Ephrin-B3 is a myelin-based inhibitor of neurite outgrowth

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The inability of CNS axons to regenerate after traumatic spinal cord injury is due, in part, to the inhibitory effects of myelin. The three major previously identified constituents of this activity (Nogo, myelin-associated glycoprotein, and oligodendrocyte myelin glycoprotein) were isolated based on their potent inhibition of axon outgrowth in vitro. All three myelin components transduce their inhibitory signals through the same Nogo receptor/p75 neurotrophin receptor/LINGO-1 (NgR1/p75/LINGO-1) complex. In this study, we considered that molecules known to act as repellants in vertebrate embryonic axonal pathfinding may also inhibit regeneration. In mice, ephrin-B3 functions during development as a midline repellent for axons of the corticospinal tract. We therefore investigated whether this repellent was expressed in the adult spinal cord and retained inhibitory activity. We demonstrate that ephrin-B3 is expressed in postnatal myelinating oligodendrocytes and, by using primary CNS neurons, show that ephrin-B3 accounts for an inhibitory activity equivalent to that of the other three myelin-based inhibitors, acting through p75, combined. Our data describe a known vertebrate axon guidance molecule as a myelin-based inhibitor of neurite outgrowth.

spinal cord injury | regeneration | axon | Eph receptor

The corticospinal tract (CST) is the major spinal axon bundle responsible for fine locomotor control. Damage to this tract contributes to the permanent loss of motor control that is the most visible hallmark of spinal cord injury (SCI). These, and other axons of the CNS damaged in SCI, do not regenerate, in part, because of the nonpermissive environment of myelin in the mature CNS (1). Constituents of this repressive activity in myelin include Nogo, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp) (reviewed in ref. 2). The recently identified Nogo receptor (NgR1) is a glycosylphosphatidylinositol-linked molecule that binds the 66-aa extracellular loop of Nogo (Nogo-66) and transduces its inhibitory activity when expressed in neurons (3). Surprisingly, MAG and OMgp also bind the NgR1 (4–6) in a complex with the p75 neurotrophin receptor and the newly characterized leucine rich repeat transmembrane protein LINGO-1. p75 and LINGO-1 are requisite signaling components for inhibition by Nogo-66, MAG, and OMgp as p75 and cerebellar granule neurons (CGNs) or dorsal root ganglion (DRG) neurons exhibit reduced sensitivity to these three inhibitors and to inhibition by CNS myelin (7), and NgR1/p75 complexes confer sensitivity to these inhibitors only in the presence of LINGO-1 (8).

Although it is thought that Nogo, MAG, and OMgp may act in the uninjured CNS to control aberrant sprouting and stabilize mature connections (9), their normal and developmental roles remain a mystery. Axon repellants such as semaphorins, slits, and ephrins play critical roles in tract formation during embryonic development (10). We previously proposed that such molecules may function as inhibitors of regeneration in the mature CNS (11). But, to date, no direct evidence for such a function has yet been found.

In mice, axons of the CST originate from layer V neurons of the motor cortex, cross to the contralateral side of the spinal cord during the first week of life, and hug the dorsal midline as they descend until they reach their targets. We, and others, reported that these neurons express the EphA4 receptor tyrosine kinase (RTK) and are prevented from inappropriate recrossing by their resultant sensitivity to the midline expression of the transmembrane ligand ephrin-B3 (12–14). Genetic ablation of ephrin-B3 in mice thus results in bilateral innervation by the CST and a kangaroo-like “hopping” phenotype. Knocking out EphA4 produces an identical phenotype, demonstrating a receptor/ligand Eph/ephrin relationship in this process (12).

The recent development of critical axon guidance molecule as a myelin-based inhibitor of neurite outgrowth and have significant implications for understanding the mechanisms underlying the lack of regeneration in the CNS.

Methods

Mouse Strains. Ephrin-B3/LacZ mice, encoding a chimeric protein with a β-gal moiety replacing the intracellular segment of ephrin-B3, and ephrin-B3 knockout mice are described in Yokoyama et al. (12). EphA4/LacZ mice, carrying a LacZ cDNA in place of a functional EphA4 allele, were provided by P. Charnay (15). p75 knockout mice containing a targeted deletion in the p75 gene are described in Lee et al. (16). Olig1 knockout mice are described in Lu et al. (17).

