NGF Controls Axonal Receptivity to Myelination by Schwann Cells or Oligodendrocytes

Jonah R. Chan,1,3 Trent A. Watkins,1,3,* José M. Cosgaya,1 ChunZhao Zhang,1 Lian Chen,1 Louis F. Reichardt,2 Eric M. Shooter,1 and Ben A. Barres1
1Department of Neurobiology
Stanford University School of Medicine
Fairchild Science Building D235
Stanford, California 94305
2Department of Physiology
University of California, San Francisco
San Francisco, California 94143

Summary

Axons dictate whether or not they will become myelinated in both the central and peripheral nervous systems by providing signals that direct the development of myelinating glia. Here we identify the neurotrophin nerve growth factor (NGF) as a potent regulator of the axonal signals that control myelination of TrkA-expressing dorsal root ganglion neurons (DRGs). Unexpectedly, these NGF-regulated axonal signals have opposite effects on peripheral and central myelination, promoting myelination by Schwann cells but reducing myelination by oligodendrocytes. These findings indicate a novel role for growth factors in regulating the receptivity of axons to myelination and reveal that different axonal signals control central and peripheral myelination.

Introduction

During vertebrate development, selected axons are myelinated by Schwann cells in the peripheral nervous system (PNS) and by oligodendrocytes in the central nervous system (CNS), while other axons remain unmyelinated in both locations. What are the controls for whether or not an axon becomes myelinated, and are the regulatory mechanisms the same in the PNS and the CNS?

It is generally thought that characteristics of the axons themselves are critical for defining whether or not they will become myelinated (Colello and Pott, 1997; Raval-Fernandes and Rome, 1998). Two observations have led to the presumption that the axonal signals that control central myelination are likely to be very similar to those that control peripheral myelination. First, it is thought that nearly all primary sensory and lower motor axons maintain the same myelinated or unmyelinated phenotype along their length through the PNS and CNS. Second, Schwann cells are capable of myelinating CNS axons in certain pathological conditions (Duncan and Hoffman, 1997), transplantation protocols (Blakemore and Franklin, 2000), and in vitro models (Bahr et al., 1991). Together, these results have suggested a model in which myelination by both Schwann cells and oligodendrocytes is controlled by common axonal signals (Colello and Pott, 1997).

Direct evaluation of this model has been difficult, primarily because little is known about the nature of the axonal signals that control myelination or how these signals are regulated. Experimentally increasing axonal target size leads to two changes in peripheral axons: an increase in axon diameter and a switch from the unmyelinated to the myelinated state (Voyvodic, 1989). These concurrent changes may reflect a causal relationship between axon diameter and myelination, although no direct evidence yet exists to prove that an increase in axon diameter is sufficient to induce myelogenesis. Importantly, these experiments demonstrate that axonal myelination signals, whether or not they include axon diameter, are subject to regulation by environmental cues encountered by developing neurons. The identities of these cues are not yet established.

TrkA-expressing DRGs are a particularly interesting system for studying the regulatory mechanisms that specify which axons will become myelinated and which will remain unmyelinated. These DRGs, which are dependent on target-derived NGF for survival early in their development, are thought to mature into the nociceptive neurons that make up 70%–80% of an adult dorsal root ganglion (Ruit et al., 1992). Their axons, which travel in both the peripheral nerves and the spinal cord, become the unmyelinated and the thinly myelinated sensory fibers whose conduction velocities (reflecting their myelination status) fall in the C and Aβ ranges, respectively. The fact that each of these fibers must decide whether or not it will become myelinated, both in the CNS and the PNS, makes these neurons an attractive model for examining the regulation of both central and peripheral myelination.

Here we exploit the maturation of these DRGs to an NGF-independent state to examine the role of NGF in regulating myelination of their axons by Schwann cells and oligodendrocytes. We find that NGF has potent effects on both peripheral and central myelination, and these effects are mediated by changes to the axonal signals that control myelination rather than by direct action on myelinating glia. Contrary to expectation, NGF inversely affected central and peripheral myelination, promoting Schwann cell myelination but inhibiting oligodendrocyte myelination. These findings are inconsistent with the notion that common axonal signals control both central and peripheral myelination and instead imply that distinct, differentially regulated axonal signals promote myelination by oligodendrocytes and Schwann cells.

