Development/Plasticity/Repair

Modulation of Semaphorin3A Activity by p75 Neurotrophin Receptor Influences Peripheral Axon Patterning

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The p75 neurotrophin receptor ($p75^{NTR}$) interacts with multiple ligands and coreceptors. It is thought to mediate myelin growth inhibition as part of the Nogo receptor complex, in addition to its other roles. Paradoxically, however, peripheral axons of $p75^{ExonIII-/-}$ mutant embryos are severely stunted. This inhibition of axon growth may be a result of neurite elongation defects in $p75^{NTR}$ mutant neurons. Here, we show that $p75^{ExonIII-/-}$ DRG neurons are hypersensitive to the repellent molecule Semaphorin3A (Sema3A). NGF modulates Sema3A activity equally well in both the $p75^{NTR}$ mutant and wild-type neurons, indicating that the hypersensitivity of $p75^{NTR}$ mutant neurons is probably not related to their NGF receptor activity. Neuropilin1 and $p75^{NTR}$ partially colocalize in DRG growth cones. After Sema3A stimulation, the degree of colocalization is dramatically increased, particularly in clusters associated with Sema3A receptor complex activation. Coimmunoprecipitation studies show that $p75^{NTR}$ interacts directly with the Sema3A receptors Neuropilin1 and PlexinA4. When coexpressed with both Neuropilin1 and PlexinA4, $p75^{NTR}$ reduces the interaction between these two receptor components. Finally, $p75^{NTR}$/Sema3A double-mutant embryos show growth similar to that observed in Sema3A-null mice. These data indicate that $p75^{NTR}$ is an important functional modulator of Sema3A activity and that, in the absence of $p75^{NTR}$, oversensitivity to Sema3A leads to severe reduction in sensory innervation. Our results also suggest that while inhibition of $p75^{NTR}$ in CNS injury may enhance nerve regeneration resulting from the inhibition of myelin-associated protein, it may also inhibit nerve regeneration through its modulation of Sema3A.

Key words: **axon guidance; development; dorsal root ganglion; DRG; knock-out mice; nerve growth factor; NGF; regeneration**

Introduction

$p75^{NTR}$ interacts with multiple coreceptors and ligands (Bandtlow and Dechant, 2004). Examples include the $p75^{NTR}$/sortilin complex, which mediates cell death by proneurotrophins, the Trk receptor/$p75^{NTR}$ complex, which mediates neuronal survival by neurotrophins and the Nogo-R/lingo/$p75^{NTR}$ complex, which mediates inhibition of regeneration by Nogo, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgP) (Nykjaer et al., 2005). Additional independent effects of $p75^{NTR}$ have also been reported (Nykjaer et al., 2005). The results of targeted mutation of the $p75^{NTR}$ show that $p75^{NTR}$ is involved in several aspects of central and peripheral innervation (Lee et al., 1992; McQuillen et al., 2002). One of the most striking characteristics of $p75^{NTR}$ mutants is a dramatic reduction in sensory and sympathetic innervation (Lee et al., 1994; Bergmann et al., 1997; Yamashita et al., 1999). The sensory innervation defect is likely to be a developmental defect, because peripheral axons from embryonic day 11.5 (E11.5)–E14.5 $p75^{NTR}$ mutant embryos are severely stunted and poorly arborized (Yamashita et al., 1999; Bentley and Lee, 2000). In adult $p75^{NTR}$ mutants, this reduction in innervation correlates with a loss of heat sensitivity and is associated with the development of ulcers in the distal extremities (Lee et al., 1992). The formation of functional sensory networks is critically dependent on the ability of neurons to extend axons to remote targets in an accurate and timely manner. Signaling molecules tightly regulate all parameters of axon trajectories to ensure the proper formation of the peripheral nervous system (Wen and Zheng, 2006). The cause of the dramatic reduction in sensory innervation in $p75^{NTR}$ mutants is not entirely clear. In growth assays, axons of $p75^{NTR}$ mutant neurons have been shown to grow slightly more slowly than those of wild-type neurons (Bentley and Lee, 2000). This effect, however, is quite small and it is difficult to understand how it could result in such a pronounced defect *in vivo*. An alternative explanation for the reduction in sensory axon growth in $p75^{NTR}$ mutant mice may be based on the enhancement of an axon growth inhibition signal. How does $p75^{NTR}$ affect axon growth inhibition? It is possible that there could be a reduction in tropomyosin-related kinase A (TrkA) signaling resulting from the reduced affinity of NGF in the absence of $p75^{NTR}$.

Sema3A's activity as a powerful negative regulator of axon growth (Togashi et al., 2006) and guidance has been well documented (Taniguchi et al., 1997; Ulupinar et al., 1999; Nakamura et al., 2000; White and Behar, 2000). In Sema3A-null mice, sen-

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sory axons have been shown to penetrate inappropriate fields and overshoot the front observed in wild-type embryos, resulting in increased growth (Taniguchi et al., 1997). A number of studies have documented the role of NGF as a negative regulator of Sema3A activity (Tuttle and O'Leary, 1998; Dontchev and Letourneau, 2002; Tang et al., 2004). It is possible that, in the absence of p75^{NTR}, sensory axon growth is inhibited because of the reduced ability of NGF to regulate Sema3A repulsion activity. In this study, we tested the hypothesis that the p75^{NTR} sensory axon growth defect is a result of this mutation's effect on Sema3A activity.

Materials and Methods

Antibodies, growth factors, and materials

NGF was obtained from Sigma (St. Louis, MO). The anti-HA, anti-myc, and anti-FLAG antibodies were purchased from Cell Signaling Technologies (Danvers, MA). Anti-FLAG was purchased from Sigma. Anti-neurofilament 2H3 (developed by Thomas M. Jesell and Jane Dodd, Columbia University, New York, NY) was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences. Anti-p75^{NTR} for the immunofluorescence studies was purchased from Millipore (Temecula, CA). The anti-p75^{NTR} (directed to the extracellular domain) used in Western blot analyses was purchased from Alamone Labs (Jerusalem, Israel). Anti-Neuropilin1 was purchased from Neuromics (Edina, MN). Secondary antibodies were obtained from Jackson Immunoresearch Laboratories (Jackson, PA).

Protease inhibitor mixture was obtained from Roche Diagnostics (Mannheim, Germany), Matrigel from BD Biosciences (Franklin Lakes, NJ) and rhodamine phalloidin and Opti-MEM from Invitrogen (Carlsbad, CA). Tissue culture reagents were purchased from Biological Industries (Kibbutz Beit Haemek, Israel). All other reagents were purchased from Sigma.

Expression plasmids

The following plasmids were generous gifts from other researchers: pFlag-CMV-NP1, pCMV-myc-PlexinA4, and p-CMV-Flag-PlexinA4 from Dr. Marc Tessier-Lavigne (Genentech, San Francisco, CA); myc-NP1 from Dr. Stephen Strittmatter; pCDNA-HA-p75^{NTR} and pFLAG-CMV2-γICD from Dr. Moses Chao; pCDNA3-p75^{ICD} (intracellular domain of p75^{NTR}) and pCDNA3-p75^{ECD} (extracellular domain of p75^{NTR}) from Dr. Yves-Alain Barde (University of Basel, Switzerland) and pCDNA3-myc-Necl5 from Dr. Elior Peles (Weizmann Institute of Science, Rehovot, Israel). pcDNA3 was obtained from Invitrogen and pEGFP-N1 was obtained from Clontech (Mountain View, CA).