Immunohistochemistry, β-Gal, and Western Analyses. Tissues were lightly perfused with 4% paraformaldehyde, equilibrated in 30% sucrose, and cryosectioned at 12 μm. Sections were either stained with X-Gal to detect β-galactosidase (β-gal) activity or immunolabeled with antibodies against β-gal (Chemicon), adenomatous polyposis coli (APC; Calbiochem), neuronal nuclei (NeuN; Chemicon), or glial fibrillary acidic protein (GFAP; DAKO). Primary antibodies were detected with goat anti-mouse or anti-rabbit Cy2- or Cy3-conjugated secondary antibodies. Detergent extraction of purified myelin preparations was carried out as described (18). Quantitative β-gal assays were carried out with 20 μl of soluble and insoluble myelin fractions by using an o-nitrophenyl β-D-galactoside (ONPG) colorimetric assay kit according to manufacturer’s instructions (Promega). Protein was quantitated by the Bradford method with a Bio-Rad kit. For analysis of myelin inhibitor levels, membranes were subjected to SDS/PAGE and transferred to nitrocellulose, and

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Abbreviations: CST, corticospinal tract; SCI, spinal cord injury; MAG, myelin associated glycoprotein; OMgp, oligodendrocyte myelin glycoprotein; NgR, Nogo receptor; CGN, cerebellar granule neuron; Nogo-66, 66-aa extracellular loop of Nogo; APC, adenomatous polyposis coli.

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myelin proteins were detected with monoclonal anti-MAG (Chemicon), or polyclonal anti-Nogo (11C7, Novartis, Basel, Switzerland), or anti-OMgp (Serotec) at 1:5,000, 1:1,000, and 1:500, respectively, in TBS/H2O/0.1% Triton X-100/H2O/5% nonfat milk with 1:10,000 diluted horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology).

In Situ Hybridization. cDNAs from RT-PCR of all eight known ephrins were subcloned into pCR2.1 (Invitrogen) or pBlue-scriptSK(−) (Stratagene) and used to create DIG-labeled cRNA probes by in vitro transcription. The probes were used on transverse sections of spinal cord and on mouse brain (as positive control; data not shown).

Neurite Outgrowth Assays. Myelin outgrowth assays were adapted from the method of DeBellard et al. (19). Purified myelin was prepared by sucrose gradient centrifugation (20), and 3 μg per well was used to coat poly(DL)-ornithine-coated glass eight-well chamber slides (LabTek II; Nunc) in a vacuum dessicator. Cells were isolated from P4-P5 mice according to the method of Brewer (21). Briefly, cortices and cerebella were dissected in Hibernate A medium (BrainBits, East Springfield, IL) supplemented with B27 (Invitrogen), cleaned of meninges, and digested for 30 min in 2 mg/ml papain (Worthington). After trituration, the neurons were purified on an Optiprep (Greiner, Nurtingen, Germany) spin gradient and plated at 2.5 × 10^4 per well. For assays involving recombinant Fc chimeric fragments, ephrin-B3/Fc, MAG/Fc, EphA4/Fc, p75/Fc, or IgG/Fc (R & D Systems) were added at 250 nM or at the concentrations indicated. Ephrin-B3/Fc was preclutered by incubation with anti-human Fcγ (Jackson Immunoresearch), a necessary step to activate the biological activity of ephrin

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Fc proteins. Cultures were grown for 2 days in Neurobasal A/B27 medium, fixed, and immunolabeled with anti-acetylated tubulin (Sigma; to visualize neurites) and an antibody against the intracellular domain of EphA4 (anti-Seki; for cortical cells only) graciously provided by D. Wilkinson (22), or with anti-phosphotyrosine (Upstate Biotechnology) and anti-Sek. Total neurite lengths from individual cerebellar and EphA4-positive cortical neurons were quantitated by using METAMORPH imaging software (Universal Imaging, Downingtown, PA) for 255–467 neurons per condition from duplicate or quadruplicate wells per experiment, and from at least three separate experiments. Experiments were performed with two different preparations of myelin, with identical results. Statistical significance was determined by using ANOVA with post hoc Fisher’s protected tests for pairwise comparisons.