Results

NGF Promotes Myelination by Schwann Cells

In order to manipulate NGF levels without altering DRG survival, we adapted the myelinating coculture systems developed by Bunge and colleagues (Kleitman et al.,...
Neuron 184

Figure 1. NGF Promotes Myelination of DRGs by Schwann Cells

(A and B) Anti-MBP immunostaining (green) of Schwann cell-DRG cocultures 6 days after addition of ascorbate in the presence of NGF (A) or the NGF scavenger TrkA-Fc (B). Scale bars equal 100 μm.

(C) Western blots of cocultures probed for myelin (P0 and MAG) and Schwann cell (S100β) proteins. β-actin served as a loading control.

(D) Injection of TrkA-Fc (2.5 μg) along the mouse sciatic nerve on the day of birth and 2 days later reduced the expression of the myelin proteins P0 and MAG at postnatal day 4 compared to the saline-injected contralateral nerve (protein levels assessed by Western blot, n = 16 per condition, % saline-injected control ± SEM, *p < 0.003, unpaired t test).

NGF Inhibits Myelination by Oligodendrocytes

To examine whether NGF also regulates central myelination of DRGs, we next seeded purified rat oligodendrocyte precursor cells (OPCs) instead of Schwann cells onto DRGs. In contrast to our Schwann cell findings, the continued addition of NGF during coculture with glia dramatically reduced myelination in OPC-DRG cocultures over 14 days (Figures 2A–2G). In the presence of NGF, there were few MBP-positive oligodendrocytes and these mostly failed to myelinate (Figure 2A). Neutralizing NGF with TrkA-Fc, however, resulted in a dense and entangled meshwork of myelinating oligodendrocytes that was difficult to assess quantitatively by immunostaining (Figure 2B). Many of the oligodendrocytes that could be clearly distinguished exhibited the multiple smooth internodes and the exclusion of MBP from the soma characteristic of mature myelinating oligodendrocytes (Figures 2C–2E), and the presence of compact myelin in these cocultures was confirmed by electron microscopy (Figure 2F). For a more quantitative analysis, we examined the protein levels of early (2',3'-cyclic nucleotide 3'-phosphodiesterase, CNPase), mid (MAG), and late (MBP) markers of oligodendrocyte differentiation (Bansal and Pfeiffer, 1985), indicating that NGF reduces the number of oligodendrocytes generated in these cocultures. Progressively greater differences in the later markers MAG and MBP reveal that NGF also inhibits or delays the maturation of the differentiated oligodendrocytes that are present. Together, these data indicate that NGF inhibits central myelination, at least in part, by reducing the generation and maturation of oligodendrocytes.

We next determined whether NGF could also affect myelination independently of its potent effects on OPC and oligodendrocyte maturation. We acutely purified mature oligodendrocytes from the developing rat brain and assessed their ability to myelinate in the presence or absence of NGF after only 3 days (Figures 2H–2J). The large majority of MBP-positive oligodendrocytes failed to extend processes and myelinate axons in the presence of NGF (Figure 2H). Neutralizing NGF with TrkA-Fc, however, modestly increased the total number

1991), utilizing the fact that embryonic NGF-dependent DRGs mature to an NGF-independent state in vitro as they do in vivo (Tong et al., 1996). We established cultures of purified NGF-dependent DRGs from embryonic day 14 (E14) rats and allowed them to grow for 2–3 weeks in the presence of NGF. At this stage, the DRGs no longer require NGF for survival or maintenance of their axons (Supplemental Figure S1 at http://www.neuron.org/cgi/content/full/43/2/183/DC1). We then seeded purified rat Schwann cells and allowed them to proliferate and align with the axons for 1 week prior to adding ascorbate to initiate myelination.

At this stage, we examined the role of NGF in myelination by either continuing to provide exogenous NGF or by removing NGF from the cocultures. The continued addition of NGF strongly enhanced the amount of myelin in these cocultures, as assessed by immunostaining for myelin basic protein (MBP; Figure 1A) and Western blots for myelin-associated glycoprotein (MAG) and the peripheral myelin protein P0 (Figure 1C). The difference between the presence and absence of exogenous NGF was made more pronounced by the addition of the NGF scavenger TrkA-Fc (Figure 1B) or an anti-NGF blocking antibody (not shown) to neutralize residual NGF or any NGF that may be produced by Schwann cells (Matsuoka et al., 1991). Consistent with our in vitro findings, neutralizing NGF by injection of TrkA-Fc along the developing sciatic nerve significantly reduced expression of myelin proteins in vivo (Figure 1D).
Differential Axonal Control of Myelination by NGF

Figure 2. NGF Inhibits Myelination of DRGs by Oligodendrocytes

(A and B) Anti-MBP immunostaining (red) of OPC-DRG cocultures 14 days after seeding OPCs in the presence of NGF (A) or the NGF scavenger TrkA-Fc (B). Scale bars equal 200 μm.

