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A TNF Receptor Family Member, TROY, Is a Coreceptor with Nogo Receptor in Mediating the Inhibitory Activity of Myelin Inhibitors

Report

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Summary

A major obstacle for successful axon regeneration in the adult central nervous system (CNS) arises from inhibitory molecules in CNS myelin, which signal through a common receptor complex on neurons consisting of the ligand-binding Nogo-66 receptor (NgR) and two transmembrane coreceptors, p75 and LINGO-1. However, p75 expression is only detectable in subpopulations of mature neurons, raising the question of how these inhibitory signals are transduced in neurons lacking p75. In this study, we demonstrate that TROY (also known as TAJ), a TNF receptor family member selectively expressed in the adult nervous system, can form a functional receptor complex with NgR and LINGO-1 to mediate cellular responses to myelin inhibitors. Also, both overexpressing a dominant-negative TROY or presence of a soluble TROY protein can efficiently block neuronal response to myelin inhibitors. Our results implicate TROY in mediating myelin inhibition, offering new insights into the molecular mechanisms of regeneration failure in the adult nervous system.

Introduction

Damaged axons in the adult CNS are unable to spontaneously regenerate upon injury, in contrast to neurons in the peripheral or embryonic nervous system. Such a failure in regeneration is believed to arise from both a decline in the intrinsic growth state of mature neurons, as well as the presence of a nonpermissive environment in the injured adult CNS preventing axon growth (reviewed in Schwab and Bartholdi, 1996; Horner and Gage, 2000). In addition to the physical barrier formed by the astrocytic scar tissue that develops at the lesion site (Morgenstern et al., 2002), inhibition by both extracellular matrix proteoglycans secreted by reactive glial cells and inhibitory molecules associated with CNS myelin have been shown to restrict axon regeneration in the adult nervous system (Schwab and Bartholdi, 1996). To date, three major inhibitory molecules in myelin have been characterized, including Nogo-A, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp), which collectively account for the majority of the inhibitory activity in myelin (reviewed by Filbin, 2003; Yiu and He, 2003; He and Koprivica, 2004). Though structurally distinct, all three myelin inhibitors signal through a common receptor complex in neurons, which includes the ligand binding Nogo-66 receptor (NgR), and two signal-transducing binding partners, p75 and LINGO-1, to exert its effects on the neuronal cytoskeleton and prevent axon growth (Domeniconi et al., 2002; Liu et al., 2002; Wang et al., 2002a, 2002b; Mi et al., 2004).

Although this receptor complex has been extensively scrutinized as a convergence point for the inhibitory signals of CNS myelin, it remains unclear whether other coreceptors may also be involved. Interestingly, while NgR and LINGO-1 are widely expressed in the adult CNS (Figure 1; Wang et al., 2002c; Mi et al., 2004), p75 expression is much more restricted. Initially identified as a low-affinity receptor for neurotrophins, p75 is highly expressed in the developing nervous system during periods of axon outgrowth and dendritic arborization, but decreases over the postnatal period and adulthood (reviewed by Roux and Barker, 2002; Chao, 2003). In fact, only subpopulations of a few types of mature neurons, such as subsets of dorsal root ganglion (DRG) neurons, have been shown to express p75 in adults (McMahon et al., 1994; Wright and Snider, 1995; Averill et al., 1995). Furthermore, though neurons from p75 mutant mice are less inhibited by myelin components in an in vitro neurite outgrowth assay, a significant amount of residual inhibitory response remains (Wang et al., 2002b). Consistent with this, studies with p75 mutant mice have failed to show any significant promotion of regeneration in an in vivo spinal cord injury model (Song et al., 2004). Together, these results suggest the possible presence of other functional homologs of p75 that allow neurons lacking this coreceptor to remain responsive to myelin inhibition.

p75 is a member of the TNF receptor (TNFR) family, a class of type I transmembrane proteins bearing significant homology in their extracellular domains, which contain one or more cysteine-rich domains (CRDs) characteristic of this receptor superfamily (reviewed by Locksley et al., 2001; Dempsey et al., 2003). However, their cytoplasmic tails are highly divergent, with a broad range of affinities for intracellular adaptors allowing for different signaling specificities. While many TNFR family members have been well characterized in the context of injury and inflammation (Locksley et al., 2001; Dempsey et al., 2003), recent studies are just beginning to unveil their role in neuronal functions (Neumann et al., 2002; Desbarats et al., 2003; Demjen et al., 2004). In this study, we provide evidence for the involvement of TROY (also known as TAJ), a newly identified member of the TNF receptor family expressed in the adult nervous system (Kojima et al., 2000), as a functional homolog of p75 in mediating myelin inhibition. Like p75, TROY can form a receptor complex with NgR and LINGO-1 and mediate the outgrowth inhibition and signaling pathways triggered by myelin inhibitors. Moreover, blocking the interaction of TROY with NgR by different dominant-negative approaches efficiently abolished neuronal responses to myelin inhibitors. These results implicate an important role for TROY in mediating

