator 400 (Nicholson Precision Instruments). Video microscopy was performed with a Zeiss inverted microscope under phase optics with a Newvicon video camera (Dage). Images were acquired at intervals ranging from 30 s to 10 min by opening a computer-driven shutter for <1 s to reduce the possibility of photo damage. Phase images were processed with the Image I processing system and stored on an optical memory disc recorder. Trajectories of individual growth cones on the membrane stripe assays were obtained from tracings of video images at 15-min intervals. Tracings were made on acetate sheets overlaid on the video monitor.

Light Microscopy

To visualize neurite outgrowth on the uniform membrane carpets, living cortical explants were fluorescently labeled after 48 h in culture. The culture medium was removed and 1 mL of a 20-μg/mL solution of the vital fluorescent dye 5-(and-6)-carboxyfluorescein diacetate in PBS [6 mg/mL stock solution in dimethyl sulfoxide (DMSO) diluted 1:300 in PBS] was added to the dish. After 5 min, the dye solution was removed and a solution of 5 mM 1,4-p-phenylenediamine (PPDA) in PBS was added to the dish to inhibit photo-bleaching. The cultures were observed using fluorescence optics. Images of fluorescently labeled explants and neurites were captured under low light levels with a camera system and stored on an optical memory disc recorder (Panasonic). Neurite outgrowth on membrane carpets was measured by levels of brightness from the fluorescence images. Several low-magnification images were obtained of the entire perimeter of the explant. Then, by means of software programs in the Image I system, brightness thresholds were set to include all fluorescently labeled neurites, whose extent was manually traced. Measurements of areas above threshold, excluding the explant itself, gave the measure of total neurite outgrowth from each explant. To assess differential neurite outgrowth on the membrane stripe assays, single phase images were obtained from either fixed or live cultures. Adopting the scale of Bahr and Wizenmann (1996), we rated random neurite outgrowth as no preference (0), slight preference (1), or clear-cut preference (2). Each case consisted of a single explant. The borders of the membrane stripes were visualized under fluorescence optics.

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RESULTS

Growth of Cortical Neurites on Uniform Spinal Cord Membrane Carpets

To determine the ability of cortical neurites to extend on membranes obtained from spinal cord of increasing postnatal ages, we measured the extent of neurite outgrowth from cortical explants plated onto uniform membrane carpets applied to Millipore filters. As shown in Figure 1, after 48 h in culture, neurite outgrowth from the 1-day-old forelimb sensorimotor cortex was robust on 1-day cervical spinal cord membranes, which represent a topographically appropriate target for forelimb cortical axons (Kuang and Kalil, 1994; Kuang et al., 1994). Neurites grew out from all regions of the explant, averaged about 815 μm in length, and tended to fasciculate in bundles. On membranes from 8-day-old spinal cord, neurite growth had noticeably declined but was still extensive. Neurites were somewhat shorter and sparser than on the 1-day-old membranes and were also less fasciculated. Membranes from 15-day spinal cord supported only sparse, short cortical neurites. On membranes from 22-day spinal cord, cortical neurite outgrowth was almost completely lacking. As shown in Figure 1, neurites did not grow out onto the 22-day membranes, but instead grew in thick bundles within the cortical explant.

The developmental decline in neurite outgrowth on older spinal membranes was apparent for gray matter alone, white matter, or a mixture of white- and gray-matter membranes. However, as shown in Figure 2, at all ages examined, membranes obtained from spinal gray matter were always a better substrate than white matter. Membranes from both gray and white matter gave intermediate values. In all cases, neurite outgrowth declined with increasing age of the spinal cord. Numbers above the bars indicate numbers of individual cortical explants examined. Error bars indicate standard errors.

Figure 2 Neurite outgrowth (measured by area) on membrane carpets from spinal cord of various compositions and postnatal ages. At all ages tested, membranes from the gray matter were a better substrate than white matter. Membranes from both gray and white matter gave intermediate values. In all cases, neurite outgrowth declined with increasing age of the spinal cord. Numbers above the bars indicate numbers of individual cortical explants examined. Error bars indicate standard errors.
port neurite growth (Fig. 3). This suggested the presence of inhibitory properties in the 15-day white matter which the 1-day membranes were unable to override.