(C–E) A higher-magnification view of a myelinating oligodendrocyte in an OPC-DRG coculture double-labeled with CC1 (C) and anti-MBP (D). Scale bars equal 100 μm.

(F) The presence of compact myelin in these cocultures was confirmed by electron microscopy.

(G) Western blots of OPC-DRG cocultures probed for myelin (MBP, MAG) and oligodendrocyte (CNPase) proteins indicate that NGF reduces oligodendrocyte maturation. β-actin served as a loading control.

(H–J) NGF also limits myelination by differentiated oligodendrocytes. Cocultures of DRGs seeded with acutely purified oligodendrocytes examined by anti-MBP immunostaining (red) after 3 days. Scale bars equal 200 μm.

(H) In the presence of NGF, many MBP-positive cells failed to extend processes, while others extended processes that failed to myelinate.

(I) In the presence of TrkA-Fc, many oligodendrocytes myelinated DRG axons only 3 days after seeding.

(J) Myelinating GalC^A2B5^ oligodendrocytes were quantified by counting the proportion of MBP^+^ cells with at least two myelinating processes (16 fields/coverslip, 6 coverslips/condition, fold-change from NGF condition ± SD, *p < 0.001, unpaired t test).

Figure 2. NGF Inhibits Myelination of DRGs by Oligodendrocytes

(A and B) Anti-MBP immunostaining (red) of OPC-DRG cocultures 14 days after seeding OPCs in the presence of NGF (A) or the NGF scavenger TrkA-Fc (B). Scale bars equal 200 μm.

(C–E) A higher-magnification view of a myelinating oligodendrocyte in an OPC-DRG coculture double-labeled with CC1 (C) and anti-MBP (D). Scale bars equal 100 μm.

(F) The presence of compact myelin in these cocultures was confirmed by electron microscopy.

(G) Western blots of OPC-DRG cocultures probed for myelin (MBP, MAG) and oligodendrocyte (CNPase) proteins indicate that NGF reduces oligodendrocyte maturation. β-actin served as a loading control.

(H–J) NGF also limits myelination by differentiated oligodendrocytes. Cocultures of DRGs seeded with acutely purified oligodendrocytes examined by anti-MBP immunostaining (red) after 3 days. Scale bars equal 200 μm.

(H) In the presence of NGF, many MBP-positive cells failed to extend processes, while others extended processes that failed to myelinate.

(I) In the presence of TrkA-Fc, many oligodendrocytes myelinated DRG axons only 3 days after seeding.

(J) Myelinating GalC^A2B5^ oligodendrocytes were quantified by counting the proportion of MBP^+^ cells with at least two myelinating processes (16 fields/coverslip, 6 coverslips/condition, fold-change from NGF condition ± SD, *p < 0.001, unpaired t test).

TrkA Activation Is Sufficient to Mimic the Effects of NGF on Myelination

We aimed to identify the functional target of NGF that mediates its strong and opposite effects in these cocultures. Neurotrophins, acting through either the p75

TrkA-Expressing DRG Axons Mediate the Effects of NGF on Myelination

To determine whether neuronal or glial TrkA was responsible for mediating these NGF effects, we first performed immunostaining for TrkA in the cocultures. Whereas nearly all neurons were clearly TrkA positive, almost no glia were immunopositive for TrkA (not shown). In addition, we found no influence of NGF on the prolifera-
Figure 3. TrkA, not p75NTR, Mediates the Effects of NGF on Myelination

(A–C) p75NTR is not required for the NGF-mediated enhancement of Schwann cell myelination. Dissociated DRG explants from p75NTR−/− mice fail to myelinate robustly without NGF (B), as determined by immunostaining for MBP (green) and Western blots (C) for myelin and Schwann cell proteins (P0, MAG, and S100β). Scale bars equal 100 μm.

(D–F) Glial p75NTR is not required for the NGF-mediated inhibition of oligodendrocyte myelination.

(G and H) TrkA-activating antibodies (RTA, 50 μg/ml) are sufficient to mimic NGF in both types of myelinating cocultures. Anti-NGF neutralizing antibodies were added to block endogenous NGF, and mouse and rabbit IgG were used as controls for anti-NGF and RTA, respectively.