Animals

Pregnant mice were obtained after overnight mating (day of vaginal plug is defined as embryonic day 0.5). For the p75^{NTR} experiments, we used the p75^{NTRExonIII}-null mutant (Lee et al., 1992) obtained from The Jackson Laboratory (Bar Harbor, ME). p75^{NTR} embryos were genotyped using the following primers: 5′ AGTATGTCCGCTCCCTGTGT, 5′ CCCTTCCTCACGAATCTAGC and 5′ ACCAAATTAAGGGCCAGCTC. Sema3A-null mice have been described previously (Behar et al., 1996). Sema3A embryos were genotyped using the following PCR primers: 5′ TGATGGCGAAAAGACTGTGT, 5′ CACACGCACAGAGGAATC and 5′ ACCAAATTAAGGGCCAGCTC.

Animal handling adhered strictly to national and institutional guidelines for animal research and was approved by the ethics committee of our institution.

Growth cone collapse

DRG explants were tested for growth cone collapse, as described previously (Behar et al., 1999; Ben-Zvi et al., 2006). Each experiment included three to four wells (each set of wells represented explants from a single embryo; 100–200 growth cones per well) and was repeated using five to seven embryos for each genotype. The growth cones were counted by an observer who was blind to the identities of the treatments.

Neurite outgrowth assay

DRG explants were grown for 20 h. At this time ($t = 0$), medium was changed and the explants were grown for an additional 6 h with or without Sema3A. Explants were photographed at $t = 0$ and after 6 h. To monitor a large number of neurites, we divided each explant into four quarters (see Fig. 1*B*, diagram) and photographed these same quarters at $t = 0$ and $t = 6$ h. The lengths of the explants' neurites were measured using Image-Pro software and the differences between the lengths at $t = 0$ and $t = 6$ were calculated (growth rate was calculated by dividing the change in length by the duration of the experiment).

COS-7 contraction assay

A COS cell contraction assay was performed essentially as described by Turner and Hall (2006), with minor modifications. COS-7 cells were maintained in DMEM supplemented with 10% fetal bovine serum. Cells were transfected with 1000 ng cDNA. In all experiments, cells were transfected with expression vectors for enhanced green fluorescent protein (pEGFP) (100 ng) and Neuropilin1 (256 ng). The other expression vectors were used as follows. PlexinA4 (385 ng), p75^{NTR} (100 or 260 ng), nectin-like 5 (Necl5; 260 ng), and pcDNA3 were used to bring the total concentration to 1000 ng. Fugene6 (Roche Diagnostics) was used as a transfection reagent in all experiments. The cells were replated on glass coverslips after 12 h. After 24 h of growth, Sema3A (1 nM) was added to each well and the cells were incubated for 40 min. Cells were then fixed in 4% paraformaldehyde, washed once in PBS and visualized for GFP. Cell images were captured using a fluorescence microscope (Olympus, Hamburg, Germany) equipped with a cooled CCD camera (Roper Scientific, Duluth, GA) and a 20× objective. The cells were counted and classified according to their morphology by an observer who was blind to the identities of the treatments.

Cell lysis, immunoprecipitations, and Western blots

Cultures were lysed in a buffer containing 20 mM Tris, 150 mM NaCl, 2 mM EDTA, 1% NP-40 and protease inhibitors. Antibodies were added to 700 μl of lysate containing 1 mg of protein. Immunocomplexes were precipitated with 150 μl of protein A Sepharose beads (Amersham Biosciences, Piscataway, NJ), washed and resuspended in SDS-PAGE loading buffer containing β–mercaptoethanol. Each immunoprecipitate was loaded in one lane of a 10-lane, 6% polyacrylamide mini-gel. Western blot analysis was performed as previously described (Ben-Zvi et al., 2006).

Sema3A

Partially purified Sema3A was prepared as described previously (Ben-Zvi et al., 2006).

Whole-mount neurofilament staining

Whole-mount immunostaining with 2H3 was performed essentially as described by Kitsukawa et al. (1997). In brief, embryos were fixed for 2 h in 4% paraformaldehyde and 10 mM PBS, pH 7.0,, washed three times in PBS plus 0.2% Triton X-100 and dehydrated through a methanol series. Endogenous peroxidase activity was quenched overnight at 4°C with 80% MeOH and 3% H_2O_2, followed by rehydration through a methanol series. After a 3 h washing with a Tris-buffered saline solution containing 0.2% Triton X-100 (PBST), the embryos were incubated with the antibody 2H3 (1:100 dilution of 2H3 hybridoma culture supernatant with PBST-containing 2% skim milk) for 3–4 d at 4°C. The embryos were then incubated for 1 d with HRP-coupled anti-mouse Ig antibody (The Jackson Laboratory; 1:200 dilution in PBST containing 2% skim milk, 2% goat serum). HRP activity was detected with diaminobenzidine.

Confocal microscopy and quantitative colocalization

DRG neurons were fixed in 4% paraformaldehyde and 10% sucrose. Cells were incubated overnight at 4°C with primary antibodies diluted in PBS containing 5% BSA, either with or without 0.1% Triton X-100. The cells were washed on the following day and then incubated with Cy3- (1:100) and fluorescein-5-isothiocyanate (FITC)-conjugated (1:200) antibodies (The Jackson Laboratory). Stainings were analyzed using a laser-scanning confocal microscope (Olympus IX70). Images were captured using a 100× oil objective (numerical aperture 1.4) at room temperature

and confocal acquisition software (Fluoview; Olympus). The interval between imaged optical sections was 0.2–0.3 μm. Sequential scanning for Cy3 (emission, 570–640 nm) and FITC (emission, 510–530 nm) was performed.

Quantitative colocalization of Neuropilin 1 (NP1) and p75^{NTR} was calculated using ImageJ software, with the colocalization threshold plug-in (written by Tony Collins, McMaster University, Hamilton, Ontario, Canada). This plug-in quantifies colocalization. We present the percentage image volume colocalized out of the total NP1 immunofluorescence signal. To calculate the degree of overlap in Sema3A-iduced NP1 clusters, we randomly chose three such clusters in 30 different images of NP1 and then tested the degree of overlap with p75^{NTR} in these clusters. To generate an image representing only the colocalized pixels, we used the RG2B ImageJ plug-in (written by Christopher Philip Mauer, Northwestern University, Chicago, IL).

Quantification of neurofilament staining

Images of embryos were obtained using an SZX12 stereomicroscope (Olympus, Hamburg, Germany) equipped with a C-4040 digital camera (Olympus). Two strategies, longest axon measurement and total axon length, were used to evaluate axon growth in the embryos.