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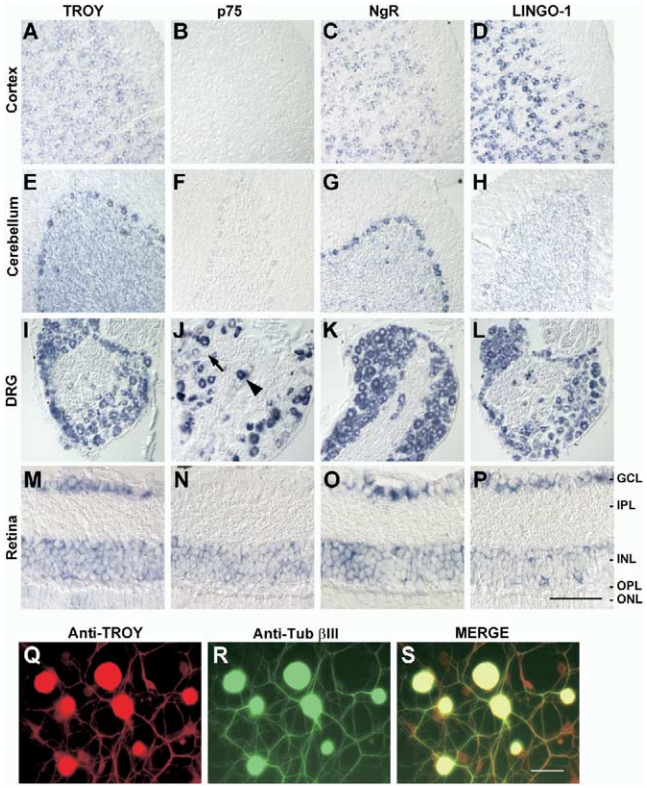

Figure 1. Expression of TROY, p75, NgR, and LINGO-1 mRNA in the Adult CNS

(A–L) In situ hybridization with antisense probes demonstrating the expression pattern of TROY (A, E, I, and M), p75 (B, F, J, and N), NgR (C, G, K, and O), and LINGO-1 (D, H, L, and P) in the adult mouse CNS, including cerebral cortex (A–D), cerebellum (E–H), dorsal root ganglia (I–L), and retina (M–P). p75 expression is limited to a subpopulation of DRG cell bodies (arrowhead), but undetectable in other DRG cell bodies (arrow), cortical neurons, cerebellar granule neurons, and most RGCs. In contrast, TROY, NgR, and LINGO-1 are expressed in most populations of cells in these CNS tissues. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer. Scale bar, 150 μM (cortex, cerebellum, and DRG); 50 μM (retina). (Q–S) Immunocytochemistry with anti-TROY antibodies demonstrating TROY expression on cell bodies and neurites of cultured DRG neurons. Neurons were costained with anti-TROY and anti-β-tubulin antibodies. Scale bar, 50 μM.

the inhibitory effects of myelin components on axon regeneration.

Results

Both p75-Positive and p75-Negative Neurons Are Inhibited by Myelin Inhibitors

The fact that p75 is not expressed in all mature neurons raises the question of why neurons lacking p75 expression also fail to regenerate after axonal injury. One possibility is that these neurons are inhibited in an NgR-independent manner. Alternatively, other functional equivalents of p75 may allow these neurons to remain responsive to myelin inhibitors. To distinguish these possibilities, we employed DRG neurons from adult rats. Consistent with previous reports (McMahon et al., 1994; Wright and Snider, 1995), we found that only 60%–70% of DRG neurons expressed detectable levels of p75 (p75+) and that this subpopulation was mostly distinct from those positively stained by Isolectin B4 (IB4+), a marker for cell surface glycoconjugates on neurons lacking p75 (Averill et al., 1995; Molliver et al., 1997). When cultured on immobilized inhibitory substrates such as the extracellular domain of Nogo-A (Nogo-66) or OMgp, most DRG neurons exhibited reduced neurite outgrowth regardless of p75 expression (see Figure S1A in the supplemental material available with this article online). This neurite outgrowth inhibition is mediated by NgR receptor complexes, as overexpression of a truncated NgR that retains the ligand binding domain but not the coreceptor binding domain (Wang et al., 2002b) resulted in enhanced neurite outgrowth on these inhibitory substrates in more than 90% of the DRG neurons including both p75–/IB4+ cells and p75+/IB4– cells (Figure S1B). Similarly, overexpressing a truncated p75 lacking its intracellular domain also allowed significant neurite outgrowth in most DRG neurons, including the p75–/IB4+ cells (Figure S1B). These results suggest that the response to myelin inhibitors in neurons lacking p75 expression may be mediated by other potential p75 functional homologs that can also be blocked by the truncated p75 in a dominant-negative manner.