**Growth of Cortical Neurites on Membrane Stripes**

Results from experiments assessing neurite outgrowth on uniform membrane carpets suggested that reduction of neurite outgrowth on membranes from older cord was due to a developmental increase in inhibitory cues in both white and gray matter of the spinal cord. To address this possibility, we confronted cortical neurites with a choice assay consisting of membrane stripes of different attractiveness. Results obtained from a total of 64 cultures in which the substrate consisted of membrane stripes from 3- or 4-day-old spinal cord alternating with membrane stripes from 22-day-old cord showed that cortical neurites observed 48 h after plating overwhelmingly (88–91% of the cases) preferred to grow on the young membranes (Table 1). Preferences by cortical neurites for young membranes was clear-cut (preference 2) regardless of whether the lanes consisted of membranes from white matter, gray matter, or both components of the spinal cord. The remaining cases showed random outgrowth (preference 0), but these were also characterized by either very little neurite outgrowth from the explants or insufficient transfer of the membranes from the filter to the coverslips. The amount of transfer of the membranes was assessed directly in phase microscopy or by the density of the beads visualized under fluorescence.

Although preferences by cortical neurites for young spinal cord membranes were usually obvious, there was some variation in patterns of neurite outgrowth. As shown in Figure 4, neurites extending laterally from the sides of the explant very often grew along the 3-day membrane stripes. In regions of 22-day-old membranes, however, few neurites extended from the explant, suggesting that they were inhibited from growth at regions adjacent to the 3-week membranes. In other cases (Fig. 5) neurites grew out from all aspects of the explant, crossing over several stripes before gradually curving onto the membrane stripe from young spinal cord. Once neurites contacted stripes from 3- to 4-day-old cord, they frequently turned and extended laterally on it. These results show that membranes from older spinal cord are not absolutely in-

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**Table 1** Results from Experiments in which Assay Consisted of 3- or 4-Day Spinal Cord Membranes Alternating with Stripes of 22-Day Spinal Cord Membranes

<table>
<thead>
<tr>
<th>First Membrane</th>
<th>Second Membrane</th>
<th>No. of Cases</th>
<th>Random Outgrowth (0)</th>
<th>Preferential Outgrowth (2)</th>
<th>Inverted Preference</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-week-old spinal cord</td>
<td>3- to 4-day-old spinal cord</td>
<td>47</td>
<td>4(9%)</td>
<td>43(91%)</td>
<td>0(0%)</td>
</tr>
<tr>
<td>3- to 4-day-old spinal cord</td>
<td>3-week-old spinal cord</td>
<td>17</td>
<td>1(6%)</td>
<td>15(88%)</td>
<td>1(6%)</td>
</tr>
</tbody>
</table>

Results show an overwhelming preference of the neurites for growth on the membranes from 3- to 4-day-old cord regardless of whether the young membranes were applied first or second in the assays.
hibitory to cortical neurite growth, as on the uniform membrane carpets, but that cortical neurites when confronted with a choice clearly prefer to grow on membranes from early postnatal cord.

Results from control experiments (data not shown) eliminated the possibility of several artifacts associated with membrane stripe assays. First, preferences by cortical neurites were the same regardless of which order the membranes were applied to the filter (Table 1). Second, borders between lanes did not by themselves affect guidance of cortical neurites. When membranes from 3-day spinal cord were alternated with stripes of the same 3-day membranes, cortical neurite outgrowth was random and uniform and neurites crossed the borders between the stripes freely. As a final control to eliminate the possibility that laminin underlying the membrane stripe assay could have influenced growth of neurites on the membranes, prior to the application of the membrane stripes the laminin coated coverslips were subjected to ultraviolet irradiation, which eliminates the growth-promoting properties of laminin. Such treatment did not change the preference of cortical neurites for growth on the 3-day membrane lanes. Finally, to confirm that growth preferences of cortical neurites reflected the presence of membrane-associated molecules rather than physical properties of the membrane stripe assays, we assessed neurite outgrowth on membranes subjected to heat, which denatured proteins in the membranes. Results showed that heat treatment virtually abolished neurite outgrowth on membranes from 8-day gray matter, which would normally be a growth-promoting substrate. This showed that neurites are indeed responding to molecular cues in the membranes.

Some variability in the results probably resulted from artifacts that could not be controlled for. First, glial cells occasionally migrated from the borders of some of the explants. Although we excluded cases in which extensive glial migration occurred, initial growth of neurites onto and across older membrane stripes was observed. Second, when the membrane stripe assay was applied to a glass coverslip, which permitted clear visualization of the all the neurites without fluorescent labeling. Images at left show positions of the membrane lanes in fluorescence. Cortical neurites clearly preferred to grow on the membranes from 3-day spinal cord.