Results

The ephrin-B3 reporter mouse was generated by the creation of a knockin allele in which the cytoplasmic tail of the molecule was replaced by a β-gal moiety (12). In these mice, we observed a postdevelopmental reduction of ephrin-B3/β-gal expression at the midline, beginning at postnatal day 5 and a concomitant appearance of expression in the white matter of both the spinal cord (Fig. 1 Ai–Al) and the corpus callosum (Fig. 5, which is published as supporting information on the PNAS web site). This expression persisted in the adult and remained especially concentrated around the mature CST (Fig. 1 Ag and Ah, arrows). In situ hybridization using an ephrin-B3-specific probe confirmed transfer of expression of ephrin-B3 mRNA from midline to white matter in WT mouse spinal cord (Fig. 1 Ai–Al). Ephrin-B3/β-gal was not detected in the peripheral nervous system (PNS) (Fig. 1 Ag Inset).

Several lines of evidence indicate that ephrin-B3 expression is restricted to myelinating oligodendrocytes in the CNS. First, immunocytochemical comparison of β-gal expression in adult spinal cord with the oligodendrocyte marker APC (Fig. 1B) showed that the ephrin-B3/β-gal chimeric protein is expressed in oligodendrocytes and their corresponding myelin membranes whereas ephrin-B3/β-gal did not colocalize with markers for astrocytes (GFAP), or neurons (NeuN) (data not shown). Second, we observed strong β-gal activity in preparations of biochemically purified myelin membranes (Fig. 1C). Finally, in situ hybridization with spinal cords from mice lacking olg1 (23), a transcription factor critical for proper maturation of oligodendrocytes and myelination, revealed a complete loss of ephrin-B3 expression (Fig. 1D).

Given its developmental function as a midline repellent and subsequent expression in postnatal myelin, we reasoned that ephrin-B3 might function as a myelin-based inhibitor of axonal outgrowth from cortical neurons, which continue to express EphA4 into adulthood (ref. 11 and Fig. 6, which is published as supporting information on the PNAS web site). Treatment of cultured postnatal cortical neurons with purified, preclustered ephrin-B3/β-gal chimeric protein (Fig. 2 Ag–Al) resulted in characteristic clustering of EphA4 receptors on the surface of the cells and concomitant tyrosine phosphorylation, indicating that the EphA4 receptor tyrosine kinase is activated by the ephrin ligand present in myelin. When cortical neurons were plated on myelin membranes from WT (Fig. 2 Aj–Al) or ephrin-B3 knockout mice (Fig. 2 Am–Al), EphA4 clustering and tyrosine colocalization were observed only with WT myelin. These data indicate the active and specific interaction between EphA4 expressing myelin membranes and EphA4 expressing cortical neurons.

To test whether activation of EphA4 receptors by ephrin-B3 in postnatal cortical neurons results in inhibition of axon outgrowth comparable with that of an established myelin-based inhibitor, we measured the neurite outgrowth of EphA4-positive cortical neurons grown in increasing concentrations of soluble ephrin-B3/β-gal or MAG/β-gal. As shown in Fig. 2B, ephrin-B3/β-gal inhibited outgrowth in a dose-dependent manner, resulting in a 40% reduction compared with untreated or IgG/β-gal-treated neurons at the highest concentrations.
analysis of our myelin preparations confirmed that this relief of inhibition from Nogo A to lose inhibitory sensitivity to Nogo-66, MAG, and OMgp (7), we found that outgrowth trends mirrored those seen with cortical neurons. Thus, ephrin-B3 is an active repulsive component of myelin for EphA4-positive neurons, which include the corticospinal neurons that are a critical population damaged in SCI.

We further observed that inhibition of p75\textsuperscript{+/-} neuron outgrowth by WT myelin was also substantially reduced compared with that of p75\textsuperscript{-/-} neurons (Fig. 3A, columns 2 and 5). However, the combined absence of ephrin-B3 in myelin and p75 in neurons resulted in greater neurite outgrowth than the removal of either inhibitory pathway individually (Fig. 3A, columns 3, 5, and 6), essentially abolishing all myelin inhibition. These data indicate that, for cortical neurons grown under our experimental conditions, the fraction of inhibition on purified myelin membranes attributable to ephrin-B3 is equivalent to that of all three p75-mediated inhibitors combined.