In previous studies, although we found that DRG neurons from p75 knockout mice were able to overcome the inhibitory effects of immobilized myelin substrates as compared to wild-type neurons (Wang et al., 2002b), average neurite lengths were still shorter than those on PDL, indicating some residual response to myelin inhibition. Yet, when the response of different subpopulations of DRG neurons (IB4+ and IB4–) from $p75^{-/-}$ mice were quantified separately, we found that the majority of the cells that could overcome myelin inhibition were IB4–, while most of the IB4+ neurons, which would represent the p75– population from wild-type animals, remained inhibited by myelin inhibitors (Figure S1C). Even within the IB4– population of DRG neurons from $p75^{-/-}$ mice, average neurite lengths on myelin substrates were still less than those on PDL

(Figure S1C), suggesting that these neurons retained some residual response to myelin inhibitors. Together, these results provide further support for the potential existence of other functional homologs of p75.

Expression of TROY in Adult CNS

In addition to p75, several other known members of the TNFR family, including TROY (Kojima et al., 2000), CD40 (Tan et al., 2002), DR6 (Pan et al., 1998), and TNFR1A (Yan et al., 2003), have been reported to be expressed in the mature nervous system. To examine the possibility of these molecules as potential p75 functional homologs, we first examined their expression patterns in the adult CNS by in situ hybridization. We found that several TNFR family members, including TROY (Figures 1A, 1E, 1I, and 1M), CD40, and DR6 (data not shown) are highly expressed in most parts of the adult CNS, including the cerebral cortex and cerebellum, as well as most DRG and retinal ganglion neurons (RGCs). This was in sharp contrast to the expression of p75, which was limited to subpopulations of DRG neurons (Figure 1J) and undetectable in cerebral cortex, cerebellum, and most RGCs (Figures 1B, 1F, and 1N). Consistent with previous studies (Wang et al., 2002c; Mi et al., 2004), both NgR (Figures 1C, 1G, 1K, and 1O) and LINGO-1 (Figures 1D, 1H, 1L, and 1P) are broadly expressed across most of these CNS regions. Immunostaining of DRG neurons in culture also showed colocalization of signals from both anti-TROY and anti-β-tubulin antibodies, demonstrating the expression of TROY in both cell bodies and neurites of these neurons (Figures 1Q–1S). Together, these results are consistent with the notion that TROY can act as a coreceptor of NgR.

TROY Can Bind NgR to Form a Receptor Complex

To determine whether TNFR members expressed in the CNS can substitute for the role of p75 as an NgR coreceptor, we first determined whether they could form a receptor complex with NgR and LINGO-1. Using a cell surface binding assay, we found that, like p75, an alkaline phosphatase (AP) fusion protein containing the extracellular domain of TROY (AP-TROY) can bind to NgR-expressing COS-7 cells (Figures 2A and 2B) with an apparent kD of ~10 nM for AP-TROY and ~80 nM for AP-p75. This is further supported by the fact that neither AP-p75 nor AP-TROY can bind to a truncated form of NgR (DN-NgR), which retains its ligand binding domain but lacks the unique carboxy-terminal region required for coreceptor binding (Figure 2A), suggesting that p75 and TROY may bind to a similar region of NgR. In contrast, neither CD40-AP nor DR6-AP showed any detectable binding in the nanomolar range (Figure 2C). When transfected into 293T cells, TROY can be coimmunoprecipitated with NgR and LINGO-1 (Figure 2D), demonstrating that TROY, like p75, is capable of forming receptor complexes with NgR and LINGO-1.