Longest axon. To estimate axon growth, we measured the length of the longest axon in the ophthalmic branch of the trigeminal nerve and normalized to the eye perimeter, or measured the longest axon in the limb, from the base of the limb, and normalized to the length of the whole limb. This parameter was measured using ImageJ software.

Total axon length. To estimate the degree of growth we measured the total axon length using stereological principles, essentially as described (Ronn et al., 2000; Mandarim-de-Lacerda, 2003). In brief, the projection of the neuronal network in each limb was superimposed on a square grid (a set of parallel lines) using Adobe (San Jose, CA) Photoshop software. The total axon length was estimated using Buffon's needle problem equation: $L = (\pi d/M2)\ I$, where L is the axon length, d the distance between the grid lines, I the number of intersections between the axons and the grid lines, and M the magnification.

Statistical analysis

Collapse assay. Two statistical methods were used. (1) Fisher's exact test for 2×2 tables (performed with SPSS 13.0 software, SPSS, Chicago, IL) was used to test the significance of differences in growth cone response. We used the actual number of growth cones (collapsed or noncollapsed) pooled from three to four experiments. (2) The Mann–Whitney U test (performed with SPSS 13.0 software) was used to test the significance of differences in the collapse assay results of the different embryos. We used the average ratio of collapsed versus noncollapsed growth cones from each treatment (six to eight embryos per treatment).

Neurite outgrowth assay. The Mann–Whitney U test with a Bonferroni correction (performed with SPSS 13.0 software) was used to test the significance of differences in the growth rate results of the embryos of the different genotypes, using the average growth rate of each embryo (three explants per embryo, three to six embryos per genotype from 3 different litters). In addition, to test the significance of the apparent trend in the growth rates of the different genotypes, we used the Jonckheere–Terpstra test (performed using StatXact; Cytel Software Corporation, Cambridge, MA).

COS-7 contraction assay. The significance of the differences between the responses of COS-7 cells expressing NP1/PlexinA4 and those expressing NP1/PlexinA4/Necl5 to Sema3A was determined using the Fisher's exact test for 2×2 tables. We used the actual number of cells (shrunk or normal) from three individual experiments. In the case of increasing concentrations of p75^{NTR} expression, we used the Cochran–Armitage trend test (performed using StatXact; Cytel Software Corporation). In both cases, individual p values and the overall p value as determined by the χ^2 test for combined probabilities are presented.

In vivo *neurofilament staining quantification.* The significance of the differences between the normalized lengths of the longest axon in various genotypes was determined using Mann–Whitney U tests, with a Bonferroni correction when appropriate. Mann–Whitney U tests were used because of the small sample size. In addition, we used the Jonckheere–Terpstra test (performed using StatXact, Cytel Software Corporation) to test the significance of the apparent trend in the total axon coverage of the different genotypes. All tests used $\alpha = 0.05$.

Results

Increased sensitivity of P75^{NTR}−/− DRG neurons to Sema3A-repellent activity

In the presence of NGF, neurons from E12.5 DRG express both TrkA and p75^{NTR}. To examine the role of p75^{NTR} in Sema3A repulsion activity, E12.5 DRG neurons from either from p75^{NTR} mutants or their wild-type littermates were explanted in the presence of 10 ng/ml NGF and their sensitivities to Sema3A were compared in a growth cone collapse assay. In the presence of 15 pM Sema3A, ~50% of wild-type DRG growth cones collapsed. In contrast, under the same conditions, 73% of the p75^{NTR} mutant DRG growth cones collapsed (Fig. 1*A*, supplemental Figure 1, available at www.jneurosci.org as supplemental material). The difference between wild-type and p75^{NTR} mutant growth cones treated with Sema3A is highly significant ($p = 2.79 \times 10^{-52}$, as determined by a Fisher's exact test for 2×2 tables with $n = 7032$). The difference between wild-type and p75^{NTR} mutant embryos is also significant ($p = 0.005$, as determined by a two-tailed Mann–Whitney U test with $n = 13$). A similar difference in growth cone collapse was detected in the presence of 7.5 pM Sema3A (Fig. 1*A*) (40% in wild-type embryos and 57% in p75^{NTR} mutant embryos). At this concentration, the difference between wild-type and p75^{NTR} mutant embryos is also significant ($p = 0.014$, two-tailed Mann–Whitney U test with $n = 24$). This result demonstrates that DRG neurons are significantly more sensitive to Sema3A growth cone collapse activity in the absence of p75^{NTR}. It is worth noting that there was no difference between wild-type and p75^{NTR} mutant growth cone collapse in the absence of Sema3A. This indicates that the oversensitivity of p75^{NTR} mutant-derived neurons is not the result of any innate tendency to collapse.

NGF is known to modulate the expression levels of NP1 in DRG neurons (Dontchev and Letourneau, 2002). To test whether the absence of p75^{NTR} somehow modulates the levels of NP1, we examined the presence of this protein in growth cones of wild-type and p75^{NTR} mutant embryos. We detected no statistically significant differences in the NPI levels of wild-type and p75^{NTR} mutant embryos (t test; $p = 0.415$) (supplemental Fig. 2, available at www.jneurosci.org as supplemental material).

Increased sensitivity of P75^{NTR}−/− and P75^{NTR}+/− DRG neurons to Sema3A-induced neurite outgrowth inhibition

To further examine the effects of p75^{NTR} with regard to the sensitivity of DRG neurons to Sema3A, explants taken from p75^{NTR} mutants, heterozygotes and wild-type littermates were examined in a neurite outgrowth assay. In this experiment, we measured the difference between neurite length at the time Sema3A was added (defined as $t = 0$) and 6 h later (see Materials and Methods for a detailed description). In Figure 1*B*, we show an example of one segment of an explant at time 0 and 6 h later (To observe a large number of neurites, we monitored four quarters of each explant, as indicated in the diagram shown in Fig. 1*B*). Neurons from each genotype (wild-type, heterozygous or homozygous for the mutation in the p75^{NTR} gene) were grown for 20 h before the addition of different concentrations of Sema3A (0, 15 and 30 pM Sema3A). The average axon growth rates, with or without Sema3A, for each genotype are shown in Figure 1*C*. In the absence of Sema3A, the growth rates of each of the genotypes were not significantly different (51 μm/h for the wild-type, 54 μm/h for heterozygotes and