Discussion

NGF Regulates the Axonal Signals that Control Myelination of TrkA-Positive DRGs

Neurotrophins have many important effects on the phenotype of developing neurons, controlling cell survival, differentiation, neurite outgrowth, and excitability, as well as synaptic plasticity and function (Lewin and Barde, 1996). The results presented here reveal a novel function for NGF in neural development, the regulation of the axonal signals that control myelination. We find that NGF, acting through neuronal TrkA, profoundly influences myelination of DRG axons by both Schwann cells and oligodendrocytes. These observations have implications for the role of neurotrophins in controlling myelination and the role of growth factors in regulating axonal signals.

The present findings reveal a second category of functions for neurotrophins in regulating myelination. Our previous results established that neurotrophins directly.
Figure 4. The Effects of NGF Are Mediated by Changes to the Axonal Signals that Control Myelination of TrkA-Positive DRGs

(A–E) The effects of NGF on myelination require TrkA-positive DRGs. (A and B) No effects of NGF or TrkA-Fc on the expression of myelin proteins in cocultures of Schwann cells (A) or cocultures of OPCs (B) with BDNF-dependent DRGs, as assessed by Western blot.

(C–E) No effects of NGF or TrkA-Fc on the numbers of oligodendrocytes or myelin internodes in cocultures of OPCs with BDNF-dependent DRGs, as assessed by immunostaining for MBP (red).

(F–H) Exposure of NGF to TrkA-expressing DRG soma or axons results in inhibition of oligodendrocyte myelination. (F) TrkA-expressing DRGs in the somal compartment of Campenot chambers extend axons through a vacuum grease seal to the neighboring chamber. OPCs were seeded in the axonal chamber only.

(G) Extensive myelination (MBP; green) of TrkA (red)-expressing axons is only seen when TrkA-Fc is present in both the axonal and somal compartments.

(H) The number of nonmyelinating MBP-positive oligodendrocytes and myelin segments were quantified by counting 6 fields/chamber, 4 chambers/condition (cells or myelin segments/field ± SEM). *p < 0.01 versus NGF in both compartments and †p < 0.05 versus TrkA in both compartments (Student-Newman-Keuls post hoc comparison after one-way ANOVA).

regulate the development of myelinating glia. Neurotrophin-3 (NT3) helps to promote the proliferation of purified OPCs (Barres et al., 1994) and the survival of oligodendrocytes (Barres et al., 1993). In addition, NT3, acting through glial TrkC, inhibits myelination by Schwann cells (Chan et al., 2001) and enhances their migration (Yamauchi et al., 2003). In contrast, BDNF, working through p75<sup>NTR</sup>, enhances peripheral myelination (Cosgaya et al., 2002). The data presented here demonstrate that neurotrophins also profoundly influence oligodendrocytes and Schwann cells indirectly by regulating the axonal signals that control their development. Collectively, these results indicate that neurotrophins are important regulators of myelination in two distinct ways: by direct action on myelinating glia and by regulation of axonal signals.

In addition to this new role for neurotrophins, our data provide the first unambiguous evidence that a specific
growth factor, NGF, can potently regulate myelination of one particular population of neurons, TrkA-expressing DRGs. This finding suggests that the extracellular cues that regulate axonal myelination signals, like those that control early neuronal survival decisions, differ for various populations of neurons, based on their complement of receptors. Two recent reports may reflect a comparable role for other growth factors in regulating the axonal signals that control myelination of specific axons. Over-exposure to glial cell line-derived neurotrophic factor (GDNF), a growth factor important in the development of a subset of nociceptors, increases the proportion of those axons that are myelinated in the PNS, although direct effects of GDNF on Schwann cells cannot be excluded (Hoke et al., 2003). In addition, BDNF-deficient mice exhibit hypomyelination of the optic nerve, suggesting that BDNF influences the axonal signals that direct myelination of TrkB-expressing retinal ganglion cell axons (Cellerino et al., 1997). While it is not certain that these two observations reflect effects of GDNF and BDNF on axons rather than glia, together they imply that the importance of NGF in controlling myelination of TrkA-expressing DRGs may point to a general role for individual growth factors in regulating the myelination signals of specific axons.

The ability of extracellular cues to regulate the axonal signals for myelination has important implications not only for understanding the normal development of myelin but also for comprehending the failure of remyelination in peripheral neuropathies and demyelinating CNS disorders such as multiple sclerosis. For instance, could reactive gliosis or an inflamed CNS environment trigger the production of growth factors like NGF that make particular axons unresponsive to remyelination by oligodendrocytes, perhaps by inducing the expression of Notch1 ligands (Wang et al., 1998)?