Like p75, TROY and NgR/LINGO-1 Can Confer RhoA Activation in COS-7 Cells

To further examine whether TROY can form a functional receptor complex with NgR and LINGO-1, we examined whether coexpression of NgR, p75, and LINGO-1 can allow nonneuronal cells to respond to myelin inhibitors by activating RhoA, a required step in the signaling pathways for myelin inhibition (Lehmann et al., 1999; Niederost et al., 2002). Consistent with previous observations (Mi et al., 2004), we found that soluble myelin inhibitors can activate RhoA in COS-7 cells cotransfected with NgR, p75, and LINGO-1 (Figure 3B). More importantly, we found that TROY can also substitute for p75 in reconstituting this functional receptor complex with NgR and LINGO-1 in responding to all three myelin inhibitors (Figures 3A–3D). As expected, all three components (NgR, LINGO-1, and p75 or TROY) were required to confer this Rho activation (Figure 3A). These results further support the role of TROY as a functional homolog of p75 in transducing the inhibitory signal of myelin inhibitors.

Blocking TROY Activity Attenuates Neuronal Response to Myelin Inhibitors

If TROY can function as a p75-like NgR coreceptor, one prediction will be that a truncated TROY lacking its intracellular domain, once overexpressed, will compete for binding of endogenous coreceptors like p75 and TROY to NgR, restricting neuronal response to myelin inhibitors in a dominant-negative manner. To test this, we generated recombinant herpes simplex viruses (HSVs) expressing either full-length (FL-TROY) or dominant-negative TROY (DN-TROY). DRG neurons infected with these viruses do not show significant changes in cell survival as indicated by Hoechst 33258 staining and neurite outgrowth on the control PDL substrate (data not shown). However, when expressed in cultured DRG neurons, the truncated, but not full-length, TROY dramatically enhanced neurite outgrowth of DRG neurons on immobilized Nogo-66 (Figure 4A) and other myelin inhibitors (data not shown), including both the IB4+ and IB4– populations.

As our results suggested that DRG neurons from *p75*$^{-/-}$ mice still retained some responsiveness to myelin inhibitors, we next examined whether such residual activity could be blocked by overexpression of DN-TROY. As shown in Figure 4B, DN-TROY resulted in further recovery from the inhibitory effects of Nogo-66 in both IB4+ and IB4– DRG neurons. However, the inhibition was not totally abolished, presumably because expression levels of the protein were not high enough to completely displace the activity of endogenous TROY and other potential coreceptors. Interestingly, expression of FL-TROY resulted in an enhanced inhibitory response of IB4– neurons to Nogo-66 compared to YFP-infected controls (Figure 4B). As these IB4– neurons express p75 in wild-type animals, these results suggest the possibility that overexpression of TROY could rescue the neuronal response to myelin inhibitors in *p75*$^{-/-}$ mice.

Based on these results, we further anticipated that a TROY-Fc fusion protein containing the extracellular domain of TROY, when supplied exogenously, would interfere with receptor complex formation and disrupt the response of cultured neurons to myelin inhibitors. As shown in Figure 4C, inhibition of neurite outgrowth from DRG neurons by Nogo-66 can be neutralized by the