Figure 4  Image taken under phase optics of neurites extending from a 1-day cortical explant plated on a membrane stripe assay in which 3-day (3 d) spinal cord membranes alternated with membranes from 3-week (3 w) spinal cord white matter. Note that the stripe assay was applied to a glass coverslip, which permitted clear visualization of the all the neurites without fluorescent labeling. Images at left show positions of the membrane lanes in fluorescence. Cortical neurites clearly preferred to grow on the membranes from 3-day spinal cord.

Figure 5  Image taken under phase optics of neurites extending from a 1-day cortical explant plated on a membrane stripe assay in which 4-day spinal cord membranes alternated with membranes from 22-day spinal cord white matter. In this case, neurites extended from the explant onto both 4-day and 22-day stripes. However, most of the neurites eventually turned and extended onto the younger membranes. Images at right show positions of the membrane lanes in fluorescence.
lanes could have been aided by glial cells. Second, transfer of the membranes to glass coverslips occasionally resulted in mixing of some of the second membranes with those in the first stripe. In these cases, there was more neurite extension across membrane lanes from older spinal cord. Variability in the transfer of the membrane stripe assays to coverslips may also explain why membrane carpets from 22-day spinal cord applied to filters were always inhibitory to neurite growth, whereas neurites were sometimes able to cross over older membrane stripes to varying degrees. Moreover, the stripe assays offered cortical neurites a favorable growth substrate of either laminin or young membranes, which could have allowed the growth cones to extend for certain distances on the unfavorable older membranes. This is consistent with previous studies in culture in which laminin-coated beads were applied to growth cones such that intermittent contact with the beads was sufficient to maintain accelerated growth (Kuhn et al., 1995).

**Rates of Neurite Growth and Behaviors of Growth Cones on Different Membrane Substrates**

Patterns of neurite outgrowth on the membrane stripe assays suggested that cortical neurites grew well on young spinal cord membranes, but poorly on membranes from older spinal cord. To assess the differences in attractiveness of membranes of various postnatal ages, we used video microscopy to observe the growth rates of cortical neurites on various substrates. The transfer of the membranes to glass coverslips made it possible to observe behaviors of cortical neurites under phase microscopy, without the necessity of staining the explants with fluorescent dyes. This approach permitted lengthy recording sessions without photo damage, which can occur during illumination of fluorescently labeled neurites and growth cones. Our initial assay consisted of membrane stripes from spinal cord at ages ranging from 3 to 22 days alternating with lanes of laminin. The use of laminin provided neurites with a growth-promoting substrate from which to approach the membrane lanes, as well as a sharp border between the lanes where behavioral changes in growth cones could be clearly observed.

Rates of cortical neurite extension were determined from video imaging of individual neurites ($n = 6$) every 10 min over time periods ranging from 2 to 12 h. Mean rates of growth were calculated from growth cone behaviors on either the laminin or membrane lane in the membrane/laminin assay. As shown in Figure 6, neurites extended rapidly on young membranes from 7-day spinal cord gray matter, averaging about 30 $\mu$m/h. In contrast, growth rates on white-matter membranes from 15-day spinal cord averaged only 5 $\mu$m/h. Rates of neurite outgrowth on laminin were about 20 $\mu$m/h. Growth rates on older membrane substrates were slower not only because of reduced rates of forward extension, but also because of more frequent growth cone pausing and/or collapsing behaviors.

The overall pattern of neurite growth on membrane stripe assays suggested that growth cones at the borders between stripes were guided by making active choices between substrates of different attractiveness. Video microscopy of growth cone behaviors at borders supported this view. As shown in Figure 7, cortical neurites were confined to laminin lanes in assays composed of 22-day-old membranes alternating with laminin. Analysis of behaviors of individual growth cones ($n = 15$) recorded every 15 s for periods of 2–16 h at these well defined border regions showed that growth cones were repelled from the 22-day-old membranes. Figure 8 illustrates the behaviors of a single growth cone on laminin approaching a membrane stripe at right angles. During the 4.5-h recording period, the growth cone repeatedly extended toward the stripe, but after extending about 10–20 $\mu$m onto the membrane lane the growth cone invariably col-
lapsed and retracted. This behavior persisted until the end of the recording period. In other cases, shown in Figure 9, cortical growth cones exhibited several different avoidance behaviors at the borders between the laminin and 22-day membrane lanes. For example, growth cones exhibited repeated extension, collapse, and retraction after contact with the membranes [Fig. 9(A–C)]. Growth cones also exhibited gradual turning behaviors after contact with the border of the membrane stripe [Fig. 9(D,E)]. In some cases [Fig. 9(F–H)], growth cones were able to cross the laminin–membrane border, usually after repeated pausing and retraction, but showed much slower growth rates on the membrane lanes.