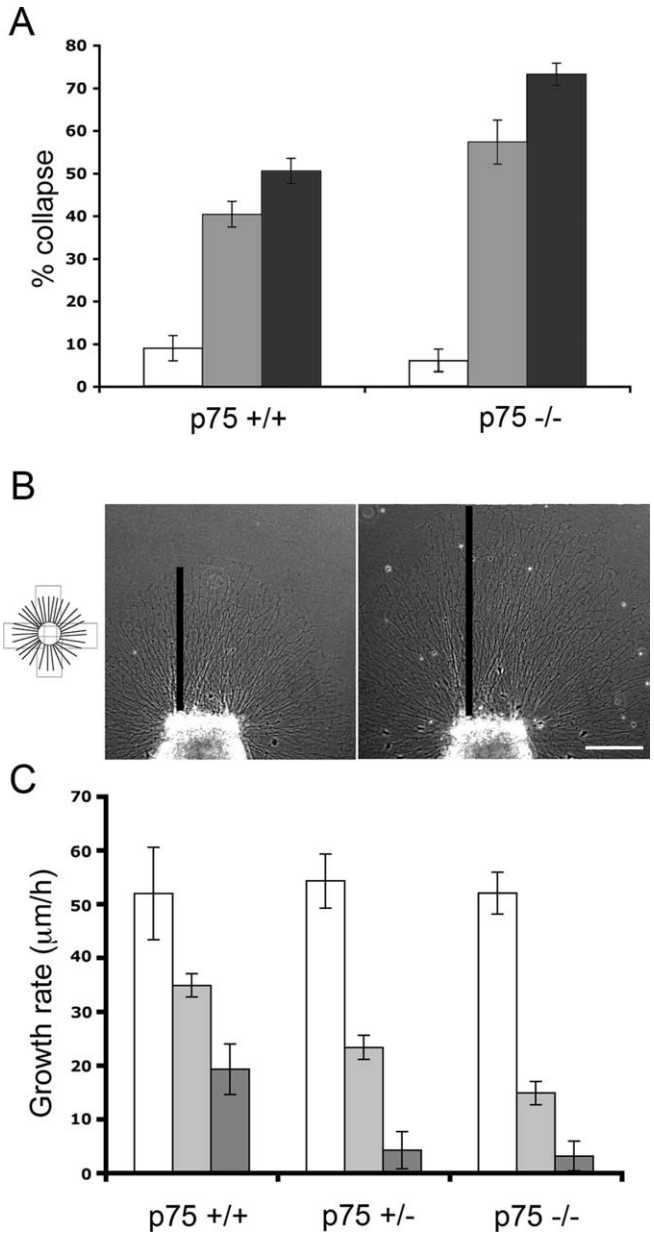

Figure 1. Increased sensitivity of p75^{NTR}−/− DRG neurons to Sema3A repellent activity. ***A***, DRG explants from E12.5 wild-type and p75^{NTR}−/− mutant embryos from the same litters were grown in the presence of 10 ng/ml NGF for 20 h, at the end of which Sema3A was added. Control cultures had no Sema3A added. After an additional incubation period of 40 min with or without Sema3A, the explants were fixed and stained with rhodamine phalloidin. Growth cone collapse results represent the means ± SEM of each treatment with no additional Sem3A (empty bar), 7.5 pM Sema3A (bright gray bar), or 15 pM Sema3A (dark gray bar) in four independent experiments. ***B***, ***C***, DRG explants from E12.5 wild-type, heterozygous, and homozygous p75^{NTR} mutants were grown as in ***A*** and treated as described in Materials and Methods. ***B***, Each explant was divided into four quarters as shown in the diagram. An example of one such quarter of an explant at $t = 0$ and $t = 6$ is shown. Scale bar, 300 μm. ***C***, Neurite outgrowth assay results represent the means ± SEM of each treatment across three independent experiments (empty bars, no Sema3A; gray bar, 15 pM Sema3A; dark gray bar, 30 pM Sema3A).

52 μm/h for homozygotes; $p = 0.548$, Mann–Whitney U-Test). In contrast, there were dramatic differences in the growth rates in the presence of 15 pM Sema3A (35 μm/h for wild-type neurons, 23 μm/h for heterozygotes and 15 μm/h for mutants). Under these conditions, heterozygous mutants exhibited an intermediate growth rate. This trend in the growth rates of the p75^{NTR} genotypes was statistically significant ($p = 0.01$, Jonckheere–Terpstra test). When neurons were grown in the presence of 30 pM Sema3A, there was a similar trend in the growth rates of the different p75^{NTR} genotypes (1.9 μm/h for wild-type neurons, 0.4 μm/h for heterozygotes, and 0.3 μm/h for mutant mice; $p = 0.049$, Jonckheere–Terpstra test). The small difference in the neurite growth of the heterozygous and homozygous mutants in the presence of 30 pM Sema3A is probably the result of saturating concentrations of Sema3A. Based on the results of our neurite outgrowth inhibition experiment, we conclude that the growth of the p75^{NTR} homozygous mutant in the presence of Sema3A is much slower then that of wild-type mice, a difference that is likely to be manifested as an extensive growth redundancy in the p75^{NTR} homozygous mutant at normal levels of Sema3A expression.

Increased sensitivity of p75^{NTR}−/− DRG neurons to Sema3A is probably not mediated by NGF

NGF is a known modulator of Sema3A-induced repulsion and, at elevated concentrations, is capable of reducing Sema3A-induced growth cone collapse (Tuttle and O'Leary, 1998; Dontchev and Letourneau, 2002). Because p75^{NTR} is a NGF receptor, it is possible that it might mediate the NGF inhibition component and that its absence could cause an increase in neuronal sensitivity to Sema3A. To test this possibility, we examined the ability of NGF to block Sema3A-induced collapse in wild-type and mutant p75^{NTR} neurons. We expected that if p75^{NTR} mediates the NGF-dependent modulation of Sema3A, then NGF will not be able to modulate the Sema3A-induced collapse response in its absence. In this set of experiments, DRG explants from wild-type and p75^{NTR} mutant embryos were grown in the presence of 2.5 ng/ml NGF for 20 h. Thirty minutes before the addition of 15 pM Sema3A, we added fresh medium with either 2.5 ng/ml or 40 ng/ml NGF. Growth cone collapse was tested in both genotypes and at both concentrations of NGF. The percentage collapse at the high NGF concentration was subtracted from the percentage collapse at the low NGF concentration to calculate the growth cone collapse inhibition induced by elevated NGF concentration. The elevated concentration of NGF resulted in similar reductions in collapse rates in both wild-type (26.58 ± 3.41% SEM) and p75^{NTR} mutant neurons (36.09 ± 2.8% SEM). (The difference between wild-type and mutant p75^{NTR} is not statistically significant ($p = 0.393$), as determined by a two-tailed Mann–Whitney U test with $n = 8$). This experiment clearly shows that p75^{NTR} is not involved in the NGF-dependent modulation of Sema3A. This finding is consistent with the idea that NGF is not the reason for increased sensitivity to Sema3A in the absence of p75^{NTR}.

p75^{NTR} disturbs Sema3A activity in a concentration-dependent manner

The Sema3A receptor is a complex of PlexinA and NP1 (Nakamura et al., 2000). Previous experiments in knock-out mice suggest that PlexinA4 and, to lesser degree, PlexinA3 are important for the repulsion activity of Sema3A (Suto et al., 2005; Yaron et al., 2005). To test the ability of p75^{NTR} to modulate Sema3A activity in a ligand-independent manner, we used the heterologous COS-7 cell contraction assay. In this assay, cDNA expression vectors for PlexinA4 and NP1 are transfected into COS-7 cells, which are challenged 48 h later with Sema3A. We modified the assay by adding p75^{NTR} or Necl5 (nectin-like-5, an unrelated neuronal receptor used as a control) expression vectors to some samples, in addition to the expression vectors for PlexinA4 and NP1. Forty-eight hours after transfection, the cells were stimulated with Sema3A (1