Cerebellar granule neurons, although not damaged in spinal cord injury, are often used as in vitro models of myelin inhibition. Although postnatal CGNs do not express EphA4, our in situ analysis showed that they do express both the EphB1 and EphB2 receptors, and so should bind and recognize B-class ephrins such as ephrin-B3 (ref. 11 and data not shown). We therefore performed parallel myelin membrane culture experiments with CGNs and found that outgrowth trends mirrored those seen with cortical neurons from p75\textsuperscript{-/-} mice and p75 knockout from neurons are all significant (P < 0.0001 for columns 2 vs. 3, 5 vs. 6, 3 vs. 6, and 2 vs. 5) as measured by a post hoc Fisher's protected test (WT, WT mouse myelin; KO, ephrin-B3 knockout myelin). Differences in neurite outgrowth due to ephrin-B3 knockout from myelin and p75 knockout from neurons are all significant (P < 0.0001 for columns 2 vs. 3, 5 vs. 6, 3 vs. 6, and 2 vs. 5) as measured by a post hoc Fisher's protected test. (D) β-gal activity of purified myelin and of detergent soluble and insoluble myelin fractions from mice containing the ephrin-B3/LacZ fusion allele is represented as absorbance at 420 nm normalized to total protein ± SD for triplicate samples. pellet, Insoluble fraction; sup., solubilized fraction.
neurite outgrowth with that seen with an IgG reagent to block the inhibitory effect of ephrin-B3 on regeneration. We attribute the source of this discrepancy to differences in experimental conditions. The neurite outgrowth assays performed by these authors used detergent extracts of myelin membranes isolated by a method that solubilizes the MAG, Nogo, and OMgp fractions (18). β-gal assays on myelin from ephrin-B3/LacZ knockin mice extracted with the same protocol showed that ephrin-B3 segregated with the insoluble membrane fraction (Fig. 3D). Thus, the ephrin inhibitory component in myelin would not be detectable in the soluble fraction of such membrane preparations.

Inhibition of CGN neurite outgrowth by the Nogo-66/MAG/OMgp fraction of myelin was shown to be relieved by the addition of excess p75/Fc chimeric protein, which acts as a competitive inhibitor for ligand binding to the NgR1/p75 complex (7). We tested whether purified EphA4/Fc protein could render similar results. We plated WT cortical or cerebellar neurons on WT myelin in the presence of EphA4/Fc, p75/Fc, or both and compared neurite outgrowth with that seen with an IgG/Fc control fragment. As shown in Fig. 4, treatment with either protein relieved about one half of the observed inhibition. These data are consistent with the previous report for p75/Fc (7) as well as with the inhibitory effects of ephrin-B3 described here. Treatment of CGNs with combined EphA4/Fc and p75/Fc proteins resulted in neurite outgrowth statistically indistinguishable from that of control neurons on polyornithine (Fig. 4B). For cortical neurons, however, the combined treatment with both proteins did not result in increased outgrowth over that with each individual Fc alone (Fig. 4A). This result is likely due to a generalized detrimental effect on growth of the relatively sensitive cortical cultures of an excessive dose of recombinant protein in the medium, as the neurite lengths of these cultures tended to decrease in the presence of high concentrations of Fc even in the absence of myelin (data not shown). We confirmed that, at the concentrations shown, none of the Fc fragments used stimulated outgrowth on polyornithine, indicating that their effects were mediated through suppression of myelin inhibition and not a generalized stimulation of neurite outgrowth (data not shown). Our results suggest that recombinant EphA4 receptor may be an effective reagent to block the inhibitory effect of ephrin-B3 on regeneration in vivo.

To determine whether any of the other known ephrins are expressed in myelin and so might contribute to its inhibitory effect on CST regeneration, we performed in situ hybridization analysis on adult spinal cord with cRNA probes to ephrins A1–A5 and B1–B3. We observed ephrin-B2 mRNA expression in a small cluster of cells near the central canal, consistent with our observed expression of ephrin-B2/β-gal fusion protein in ephrin-B2/lacZ mice (unpublished observations). However, only ephrin-B3 is expressed throughout the spinal cord white matter, and so is the only apparent ephrin candidate for a role in myelin inhibition (Fig. 7, which is published as supporting information on the PNAS web site). This result does not exclude possible roles for other ephrins and their Eph receptors in inhibition of regeneration after injury. For example, Bundsen et al. (27) observed that ephrin-B2 is up-regulated in astrocytes and regulates astrocyte–meningeal fibroblast interactions after SCI in mice. Additionally, EphA (28) and EphB3 (29, 30) receptors are up-regulated in cells surrounding injury sites in rats, suggesting that these molecules may also contribute to the nonpermissive environment in chronic SCI.