In the final set of experiments, we used membrane stripe assays consisting of alternating stripes of 3- and 22-day-old membranes to measure changes in behaviors and in rates of neurite growth at border regions when growth cones crossed from one stripe to another. Measurements of growth rates of individual neurites during time periods of 2–5 h (n = 10) as they crossed from stripes of different ages (Fig. 10) showed that in most cases, growth cones changed their behaviors. In general, growth cones collapsed or turned to avoid membrane stripes of 22-day-old white matter, whereas they extended relatively smoothly on stripes from 3-day spinal cord gray matter. Avoidance behaviors were similar to those observed at the borders of the laminin/22-day membrane stripes described above. Growth cones exhibited pausing, retracting, turning, and reduced rates of extension when they grew from a 3-day to a 22-day stripe (n = 6). However, when growth cones grew from older to younger membranes (n = 4), they freely crossed the border between the membranes and either accelerated in their growth rates or showed no change in their behaviors or rates of growth.

**DISCUSSION**

In this study, we investigated cortical neurite growth on membranes from white and gray matter of the spinal cord at varying ages. We found that neurite growth declined with increasing age of the spinal cord from which the membranes derived. Although white-matter membranes were always less supportive of neurite growth than gray matter at all ages examined, gray-matter membranes also showed a developmental decline in the ability to support neurite outgrowth. Observations of growth cone behaviors on membrane stripe assays revealed collapsing and turning behaviors as well as reduced rates of extension at border regions, which suggested that growth cone preferences for lanes of laminin or young membranes were at least in part the result of inhibitory responses to molecules in the older membranes. This view is supported by the finding that membrane mixtures from 1- and 15-day cord were almost completely inhibitory to neurite outgrowth. Taken together, these results suggest that molecules inhibitory to cortical neurite growth are present not only in white-matter axon tracts, but also in the gray matter of the spinal cord.

**Membranes from Spinal White and Gray Matter Can Inhibit Cortical Neurite Growth**

Previous studies in vivo and in vitro have focused on the role of myelin in inhibiting growth of axons in the spinal cord (reviewed in Schwab et al., 1993; Schwab and Bartholdi, 1997). Regions of the CNS in which sprouting continues into adulthood have been correlated with a low degree of myelination (Kapfhammer and Schwab, 1994), and elimination of myelin from the rat spinal cord early in development significantly enhanced sprouting of primary afferents (Schwegler et al., 1995). Myelin has been shown to contain molecules that inhibit growth of neuronal processes (Carroni and Schwab, 1988; McKerracher et al., 1994; Mukhopadhyay et al., 1994; Tang et al., 1997). Thus, experiments in vivo have used monoclonal antibodies to neutralize the inhibitory activity of myelin-associa-
ated molecules and thereby enhance the long-distance regeneration of lesioned corticospinal axons in adult rats (Schnell and Schwab, 1990, 1993; Bregman et al., 1995; Z’Graggen, 1998). Cessation of corticospinal outgrowth in the dorsal columns of the hamster spinal cord is consistent with the appearance of myelinated fibers in the corticospinal tract (Reh and Kalil, 1982). The capacity for sprouting (Kuang and Kalil, 1990) or regeneration of corticospinal axons (Kalil and Reh, 1982) also decreases during development and ceases by about 3 weeks postnatal, when the corticospinal tract contains many myelinated fibers. These time periods are consistent with the present findings, which showed a decline in cortical neurite growth on spinal membranes. The cessation of sprouting in vivo by 22 days postnatal correlates well with the cessation of neurite outgrowth on membranes from 22-day cord demonstrated in the present study, and suggests that inhibitory myelin-associated molecules may contribute to this decline in growth.