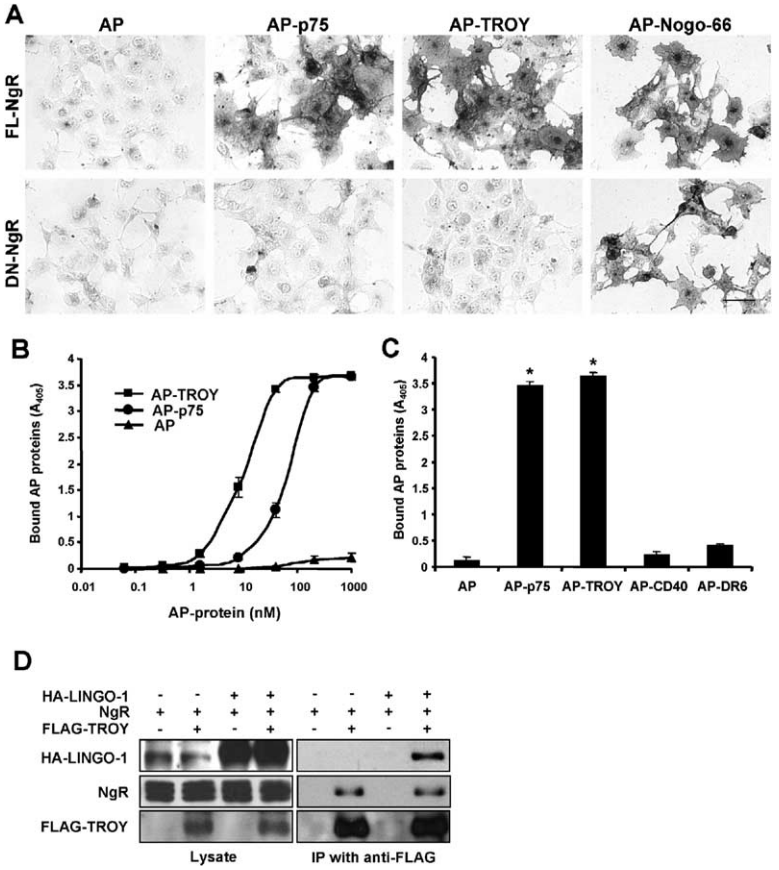

Figure 2. TROY Binds NgR with Similar Affinity as p75

(A) Visualization of cell surface binding of AP, AP-p75, AP-TROY, and AP-Nogo-66 fusion proteins to adherent COS-7 cells expressing full-length or truncated NgR. While AP-Nogo-66 can bind both the full-length and truncated forms of NgR, AP-p75 and AP-TROY selectively binds full-length NgR. Scale bar, 50 μM.

(B) Concentration-dependent cell surface binding of AP, AP-p75, and AP-TROY fusion proteins to adherent COS-7 cells expressing full-length NgR. In comparison to AP, both AP-TROY and AP-p75 bind to NgR in a concentration-dependent manner, with an apparent kD of ~10 ± 2 nM for AP-TROY and ~80 ± 15 nM for AP-p75.

(C) Quantification of cell surface binding of alkaline phosphatase (AP) alone and AP-p75, AP-TROY, AP-CD40, and AP-DR6 fusion proteins (200 nM) to adherent COS-7 cells expressing full-length NgR. While AP-p75 and AP-TROY can bind to NgR, AP-CD40 and AP-DR6 show no significant binding as compared to AP controls. Statistical analysis was done by Student's t test ($p < 0.005$).

(D) NgR, TROY, and LINGO-1 form receptor complexes. Coimmunoprecipitaton of LINGO-1, p75, and NgR1 by anti-FLAG antibody from COS-7 cells coexpressing combinations of NgR, HA-LINGO-1, or FLAG-TROY. Expression of individual proteins was detected by Western blotting analysis with specific antibodies as indicated.

addition of excess recombinant TROY-Fc, but not control IgG, in the immobilized substrate. Similar results were also obtained from experiments using other myelin inhibitors or solubilized myelin as substrates (data not shown). These results further substantiated the involvement of TROY in mediating the inhibitory activity of myelin inhibitors.

Discussion

Until now, available evidence has suggested that the majority of the inhibitory activity associated with CNS myelin signal via a common receptor complex consisting of NgR, LINGO-1, and p75. Yet, in contrast to NgR and LINGO-1, p75 is only expressed in limited populations of mature neurons in the adult CNS, even after injury (Roux and Barker, 2002; Chao, 2003). In the current study, we have demonstrated that TROY, another TNF receptor family member broadly expressed in the adult CNS, can substitute for p75 as a coreceptor of NgR and LINGO-1 and mediate the inhibitory effects of myelin inhibitors. Like p75, TROY binds NgR with similar affinity, and together with LINGO-1 can reconstitute the activation of RhoA, a required step in the signaling pathways of myelin inhibition. Additionally, both overexpression of a truncated form of TROY lacking its intracellular domain and addition of a TROY-Fc fusion protein can block neuronal response to myelin inhibitors in a dominant-negative manner. Together, these results suggest that TROY may act as a functional homolog of p75.

In addition to p75 and TROY, several other TNFR members have also been shown to be expressed in the adult CNS. Our studies, however, failed to demonstrate the ability of these TNFR members to bind NgR with high affinity, suggesting that p75 and TROY may be unique among their relatives in mediating myelin inhibition. Nevertheless, these in vitro binding results do not preclude the possibility that other TNFR members may also act as NgR coreceptors under physiological conditions. Interestingly, although TNFR members had been well characterized in the context of inflammation and cell survival, recent studies are now implicating their involvement in axonal growth as well, although the underlying mechanisms remain unclear. For example, cross-linking Fas (also known as CD95) on cultured sensory neurons has been shown to induce neurite outgrowth (Desbarats et al., 2003). Consistently, functional recovery after sciatic nerve injury is delayed in Fas-deficient mice and accelerated by local administration of antibodies against Fas (Desbarats et al., 2003). On the other hand, TNF seems to inhibit the neurite outgrowth of cultured hippocampal neurons through the TNF receptor, as suggested by studies in TNF receptor-deficient mice (Neumann et al., 2002). It will be important in the future to determine whether these phenomena are mediated by the NgR receptor complex as well.