**NGF Inversely Regulates Myelination by Oligodendrocytes and Schwann Cells**

It has generally been thought that there is a common axonal signal (or set of signals) that controls myelination by both Schwann cells and oligodendrocytes (Colello and Pott, 1997). This model is based on the observation that axons usually maintain the same myelinated or unmyelinated status in the CNS as they do in the PNS, as well as the finding that Schwann cells can, in some circumstances, myelinate central axons (Blakemore, 1977) and oligodendrocytes peripheral axons (Weinberg and Spencer, 1979). Together, these observations demonstrate that those axons that present signals that promote myelination by oligodendrocytes also frequently present signals that promote myelination by Schwann cells. The prevailing hypothesis has been that this consistent pairing along the same axons of the signals for central myelination with the signals for peripheral myelination reveals that these signals are in fact the same or very similar. Our unexpected results that NGF inversely regulates central and peripheral myelination are inconsistent with this dominant view, since a single growth factor cannot simultaneously enhance and suppress a common signal. This experimental uncoupling of the conditions under which an axon promotes myelination by Schwann cells and the conditions under which an axon promotes myelination by oligodendrocytes provides evidence that, instead of simply presenting a common set of signals to control myelination along their entire course through the PNS and CNS, axons can specifically modulate their receptivity to myelination by Schwann cells or oligodendrocytes.

Remarkably, despite the fact that peripherally myelinated axons typically continue to be myelinated after passing into the CNS, our data indicate that central and peripheral axonal signals are not always regulated in the same way by a single extracellular cue, at least in the case of TrkA-expressing DRG axons. This raises the question of how, in the context of a potent NGF signal that inversely regulates central and peripheral myelination, some of these axons, the Aδ fibers, are myelinated in both the CNS and PNS (Rethelyi et al., 1982). Although we (Supplemental Figures S3A and S3B at http://www.neuron.org/cgi/content/full/43/2/183/DC1) and others (Korsching and Thoenen, 1985) have found that NGF is more highly expressed in the early postnatal sciatic nerve than in the spinal cord, our finding that NGF regulates axonal signals throughout the DRG makes it unlikely that axons simply respond to local differences in NGF levels to present different signals in the PNS and CNS. We do, however, find that NGF levels in the sciatic nerve decrease in the second postnatal week, suggesting a potential explanation for the relatively late myelination of TrkA-positive fibers within the spinal cord (Supplemental Figures S3C and S3D; Schwab and Schnell, 1989). In this model, peripheral myelination proceeds when NGF levels are high in the sciatic nerve, followed by myelination in the CNS after peripheral NGF expression drops. This hypothesis provides a potential resolution for how Aδ fibers can promote both central and peripheral myelination despite the inverse effects of NGF on axonal signals.

This proposal, however, seems to offer little insight into how TrkA-expressing C fibers remain unmyelinated in both the PNS and the CNS. One possibility is that not all Aδ and C fibers maintain the same myelination status in both the PNS and CNS after all. Consistent with the hypothesis that a fiber that receives relatively little NGF should both inhibit peripheral myelination and promote central myelination, a fraction of C fibers acquire myelin only after passing into the CNS (Berthold et al., 1993). It is also likely that additional extracellular cues in the CNS or PNS can modulate the effects of NGF on selected axons so that the appropriate myelinated or unmyelinated phenotype is attained for each fiber along its entire length. In any case, the inverse regulation of central and peripheral myelination by NGF indicates that axons do not simply express or suppress a universal myelination signal to dictate whether or not local glia myelinate them. The precise mechanisms by which Aδ and other myelinated sensory and motor fibers ultimately promote (and unmyelinated fibers ultimately inhibit) myelination in both the CNS and the PNS will be an intriguing topic of future study.

**What Are the Identities of the Axonal Signals that Control Myelination?**

Despite the prominent role for axonal signals in dictating the development of myelinating glia, the identities of
myelination, with ATP inhibiting myelination by
Experimental Procedures
fects are a few previously described mediators of neu-
neuronal-glial interactions. The neuregulins are axonal signals that have complex, context-dependent effects at multi-
ple developmental stages of both Schwann cells (Garratt et al., 2000; Michailov et al., 2004) and oligodendrocytes
(Adikofer and Lai, 2000; Colognato et al., 2002). Pu-
rinergic compounds released by DRGs in an activity-
dependent manner also influence both peripheral and
central myelination, with ATP inhibiting myelination by
Schwann cells (Stevens and Fields, 2000) and adenosine
promoting differentiation of oligodendrocytes (Stevens et al., 2002).