Elimination of myelin in vivo, however, permitted the regrowth of only a limited number of lesioned

Figure 8  Sequence of video images of neurites extending from a cortical explant onto a stripe assay consisting of alternating lanes of laminin and 22-day spinal cord white matter. Growth cone indicated by arrow repeatedly extended toward the membrane lane but collapsed and retracted after contact with it. During this 4.5-h sequence, the growth cone never grew for any significant distance onto the membrane lane. Times in minutes are indicated on each video frame.
corticospinal axons. Moreover, elimination of the inhibitory myelin-associated glycoprotein (MAG) in mutant mice resulted in no significant regeneration in the adult lesioned corticospinal tract (Bartsch et al., 1995; Li et al., 1996). These results suggest that inhibitors of neurite outgrowth in the mature CNS are not limited to myelin-associated proteins alone, and/or that mature neurons are unable to recapitulate their growth program. Several recent studies in vitro have demonstrated that membranes from CNS gray matter also contain inhibitory molecules. Some of these molecules are associated with oligodendrocytes which are distributed throughout CNS gray matter. Recently, it was shown that axonal growth cones of

Figure 9 Trajectories of growth of individual growth cones at borders between laminin (Lam) and 22-day white matter membranes (22W) during time periods of 1.5–4 h. Circles indicate positions of the neck of the growth cones at 15-min intervals obtained by tracing them on acetate sheets overlaid on the video monitor. Asterisk indicates starting position of the growth cone. Percent time that the growth cone spent in extension (E), pausing (P), and retraction (R) behaviors is shown for each growth cone. (A–C) Growth cone was inhibited from growth onto the membranes after repeated pausing or retraction. (D,E) Growth cone gradually turned away from the membrane lane after contact with the border. (F–H) Growth cones crossed the laminin–membrane border after extensive pausing behaviors (F,G).
Figure 10  Trajectories of growth of individual growth cones at borders between stripes of 3-day gray-matter and 22-day white-matter membranes during time periods of 2–5 h. Circles indicate positions of the neck of the growth cones at 15-min intervals. Asterisk indicates starting position of the growth cone. Percent time that the growth cone spent in extension (E), pausing (P), and retraction (R) behaviors is shown for each growth cone. Growth cones growing from 3-day membranes across a border onto 22-day membranes exhibited behaviors similar to those seen at the laminin/22-day membrane borders. Growth cones were capable of extending onto the 22-day membranes but then exhibited extensive pausing behaviors.
cultured hippocampal neurons collapsed upon contact with oligodendrocytes (Shibata et al., 1998) and that beads coated with MAG, an inhibitory component of oligodendrocytes, also induced collapse of axonal growth cones. However, not all inhibitory activity in CNS gray matter is associated with MAG. For example, gray-matter membranes of adult rat cerebral cortex (Ghosh and David, 1997) were found to induce growth cone collapse, but biochemical analysis revealed that the inhibitory activity in the membranes was not related to myelin-associated inhibitors. Results from the present study demonstrate that in stripe assays, membranes from gray matter of the 3-week-old spinal cord also have strong inhibitory effects on cortical neurite outgrowth, resulting in avoidance behaviors by cortical growth cones. This is consistent with previous studies of neurite outgrowth on cryostat brain sections (Halloran and Kalil, 1996), which demonstrated that the ability of CNS gray matter to support outgrowth from cortical explants declines during development. It is therefore possible that some of the components inhibitory to cortical neurite growth may be associated with the gray matter of the spinal cord. Some of these cues may be associated with oligodendrocytes, but others may be associated with neuronal membranes.

**Guidance of Cortical Growth Cones by Inhibitory Cues in the Developing Spinal Cord**

Results from this study are in accord with findings in vivo showing that inhibitory components in the spinal cord at increasing postnatal ages diminish developmental and regenerative axon outgrowth (reviewed in Schnell and Bartholdi, 1996). An advantage to the present in vitro approach is that neurites growing out of the explant from newborn cortex are capable of growth, whereas in vivo diminished axon growth in older animals may result from intrinsic loss of growth potential in the axons as well as changes in the cellular environment. Importantly, the present study shows a decline in the ability of cortical neurites to grow on membranes from gray matter of increasing ages, possibly as a result of a developmental increase in inhibitory cues associated with gray matter targets in the spinal cord. What functions might these inhibitory molecules serve in vivo? Results from previous studies show that corticospinal axons establish connections by interstitial branches (Kuang and Kalil, 1994; O’Leary and Terashima, 1988) rather than by guidance of the primary axonal growth cone. Thus, developmentally regulated inhibitory molecules in the spinal gray matter may serve guidance functions by limiting corticospinal axon branching and formation of terminal arbors to appropriate stages of development. Inhibitory molecules may also limit lesion induced sprouting in the mature spinal gray matter. Thus, attempts to promote axon regeneration must take into account factors that not only inhibit axon outgrowth in white-matter tracts, but also limit axon branching in gray-matter targets.

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