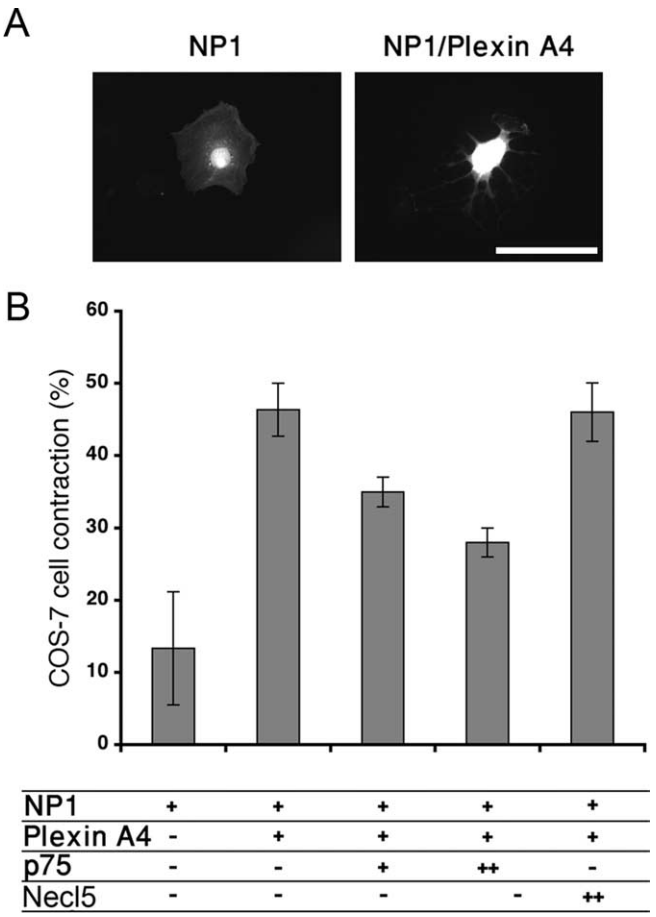

Figure 2. Expression of P75^{NTR} in COS-7 cells reduces Sema3A activity via a PlexinA4/NP1 complex in a concentration-dependent manner. COS-7 cells were transfected with cDNA as indicated and then stimulated with Sema3A (1 nM) 48 h later for 40 min. ***A***, ***B***, Examples of collapsed and noncollapsed transfected cells visualized using GFP expression. Scale bar, 100 μm. ***C***, The percentage of transfected cells that collapsed after Sema3A treatment was determined by fluorescence microscopy. Results represent the means ± SEM of three independent experiments. Cells were transfected with a total of 1 μg cDNA for all treatments. Ten percent of the DNA was pEGFP-N1, 38.5% was PlexinA4, and 26.5% was NP1. p75^{NTR}+ and p75^{NTR}++ indicate that 10 and 26% of the total DNA were p75^{NTR}, respectively. Necl5++ indicates that 26% of the total DNA was a Necl5 expression vector. In each transfection, the total DNA content was brought to 1 μg using pCDNA3 expression vector.

nM) for 40 min. We counted transfected cells to determine the percentage that had undergone morphological collapse (examples of collapsed and noncollapsed cells can be seen in Fig. 2*A*). Increasing concentrations of p75^{NTR} cDNA reduced the rate of Sem3A-induced collapse (Fig. 2*B*). This decrease was significant (tested in three individual experiments, $p = 0.023$, $p = 0.004$, and $p = 0.054$, as determined by a Cochran–Armitage trend test with $n = 108$, $n = 222$ and $n = 163$, respectively; overall significance is $p = 0.0005$, as determined by a χ^2 test for combined probabilities). In contrast, expression of Necl5 had no significant effect on the contraction response (tested in three individual experiments, $p = 1$, $p = 0.737$, and $p = 0.697$, as determined by a Fisher's exact test for 2×2 tables with $n = 105$, $n = 170$ and $n = 113$, respectively; overall significance was $p = 0.985$, as determined using a χ^2 test for combined probabilities) (Fig. 2*B*). These experiments were done in the absence of NGF, supporting the hypothesis that inhibition of collapse by p75^{NTR} is ligand independent.

p75^{NTR} and NP1 colocalize in growth cones

To begin testing whether p75^{NTR} can affect the Sema3A receptor complex directly, we first tested whether NP1 and p75^{NTR} are colocalized in growth cones. In these experiments, primary sensory neurons and DRG explants from E13.5 embryos were cultured for 20 h with 10 ng/ml NGF. The cultures were then treated with (or without) 30 pM Sema3A for 5 min and then fixed. The neurons were stained with anti-p75^{NTR} and anti-NP1 (Fig. 3). Anti-PlexinA4 was not tested because of its apparent unavailability. In control cultures, confocal laser microscopy revealed partial colocalization of p75^{NTR} and NP1 in axon shafts and growth cones (Fig. 3*A*). Using the ImageJ colocalization threshold plug-in software (for details, see Material and Methods), we found that the degree of colocalization in growth cones is ~34% (Fig. 3*C*) (this result may represent a basal colocalization level of two diffusible antigens in a limited area of the growth cone). Sema3A induces rapid morphological changes that may affect the degree of colocalization nonspecifically. To circumvent this, we tested only noncollapsed growth cones. In Sema3A-treated growth cones, the degree of colocalization is ~55% (Fig. 3*B*,*C*). Sema3A stimulation induces a rapid formation of membrane clusters (before growth cone collapse) that includes NP1, Plexin and the signaling molecule rac1 (Fournier et al., 2000). In our system, Sema3A also induces clusters of NP1. We found that the degree of p75^{NTR}/NP1 colocalization in these clusters is higher then the overall colocalization and reaches ~74%. Together, the colocalization analysis indicates p75^{NTR} is found to a large degree in close proximity to NP1, most prominently in clusters believed to be involved in Sema3A receptor complex signaling.

p75^{NTR} can form a complex with the Sema3A receptor

Because our confocal microscopy work indicated that p75^{NTR} and NP1 are partly colocalized in DRG axons, we tested whether p75^{NTR} can also form a complex with the Sema3A receptor components. We transfected human embryonic kidney 293 (HEK293T) cells to coexpress HA-tagged p75^{NTR} and Flag-tagged NP1 (Fig. 4*A*). We found that HA-p75^{NTR} can be immunoprecipitated from cell extracts with immobilized anti-Flag antibody. We also observed that immunoprecipitation with antibodies to HA coimmunoprecipitated HA-p75^{NTR} and Flag-NP1. Total IgG failed to immunoprecipitate either HA-p75^{NTR} or Flag-NP1. To further test the specificity of immunoprecipitation, we coexpressed Flag-NP1 with myc-Necl5. Myc-Necl5 and Flag-NP1 did not coimmunoprecipitate, supporting the specificity of the interaction of NP1 and p75^{NTR} (Fig. 4*B*). To test whether p75^{NTR} can form a complex with PlexinA4, we transfected HEK293T with HA-p75^{NTR} and Flag-PlexinA4 (Fig. 4*C*). Anti-Flag antibody immunoprecipitated both Flag-PlexinA4 and HA-p75^{NTR}. Similarly, immunoprecipitation of HA-p75^{NTR} resulted in immunoprecipitation of Flag-PlexinA4. Total IgG failed to immunoprecipitate either HA-p75^{NTR} or Flag-PlexinA4. When we coexpressed Flag-PlexinA4 with myc-Necl5, the two proteins did not coimmunoprecipitate (data not shown).