In light of the structural basis for the ability of NgR to

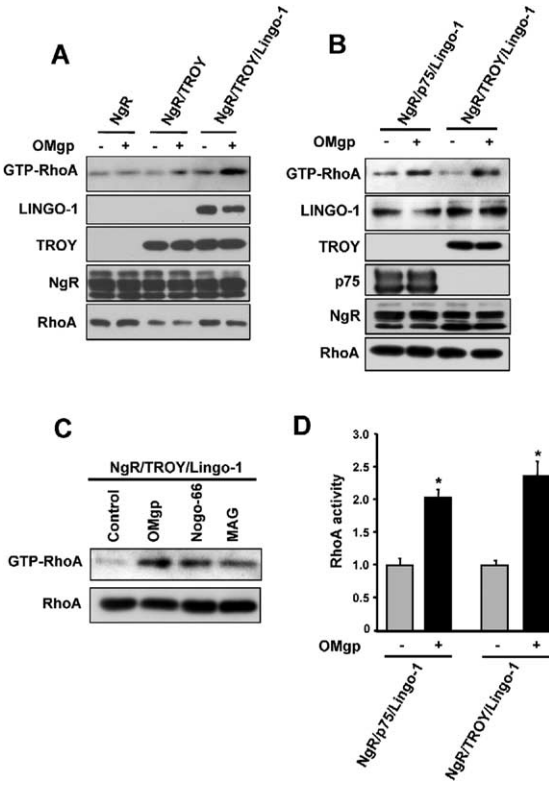

Figure 3. NgR, TROY, and LINGO-1 Expression Confers RhoA Activation by Myelin Inhibitors in COS-7 Cells

(A–C) RhoA activation as detected by GST-RBD pull-down assays in COS-7 cells transfected with plasmids expressing NgR, NgR/TROY, NgR/TROY/LINGO-1 (A), and NgR/TROY/LINGO-1 or NgR/p75/LINGO-1 (B) after treatment with OMgp (100 nM), or NgR/TROY/LINGO-1 after treatment with OMgp, Nogo-66, MAG (all at 100 nM) (C) for 10 min. Expression of individual proteins was shown by Western blot analysis with specific antibodies.

(D) Quantification of RhoA activation in cells expressing either NgR/p75/LINGO-1 or NgR/TROY/LINGO-1 after treatment with OMgp. Results from densitometric analysis are expressed as "fold activation" of basal activity (means ± SEM) and were obtained from three independent experiments. Statistical analysis was done by Student's t test ($p < 0.01$) comparing RhoA activity of cells treated and not treated with OMgp. NgR/TROY/LINGO-1-expressing cells do not show a significant difference in RhoA activation when compared to NgR/p75/LINGO-1-expressing cells.

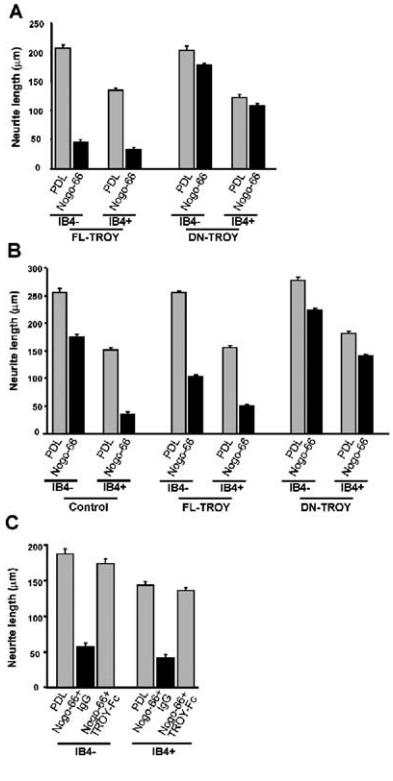

Figure 4. Blocking TROY Activity Attenuates Neuronal Response to Myelin Inhibitors

(A) A truncated TROY lacking its intracellular domain attenuates the neurite outgrowth inhibition by immobilized Nogo-66 in both IB4+ and IB4– DRG neurons. Average neurite lengths (means ± SEM) of rat DRGs infected with HSVs expressing either full-length (FL-TROY) or dominant-negative TROY (DN-TROY) and cultured on PDL or Nogo-66 were quantified. Statistical analysis was done by one-way ANOVA ($p < 0.0001$). All groups grown on Nogo-66 are significantly different from those grown on PDL. For both IB4+ and IB4– neurons cultured on Nogo-66, those infected with DN-TROY viruses are significantly different from those infected with FL-TROY.