Among the many potential candidates for these ef-
fects are a few previously described mediators of neu-
ron-glial interactions. The neuregulins are axonal signals that have complex, context-dependent effects at multi-
ple developmental stages of both Schwann cells (Garratt et al., 2000; Michailov et al., 2004) and oligodendrocytes
(Adikofer and Lai, 2000; Colognato et al., 2002). Pu-
rinergic compounds released by DRGs in an activity-
dependent manner also influence both peripheral and
central myelination, with ATP inhibiting myelination by
Schwann cells (Stevens and Fields, 2000) and adenosine
promoting differentiation of oligodendrocytes (Stevens et al., 2002).

Another possible mechanism involves an increase in
axon diameter, at least in the promotion of peripheral
myelination. NGF stimulates an increase in axon diam-
eter (Markus et al., 2002), and there is a tight correlation
between axon diameter and the degree of myelination
both in vivo and in vitro (Windebank et al., 1985). This
strong connection has led to speculation that an in-
crease in axon diameter is the key axonal signal that
induces myelination (Voyvodic, 1989). This interpreta-
tion, while plausible, is not the only potential explana-
tion for this correlation. First, myelination itself causes
an increase in axon diameter (de Waegh et al., 1992; Garcia et al., 2003). Second, NGF may concurrently increase both axon diameter and other axonal signals that pro-
mitate peripheral myelination (Colell and Pott, 1997;
Frah and Dockery, 1998). As a result of these ambigu-
ilities, our data leave open the question of whether an
increase in axon diameter in response to NGF is the
axon signal that is sufficient to induce myelination in
our Schwann cell-DRG cocultures.

Whatever the case may be for Schwann cells, changes
in axon diameter cannot explain all of the effects of NGF.
Despite increasing axon diameter, NGF reduces the
amount of oligodendrocyte myelin. This surprising un-
coupling of two phenomena that are usually tightly cor-
related (increased axon diameter and the promotion of
myelination) provides evidence that not only is an in-
crease in axon diameter insufficient to stimulate myelin-
ation by oligodendrocytes, but axon diameter and cen-
tral myelination signals need not be similarly regulated
by an individual growth factor like NGF.

The definitive characterization of the axonal signals
for myelination, like the identification of growth factors
that control their expression, may offer beneficial in-
sights relevant to promoting remyelination in both multi-
ples sclerosis and peripheral neuropathies. The profound
regulation of these signals by a single growth factor,
NGF, in our improved coculture systems could provide
a helpful experimental foothold for discovering the key
axonal signals that control the development of both
Schwann cells and oligodendrocytes.

Conclusion
The findings we have described reveal a novel role for
NGF in myelination and challenge a long-standing belief
regarding the axonal control of myelination. Previous
studies had suggested that the axonal signals that con-
trol myelination by Schwann cells were likely to be very
similar to those for oligodendrocytes. By demonstrating
that NGF regulates the axonal signals that control my-
elination of TrkA-positive DRGs and that Schwann cells
and oligodendrocytes respond strongly and oppositely
to these NGF-regulated signals, our results indicate that
there is in fact differential axonal control of central and
peripheral myelination. This unexpected finding pro-
vides an interesting new framework for understanding
the normal regulation of myelination and for developing
new therapies to promote remyelination after disease
or injury.

Experimental Procedures
Step-by-step protocols for all procedures are available upon request
to barres@stanford.edu or eshooter@stanford.edu.

Purified Schwann Cell-DRG Cocultures
Schwann cell-DRG cocultures were established as described pre-
viously (Chan et al., 2001), based on classic techniques (Kleitman
er al., 1991). Briefly, NGF-dependent, TrkA-expressing DRGs from
embryonic day 14 (E14) Sprague-Dawley rats were purified and
maintained in the presence of NGF (100 ng/ml; Serotec) for 2–3
weeks prior to seeding of Schwann cells, OPCs, or oligodendro-
cytes. Alternatively, the minority population of BDNF-dependent,
TrkB-expressing DRG neurons were isolated and maintained by the
addition of BDNF (100 ng/ml; Regeneron Pharmaceuticals) in the
absence of NGF, with a 7-fold greater initial plating density and
additional fluoroodeoxyuridine (FUD) treatment necessary to attain
equivalently dense and pure DRGs.