To learn more about the interactions between NP1, PlexinA4 and p75^{NTR}, we tested whether the intracellular (p75$^{NTR\text{-}ICD}$) or extracellular (p75$^{NTR\text{-}ECD}$) domain is important for the interactions involving PlexinA4 and NP1. As a first step, we coexpressed myc-PlexinA4 with either the Flag-p75$^{NTR\text{-}ICD}$ or the p75$^{NTR\text{-}ECD}$ (the full-length p75^{NTR} was used as a control in each experiment) (data not shown). As shown in Figure 4*D*, the extracellular domain of p75^{NTR} was sufficient for the interactions with PlexinA4. In contrast, no interaction was detected between the intracellular domain of p75^{NTR} and PlexinA4. To test which part

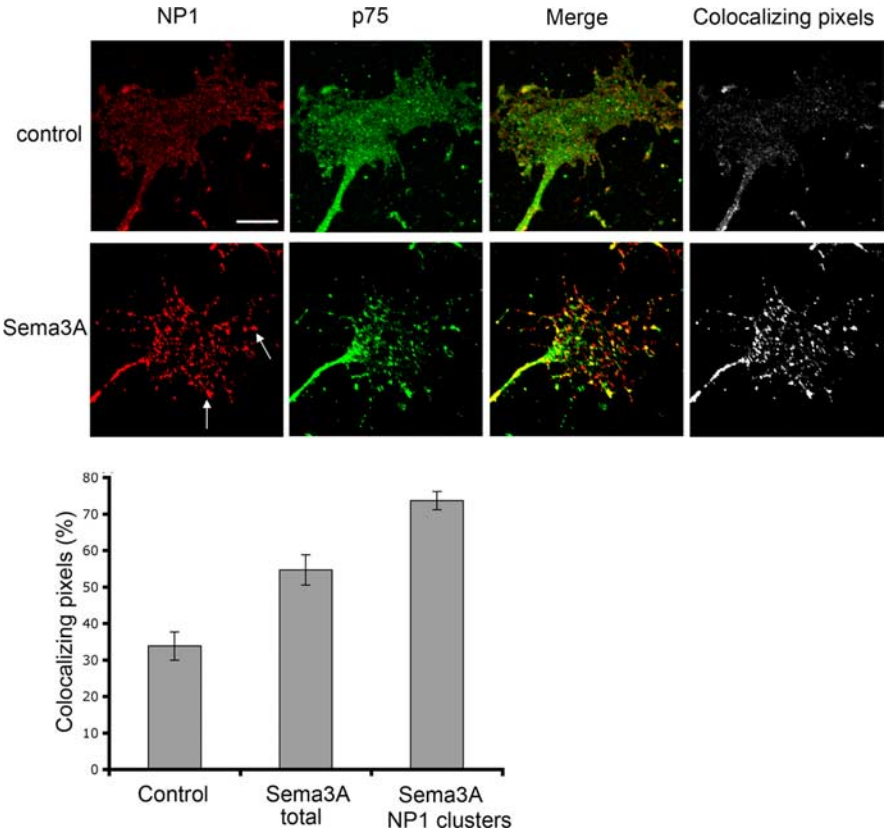

Figure 3. Colocalization of NP1 and p75^{NTR} in DRG axons. Sensory axons from E13.5 were stained with anti-p75^{NTR} and anti-NP1 and analyzed by confocal microscopy. Scale bar, 10 μm. For the control experiments, FITC-donkey anti-goat was incubated with rabbit anti-p75-labeled neurons. In addition, Cy3-donkey anti-rabbit was incubated with goat anti-NP1-labeled neurons. Acquisition parameters were defined so that background staining would show no detectable signal (data not shown). Representative optical section images of growth cones with (bottom) or without treatment (top) of 30 pM Sema3A are shown. A clear, but incomplete overlap between p75^{NTR} and NP1 is shown in the image of the overlap pixels (right). After Sema3A treatment, the reorganization of NP1 into clusters is visible (examples of such clusters are indicated by arrows). Quantification of the degree of overlap between NP1/p75 was measured as described in the Materials and Methods section. Each data point represents a mean ± SEM of 30 growth cones from three independent experiments.

of p75^{NTR} interacts with NP1, we coexpressed myc-NP1 with either the Flag-p75$^{NTR\text{-}ICD}$ or the p75$^{NTR\text{-}ECD}$. As shown in Figure 4*E*, the extracellular domain of p75^{NTR} was sufficient for the interactions with NP1. In contrast, we did not detect any interaction between NP1 and the intracellular domain of p75^{NTR}.

To further test whether p75^{NTR} interacts directly with the Sema3A receptor, we tested whether the interaction between p75^{NTR} and NP1 could be detected *in vivo*. In these experiments, we tested tissues of the DRG, spinal cord and brain. In addition, we examined DRG neuron cultures with or without Sema3A treatment. In these experiments, we immunoprecipitated p75^{NTR} and tested the levels of NP1. We were unable to detect coimmunoprecipitation of NP1 in these experiments (data not shown). (We did not repeat the experiments with anti-PlexinA4 because of a lack of a suitable commercial antibody.) Our inability to detect this interaction is likely attributable to a technical difficulty. One reason for this difficulty may be the partial colocalization and the dynamic nature of the interaction between p75^{NTR} and the Sema3A complex (Fig. 3). Additional interactions of p75^{NTR} with other molecules in these neurons (i.e., TrkA) may also interfere with stable coimmunoprecipitation. Together, it is quite possible that, in this case, coimmunoprecipitation is below the threshold level that can be detected using this technique.