(B) Dominant-negative TROY blocks the residual inhibitory response of DRG neurons from $p75^{-/-}$ mice to Nogo-66. Average neurite lengths (means ± SEM) of mouse DRGs infected with HSVs expressing YFP (control), full-length TROY (FL-TROY), or dominant-negative TROY (DN-TROY) on PDL or Nogo-66 were quantified. Statistical analysis was done by one-way ANOVA ($p < 0.0001$). On Nogo-66, both IB4+ and IB4– neurons expressing DN-TROY show significant differences from those expressing YFP (Control) and FL-TROY, and IB4– neurons expressing FL-TROY show a significant difference from IB4- cells expressing YFP.

(C) TROY-Fc blocks the neurite outgrowth inhibition by immobilized Nogo-66 in both IB4+ and IB4– DRG neurons. Average neurite lengths (means ± SEM) of rat DRGs on PDL or Nogo-66 in the absence or presence of excess amounts of TROY-Fc were quantified. Statistical analysis was done by one-way ANOVA ($p < 0.0001$). For groups cultured on Nogo-66, those with the addition of TROY-Fc are significantly different from groups cultured with the addition of control IgG.

interact with multiple binding partners (He et al., 2003; Barton et al., 2003), it is not surprising that it can also interact with more than one coreceptor. Yet, it remains unclear whether these molecules provide functional redundancy or whether they permit distinct signaling properties in different cells. Recent studies suggest that p75 is a component of three distinct receptor platforms (with Trk receptors, NgR/LINGO-1, and sortilin) that bind different ligands (neurotrophins, myelin inhibitors, and proNGF) and, under different conditions, facilitate cell survival, growth inhibition, or cell death, respectively (reviewed by Barker, 2004). Analogously, TNF receptor family members may also be present in different complexes to mediate various functions. While the function of TROY remains to be clarified, determining the portion of p75, TROY, and perhaps other TNF receptors present in NgR receptor complexes will help shed some light on how myelin inhibition may be regulated at the receptor level.

The implication that more than one TNF receptor member may be involved in myelin inhibition adds a new level of complexity to designing therapeutic strate-

gies for treating CNS injury. Following injury, microglia and astrocytes at the lesion site can secrete TNF and various other cytokines to induce an inflammatory response (Tchelingerian et al., 1993; Renno et al., 1995). At the same time, TNF receptor family members are expressed not only in neurons but also in glial cells like oligodendrocytes (Arnett et al., 2001; Matysiak et al., 2002). It remains unclear whether these inflammatory responses are harmful or beneficial for axon regeneration or functional recovery. On the one hand, macrophage activation and other inflammatory responses have been implicated as a potential approach to activate the intrinsic regenerative ability of injured neurons (Yin et al., 2003; Fischer et al., 2004; Ma et al., 2004). However, these effects often result in the death of injured neurons and supporting glial cells. For example, a recent study demonstrated that neutralization of Fas ligand, but not TNF, could significantly decrease apoptotic cell death and promote axon regeneration and functional recovery after spinal cord injury (Demjen et al., 2004). Further characterization of the underlying mechanisms of these findings and their relation to myelin inhibition may provide important insights into designing therapeutic strategies to block myelin inhibition and cell death in the context of CNS injury.

Experimental Procedures

Generation of Recombinant Proteins and Viruses

AP, Fc, AP-Nogo-66, and AP-OMgp were prepared as described previously (Wang et al., 2002a, 2002b). For generation of recombinant HSV, cDNA sequences of full-length and truncated (aa 30–193) mouse TROY were first subcloned into pFLAG-CMV-1 (Sigma) to express FLAG-TROY fusion proteins and were ligated to the pHSVprPUC vector (kindly provided by R. Neve) for HSV production. Similarly, cDNA sequences of full-length and truncated (a.a 30–271) rat p75 were subcloned into pSECTAG-A (Invitrogen) to express *myc*-p75 fusion proteins and were ligated to the pHSVprPUC vector. These plasmids were then transfected into 2-2 cells and then superinfected with herpes simplex 5dl1.2 helper virus 1 day later (Neve et al., 1997).