Purified postnatal rat sciatic nerve Schwann cells were seeded
directly onto DRGs, and myelination was initiated after 1 week by
the addition of ascorbic acid (50 µg/ml). NGF (100 ng/ml), the TrkA-
Fc receptor chimera (1 µg/ml; Regeneron Pharmaceuticals), the anti-
NGF blocking antibody (clone 27/21, 1 µg/ml; Chemicon Interna-
tional), or the anti-TrkA antibody (RTA, 50 µg/ml) were added at the
time of ascorbate addition. Mouse IgG (Sigma) and rabbit IgG
(Sigma) were used as controls for 27/21 and RTA, respectively. In
some experiments, recombinant human Fc (1 µg/ml; R&D Systems)
was added along with NGF as a control for TrkA-Fc. Factors or
neutralizing agents were replenished with a medium change every
3 days.

Myelinating Mouse DRG Dissociated Explant Cultures
Dissociated explants were established using E14 mice (wild-type
or p75<sup>−/−</sup> mice in a 129S3/SvImJ background; obtained from The
Jackson Laboratory) as a modification from the traditional explant
cultures as described previously (Cosgaya et al., 2002). Axonal pro-
cesses and endogenous Schwann cells were allowed to grow and
establish themselves for approximately 7 days prior to the initiation
of myelination with ascorbic acid.

Preparation of Immunopanning Dishes
Petri dishes containing secondary antibodies (goat anti-mouse
IgG(H+L) or anti-mouse IgM; Jackson Laboratories) or Protein A
(16.6 µg/ml; Sigma; for anti-GalC) were incubated overnight. Dishes
were rinsed and incubated at room temperature with primary anti-
odies. Primary antibodies included Ran-2 hybridoma supernatant,
A2B5 ascites, O4 hybridoma supernatant, anti-GalC (Ranscht et al.,
1982), MRC-OX7 anti-Thy1.2 hybridoma supernatant, and mouse anti-mouse Thy1.2 (Serotec).

**Purified OPC-DRG Cocultures**

Oligodendrocyte precursor cells (OPCs) were purified to >99.5% homogeneity from 5- to 8-day-old (P7-P8) rat brain cortices by immunopanning as previously described (Wang et al., 2001). Briefly, cerebral hemispheres were diced and digested with papain at 37°C. Following gentle titration, cells were resuspended in a panning buffer containing insulin (5 μg/ml) and then incubated at room temperature sequentially on three immunopanning dishes: Ran-2, anti-GalC, and O4. O4+GalC+OPCs were released from the final panning dish with trypsin (Sigma). OPCs were seeded onto coverslips containing purified DRGs in a small volume at a density of 200,000 OPCs per coverslip and incubated overnight to facilitate attachment. The following day, coverslips were transferred into wells containing 2 ml of medium with the appropriate factors (NGF, anti-NGF, TrkA-Fc, or RTA). For purification of p75NTR OPCs, P8-P9 mouse pups were used and the Ran-2 panning dish was replaced with an anti-mouse Thy1.2 dish. Cocultures were maintained for 14–15 days, with fresh medium provided every 3 days.

**Purified Oligodendrocyte-DRG Cocultures**

Mature oligodendrocytes were purified from P10 rat brain cortices, as a modification from the immunopanning protocol for the purification of OPCs. Astrocytes, macrophages, OPCs, and immature oligodendrocytes were negatively selected by sequential incubation on Ran-2 and two A2B5 panning dishes, prior to positive selection of oligodendrocytes by anti-GalC. GalC- A2B5+ oligodendrocytes were released from the final panning dish by treatment with trypsin (Sigma), resuspended in medium, and plated in a small volume on 24-day-old cultures of NGF-purified DRGs, which had been maintained in either NGF (100 ng/ml) or TrkA-Fc (1 μg/ml) 3 days prior to seeding. Cocultures were analyzed by immunostaining for MBP after 2 days. The proportion of mature oligodendrocytes that were myelinating was determined by counting the total number of MBP-immunopositive cells and the number displaying at least two processes that were clearly ensheathing axons over 16 low-magnification fields covering the majority of the axon-dense, DRG soma-sparseregions of each coverslip.

**Western Blot Analysis**

Samples from cocultures and sciatic nerves were prepared for Western blot as described (Cosgaya et al., 2002) and probed with specific antibodies against neurotrophin receptors (anti-TrkB [Transduction Labs] and anti-TrkA [RTA]) and/or mouse monoclonal antibodies against myelin proteins (anti-MAG, anti-MBP, anti-CNPase [Chemicon], and anti-P0 [gift from Juan J. Archelos, Karl-Franzens-Universität Graz, Austria]). Additionally, an antibody against β-actin (Sigma) was used as a loading control, and an antibody against S100β (DakoCytomation) was used as a control to ensure similar numbers of Schwann cells in different conditions. The three lanes under each experimental condition in the figures are from three separate wells with the respective cocultures. Blots are representative of multiple experiments and were typically stripped and reprobed.