p75^{NTR} disturbs the formation of the PlexinA4/NP1 complex

The Sema3A receptor is a complex of PlexinA and NP1, which have been shown to coimmunoprecipitate (Takahashi et al., 1999; Tamagnone et al., 1999). It is most likely that the interaction between these two proteins is a necessary event in Sema3A activity. Because we found that p75^{NTR} can form a complex with both NP1 and PlexinA4, we wondered whether the interaction of p75^{NTR} with these components could affect their interaction with each other. To test this, we transfected HEK293T with Flag-PlexinA4, myc-NP1 and increasing amounts of HA-p75^{NTR} or myc-Necl5 (Fig. 5). We found that expression of HA-p75^{NTR} resulted in reduced coimmunoprecipitation of NP1 by Flag-PlexinA4 (in five independent experiments). We normalized the levels of NP1 and PlexinA4 to their expression levels in the lysate. We then divided the normalized values of NP1 by the normalized values of PlexinA4. The result of this calculation in the absence of p75^{NTR} was defined as 100% coimmunoprecipitation. The highest p75^{NTR} concentration resulted in a 35% reduction in coimmunoprecipitated NP1. This difference is statistically significant (Mann–Whitney *U* tests, $p = 0.043$). The lower p75^{NTR} concentration resulted in a reduction of 19.7%. As at the high p75^{NTR} concentration, the difference with and without p75^{NTR} is statistically significant at the lower concentration, as well (Mann–Whitney *U* tests, $p = 0.034$). In these same coimmunoprecipitation experiments, HA-p75^{NTR} immunoprecipitated with Flag-PlexinA4 when a higher concentration of the p75^{NTR} expression vector was used, indicating that HA-p75^{NTR} competes with NP1 for PlexinA4 interaction. The fact that, at the lower levels of p75^{NTR} expression, only small amounts of this protein were immunoprecipitated with PlexinA4 may be explained by the formation of a p75^{NTR}/NP1 complex, which could not be detected in this experimental paradigm. These data support a model whereby p75^{NTR} interferes with the formation of the NP1/PlexinA4 complex. Necl5 had no effect on the ability of PlexinA4 to immunoprecipitate NP1 (data not shown), confirming the specificity of this effect of p75^{NTR}. These results suggest that the interaction of p75^{NTR} with plexin or NP1 can reduce the formation of the Sema3A receptor complex, thereby reducing Sema3A activity.

Growth inhibition of peripheral sensory projections in p75^{NTR} mutant embryos is alleviated in the absence of Sema3A

Using whole-mount antineurofilament immunohistochemistry, it was shown that peripheral nerves at E12.2–14.5 are severely stunted in the absence of p75^{NTR} (Yamashita et al., 1999; Bentley and Lee, 2000). To test whether Sema3A contributes to this growth inhibition in p75^{NTR} mutant embryos,

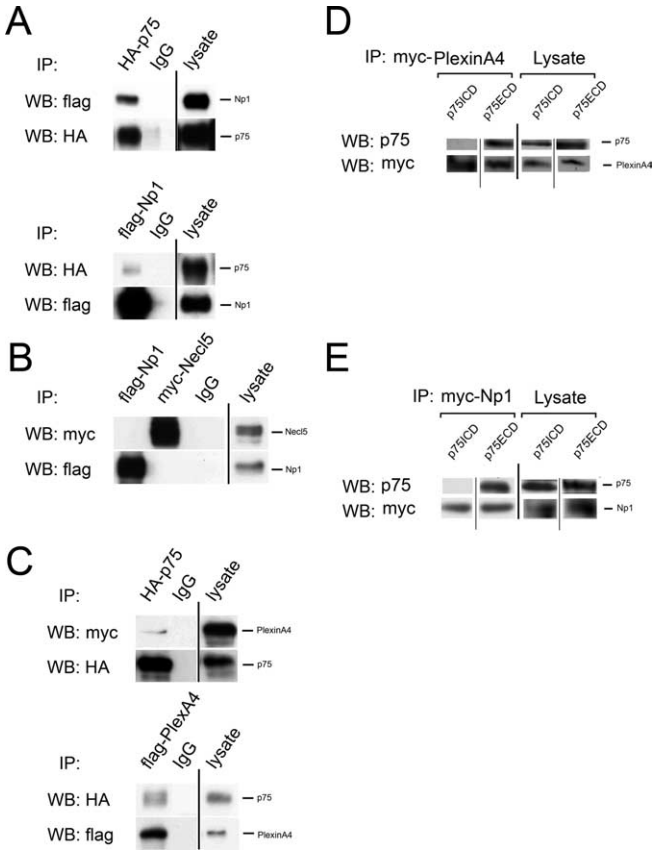

Figure 4. p75 NTR immunoprecipitates with NP1 and PlexinA4. ***A–E***, HEK293T cells were transfected with HA-tagged p75 NTR and Flag-tagged NP1 (***A***), myc-Necl5 (nectin-like-5) and Flag-tagged NP1 (***B***), HA-tagged p75 NTR and Flag-PlexinA4 or myc-PlexinA4 (***C***), myc-PlexinA4 and p75 NTR-ECD (extracellular and transmembrane domains) or Flag-p75 NTR-ICD (intracellular and transmembrane domains) (***D***), or myc-NP1 and p75 NTR-ECD or Flag-p75 NTR-ICD (***E***). The cells were lysed 48 h after transfection and tested in a coimmunoprecipitation assay. The starting material (lysate) is shown at the right of each panel, the immunoprecipitation antibody is indicated above each lane, and the Western blot (WB) antibody is indicated to the left of each panel. ***A***, Cell lysates were immunoprecipitated with anti-HA-p75 (top) and blotted with anti-Flag, and then with anti-HA-p75 NTR. In a separate sample, the cell lysates were immunoprecipitated with anti-Flag-NP1 and blotted with anti-HA, and then with anti-Flag (bottom). Total IgG was also used to immunoprecipitate cell lysates as a control (center lanes). ***B***, Cell lysates were immunoprecipitated with anti-Flag-NP1 or anti-myc-Necl and blotted with anti-Flag or anti-myc as indicated. ***C***, Cell lysates were immunoprecipitated with anti-HA-p75 NTR, resulting in immunoprecipitation of myc-PlexinA4 (top). Likewise, immunoprecipitation of Flag-PlexinA4 resulted in immunoprecipitation of HA-p75 NTR (bottom). Total IgG failed to immunoprecipitate either HA-p75 NTR or Flag-PlexinA4. ***D***, ***E***, Cell lysates were immunoprecipitated with anti-myc-PlexinA4 (***D***) or anti-myc-NP1 (***E***) and blotted with anti-p75 extracellular domain antibody in the case of p75 NTR-ECD, or anti-Flag in the case of Flag-p75 NTR-ICD. To monitor the initial amounts of myc-Plexin A4 (***D***) or myc-NP1, the membranes were reblotted with anti-myc antibody.