Discussion

The prevailing view of myelin-based inhibition of axonal regeneration has been that the three known inhibitors in myelin (Nogo, MAG, and OMgp) account for most, if not all, of the inhibitory activity found in myelin. This theory, based on extensive cell culture evidence, is at odds with reports that MAG, Nogo, p75, and NgR1 knockout mice demonstrate modest or undetectable neuronal regenerative properties in the CST after experimentally induced SCI (26, 31–37). A number of possible explanations may account for this discrepancy, including the existence of NgR1/p75 independent mechanisms of action of the known myelin inhibitors. For example, the N-terminal domain of Nogo ("amino-Nogo") inhibits neurite outgrowth without binding the NgR1 (3). Additionally, other inhibitory molecules in the extracellular environment, such as chondroitin sulfate proteoglycans (18) or other, non-ephrin, axon guidance molecules, likely contribute to failure to regenerate. For example, the transmembrane semaphorin Sema4D/CD100 is expressed by myelinating oligodendrocytes and can inhibit neurite outgrowth from cerebellar neurons (38), although the identity of the target axon population for this repellent during development and whether it exerts inhibitory activity upon axonal populations damaged in spinal cord injury remain to be elucidated. Finally, the p75-related TROY/TAI is expressed in adult cortex at higher levels than p75 and may substitute functionally for p75 in the NgR1-signaling complex in the adult (39, 40). Our results suggest that one
additional factor contributing to failure of CST regeneration in Nogo, MAG, NgR1, and p75 knockout mice may be the heretofore undetected influence of ephrin-B3. Goldsmith et al. (41) recently reported robust regeneration and functional recovery of EphA4 knockout mice after lateral hemisection of the thoracic spinal cord, despite the presence of unperturbed NgR1/p75 signaling. This report complements our present finding that the ephrin-B3/EphA4 system is active in myelin inhibition, regeneration, and functional recovery after SCI. Goldsmith et al. (41) report that EphA4 is produced by reactive astrocytes after SCI, and in EphA4 mutants, reduction of the glial reactivity results in reduced scarring. Thus, up-regulation of this and other Ephs in astrocytes and in myelin may make an important inhibitory contribution in SCI although whether by direct interaction with injured axons or by indirect modulation of the glial scar remains to be established. Our in vitro data indicate that recombinant EphA4/Fc improves growth on myelin (Fig. 4). However, these studies were not performed in the presence of reactive astrocytes and therefore do not provide a direct comparison with the in vivo data. Additional in vitro reconstitution studies and in vivo studies using mice with multiple gene knockouts, or conditional knockouts, in a bilateral injury model will be required to address the full extent to which both ephrin-B3 and other myelin-based factors contribute to the lack of regeneration in SCI.

Our results provide evidence that molecules with known repulsive activity toward specific axon types during embryonic development maintain inhibitory activity in the adult mouse spinal cord. This finding implies that the regeneration of different populations involved in SCI may each be subject to unique combinations of inhibitory molecules. Such a model is consistent with the selective regeneration of rubrospinal and raphespinal, but not corticospinal, tracts seen in NgR1 knockout mice (37). If so, these differences may be taken into account when assessing the effects of manipulating different molecules to stimulate regeneration in vivo. There is a large body of information on the roles and mechanisms of Eph/ephrin function in developmental axon repulsion that can be leveraged in the study of postdevelopmental injury. Studies that have identified specific domains and residues of Eph receptor tyrosine kinases that are required for their developmental activities can be extended to determine their involvement in inhibitory signaling after injury, thereby revealing targets for regeneration-based therapies. For example, both EphA4 and NgR1/p75 activate the small GTPase RhoA, indicating a potential convergence of their signaling pathways (2). The CST regeneration after SCI observed with pharmacological inhibition of Rho or Rho Kinase (ROCK) may therefore be attributable to partial inhibition both of Eph and NgR1/p75-mediated pathways (42). Thus, if it holds true that regeneration after injury recapitulates developmental axon growth, then our report represents a step toward the use of the tools of developmental biology to combat the lack of regeneration in the CNS.

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