**Immunocytochemistry**

Immunocytochemistry of Schwann cell-DRG cocultures was performed as described (Cosgaya et al., 2002). Briefly, cultures were fixed and dehydrated and then were permeabлизed and blocked in 20% goat serum and 0.1% Triton X-100. Myelin internodes were visualized with a mouse monoclonal anti-MBP antibody (Chemicon, 1:500) followed by incubation with Alexa Fluor 488 anti-mouse IgG secondary antibodies (Molecular Probes, 1:2000). Cellular nuclei were examined with DAPI. Immunocytochemistry of OPC-DRG and oligodendrocyte-DRG cocultures was performed similarly, with the primary differences being the use of 50% goat serum and 0.4% Triton X-100 for blocking and permeabilization, respectively. In addition to immunostaining for MBP to visualize myelin (IgG3, Chemicon, 1:500), cocultures were counterstained with mAb clone O1 (APC-7, OncoGene Research Products, 1:100) to simplify identification of axons and oligodendrocyte cell bodies as well as DAPI to label nuclei (not shown). Consequently, Alexa 488-goat anti-mouse IgG2a and Alexa 594-goat anti-mouse IgG1 (Molecular Probes, 1:500) were used as secondary antibodies.

**Injections in Mouse Sciatic Nerves**

Injections were performed as previously described (Cosgaya et al., 2002). TrkA-Fc (2.5 μg) was injected subcutaneously, starting from the caudal portion of the greater trochanter region and running parallel along the sciatic nerve. The contralateral leg served as a control with the injection of saline. Injections were performed on newborn mouse pups (129/Sv/SvJmJ) and reinfected 2 days later. Finally, nerves were extracted and processed for Western analysis. β-actin was used as an internal control but was measured separately from the myelin proteins due to differences in the sensitivity of detection. Injections with other Trk-Fc constructs were used to demonstrate specificity of TrkA-Fc action (Cosgaya et al., 2002).

**Electron Microscopy**

Electron microscopy was performed at The Cell Sciences Imaging Facility at Stanford University Medical Center. Briefly, 15-day-old OPC-DRG cocultures were fixed in 2% glutaraldehyde in sodium cacodylate buffer at 4°C. Following treatment with 1% osmium tetroxide and 1% uranyl acetate, samples were embedded in epon. Sections were taken between 75–90 nm, picked up on formvar/carbon-coated 75 mesh Cu grids, and stained for 20 s in 1:1 saturated uranyl acetate in acetone followed by staining in 0.2% lead citrate. Images were acquired with the JEOL 1230 TEM at 80 kV.

**Camperon Chambers**

Camperon chambers (Tyler Research Corp.) were prepared as previously described (Camperon, 1992). DRGs were plated into a side compartment, and axons were allowed to grow at least 3 weeks into the second compartment in the presence of NGF to select TrkA-expressing DRGs and cycles of FdU to eliminate nonneuronal cells. OPCs were then seeded into the axonal compartment and cocultures were maintained for 13 days with either NGF in both compartments, TrkA-Fc in both compartments, or a combination of NGF and TrkA-Fc in either of the compartments. The integrity of the seal that prevents bulk flow of small solutes between compartments was examined quantitatively throughout the experiment by monitoring the ability of phenol red in the somal compartment to diffuse to the phenol red-free axonal compartment. Media collected during feeding every 3 days was measured for its absorbance at 560 nm, with any absorbance from the axonal compartment indicating leakage of phenol red from the somal compartment. Only those chambers that displayed both significant axon growth into the second compartment and no signs of leakage were further analyzed for the numbers of myelin segments and nonmyelinating oligodendrocytes by immunostaining for MBP and TrkA.

**Acknowledgments**

We thank Regeneron Pharmaceuticals for generously providing reagents, John J. Perrino for his expertise with electron microscopy, and Denise A. Chan for helpful comments. B.A.B. acknowledges the helpful contribution of discussions with colleagues at the Myelin Repair Foundation. This work was supported by grants from NIH (NS04270 to E.M.S. and R01 EY10257 to B.A.B.), the Muscular Dystrophy Association (to E.M.S.), the McGowan Charitable Trust (to E.M.S.), an NSRA postdoctoral fellowship (to J.R.C.), and an HHMI predoctoral fellowship (to T.A.W.).

Received: February 17, 2004
Revised: May 19, 2004
Accepted: June 16, 2004
Published: July 21, 2004

**References**


Differential Axonal Control of Myelination by NGF