For generation of lentiviruses, cDNA sequences of full-length and truncated mouse TROY were subcloned into pSECTAG-A (Invitrogen) to express *myc*-tagged fusion proteins and then were ligated to the HRST-IRESeGFP vector (kindly provided by R. Mulligan). Lentiviral vectors for full-length and truncated p75, as well as full-length and truncated NgR, have been described previously (Wang et al., 2002b). Lentiviral constructs were cotransfected with vesicular stomatitis virus glycoprotein (VSV-G) and HIV-1 packaging vector Δ8.9 into 293T cells to generate recombinant lentiviruses (Wang et al., 2002b). For both lentiviruses and HSVs, pilot tests were performed to ensure that ~90% of the cultured neurons were infected prior to performing experiments.

Rho Activation Assay

For Rho activation assays, transfected COS-7 cells were cultured overnight and subsequently stimulated with individual inhibitors or their respective controls. GTP-Rho was precipitated using beads with the GST-Rho binding domain (RBD) of Rhotekin and was detected with an anti-Rho antibody (Santa Cruz) following the manufacturer's instructions (Upstate Biotechnology). The intensity of the bands were quantified by densitometric analysis using Scion Image software (Scion, Frederick, MD), and amounts of GTP-bound RhoA were normalized to the total amounts of RhoA in cell lysates.

Neurite Outgrowth Assay

Neurite outgrowth assays were performed as described previously (Wang et al., 2002a, 2002b). Briefly, 4-week-old mouse or rat DRGs were dissected, dissociated, and plated onto immobilized substrates (PDL, AP-Nogo-66 [100 ng/cm^2], AP-OMgp [100 ng/cm^2], MAG-Fc [100 ng/cm^2], and myelin [50 ng/cm^2]). For viral infections, cells were trypsinized 3 days (for lentiviruses) or 1 day (for HSVs) after infection and were replated onto the immobilized substrates described above. Cells were cultured for 24 hr before fixation with 4% paraformaldehyde and staining with a neuronal-specific anti-β-tubulin III antibody (Tuj-1, Covance). The average lengths of the longest neurite in individual neurons were measured from at least 150 neurons per condition, from duplicate wells and from three independent experiments, and were quantified as described previously (Wang et al., 2002a, 2002b). Immunohistochemistry was performed using antibodies against p75 (Chemicon), TROY (Santa Cruz Biotechnology), or Alexa Fluor 488-conjugated IB4 (Molecular Probes).

AP Binding Assay and Immunoprecipitation

The cDNA sequence for the extracellular domain of mouse TROY (aa 30–169), CD40 (aa 26–193), and DR6 (aa 42–351) were subcloned into the expression vector APTag-5 (a gift of Dr. J. Flanagan) for expression of AP-TROY, AP-CD40, and AP-DR6 fusion proteins in 293T cells. The construction and generation of recombinant AP-Nogo-66 and AP-p75 were described previously (Wang et al., 2002b). Cell surface binding was performed by incubating the indicated concentrations of each AP fusion protein with COS-7 cells expressing full-length or truncated human NgR. For visualization of bound AP fusion proteins, nitro blue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate (NBT/BCIP) was used as AP substrate. For quantification of bound proteins, p-nitrophenyl phosphate was employed as the substrate for measurement at an absorbance of 405 nm. Apparent kD was calculated with Graphpad Prism software by nonlinear regression using a one-site binding model. Results were obtained from three independent experiments.

In Situ Hybridization

Mouse cDNA sequences for TROY (bp 88–504), CD40 (bp 76–579), DR6 (bp 124–1053), p75 (bp 795–1254), LINGO-1 (bp 1–1000), and NgR (bp 97–923) were subcloned into pBluescript II SK+ (Stratagene). In vitro transcription was performed from linearized plasmids using T7 and T3 RNA polymerase (Promega) with digoxigenin-UTP (Roche) for synthesis of sense and antisense probes, respectively. Frozen sections of brain, DRGs, and retina were prepared from 4-week-old adult C57BL/6 mice. In situ hybridization was performed as described previously (He and Tessier-Lavigne, 1997). After hybridization, mRNA localization was detected with AP-conjugated anti-digoxigenin Fab fragments followed by visualization with NBT/BCIP.

Supplemental Data

Supplemental Data including a supplemental figure can be found online at http://www.neuron.org/cgi/content/full/45/3/345/DC1/.

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