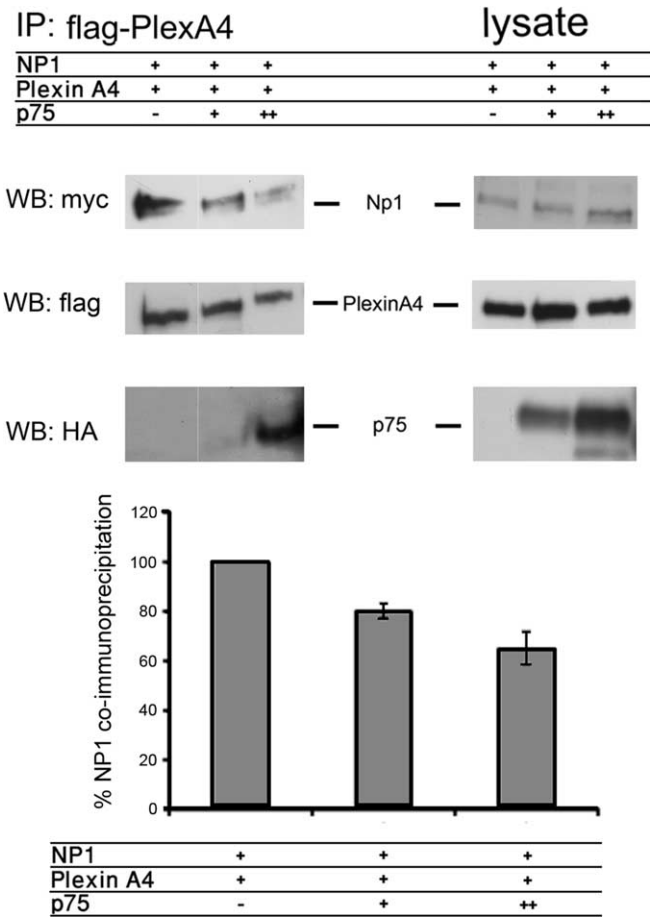

Figure 5. p75 NTR neurotrophin receptor disrupts the formation of the Plexin A4/NP1 complex. Top, HEK293T cells were transfected with 4 μg myc-tagged NP1 and 6 μg Flag-tagged PlexinA4. In addition, the cells were transfected with increasing amounts of HA-tagged p75 NTR (+ represents 4 μg of p75 NTR; ++ represents 10 μg). For all transfections, total DNA was brought to 20 μg using a pCDNA3 expression vector. The cells were lysed at 48 h after transfection and PlexinA4 was immunoprecipitated from lysates with anti-Flag antibodies. Blots were probed with anti-myc-NP1 and then reprobed, first with anti-HA-p75 NTR, and then with anti-Flag-PlexinA4. Aliquots of the total cell lysates were directly probed with anti-myc-NP1, anti-Flag-PlexinA4 and anti-HA-p75 NTR as a control. (This figure was prepared by combining two parts of the same membrane to exclude a nonrelevant lane). Bottom, Quantification of NP1 coimmunoprecipitated by PlexinA4. We normalized the levels of NP1 and PlexinA4 to their expression levels in the lysate. We then divided the normalized values of NP1 by the normalized values of PlexinA4. The result of this calculation in the absence of p75 was defined as 100% coimmunoprecipitation. Results represent the means ± SEM of five independent experiments.

we used double heterozygous mice (p75 NTR+/−; Sema3A+/−) to generate wild-type, homozygous and mutant E12.5 embryos. To evaluate the effects of these different genotypes on axon growth, we focused on the limbs and the ophthalmic branch of the trigeminal ganglia of E12.5 embryos. In the limbs, we evaluated growth by measuring the longest axon normalized to the length of the measured limb. In the case of the trigeminal projections, we used the longest length criteria normalized to the eye perimeter (for details of these measurements, see Materials and Methods). As expected, peripheral nerves in hind limbs (Fig. 6*A*,*B*), forelimbs (Fig. 6*C*, supplemental Fig. 3, available at www.jneurosci.org as supplemental material), and trigeminal ganglia (Fig. 6*D*, supplemental Fig. 4, available at www.jneurosci.org as supplemental material) of p75 NTR mutant mice are all severely stunted compared with those of the control wild-type littermates [$p = 1.25 \times 10^{-4}$ (hind limb), $p = 8.1 \times 10^{-5}$ (forelimbs), and $p = 0.008$ (trigeminal ganglion), as determined by a one-tailed Mann–Whitney U test with seven wild-type and four p75 NTR mutant embryos]. As described in a previous study, peripheral axons in Sema3A-null mice overshot the front observed in wild-type embryos (Fig. 6*A*, supplemental Figs. 3, 4, available at www.jneurosci.org as supplemental material). In p75 NTR mutant embryos, which are also Sema3A−/−, the peripheral axons overshot the front observed in wild-type embryos. The innervation pattern in these mice is, in most cases, similar to that found in the Sema3A-null embryos (Fig. 6*A*, supplemental Figs. 3, 4, available at www.jneurosci.org as supplemental material). The differences between p75 NTR−/− and p75 NTR/

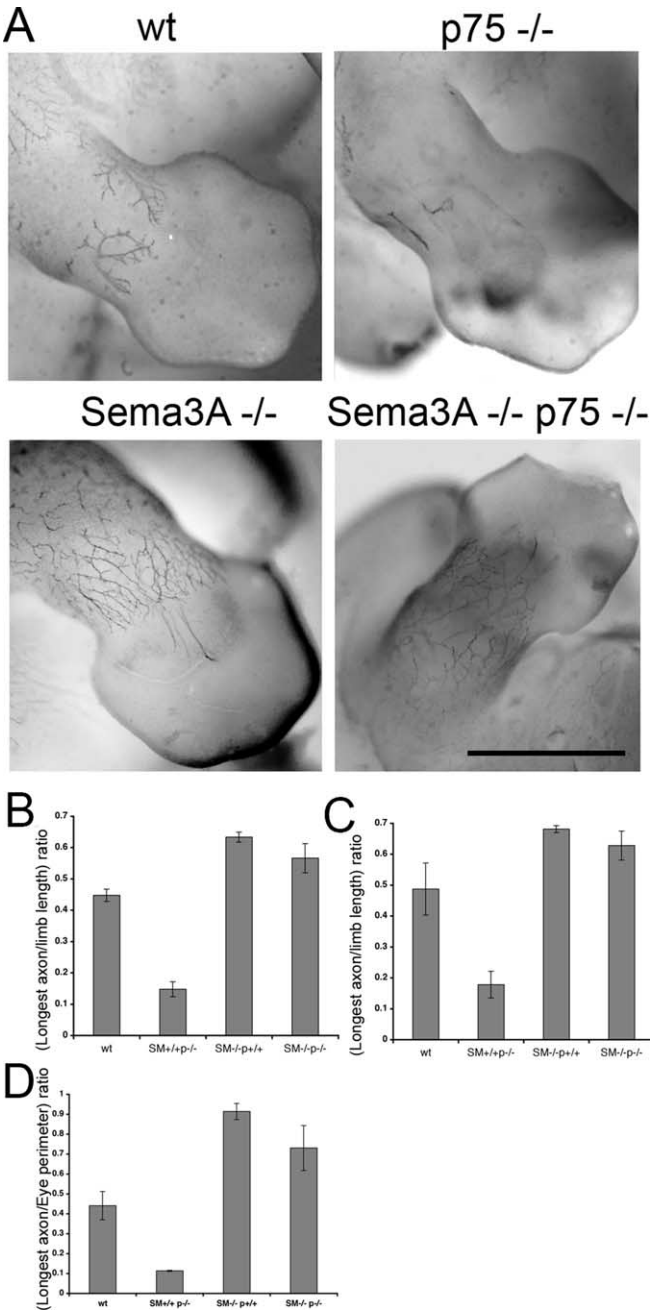

Figure 6. Growth inhibition of peripheral sensory projections in p75^{NTR} mutant embryos is alleviated in the absence of Sema3A. Whole-mount anti-neurofilament immunohistochemistry was performed on E12.5 embryos. ***A***, Peripheral nerves in hind limbs. Peripheral nerves in p75^{NTR} mutant mice are severely stunted (top right), compared with those of control wild-type littermates (top left). In contrast, in p75^{NTR} mutant mice that were also Sema3A-null (bottom right), peripheral axons overshot the front observed in wild-type embryos, similar to the overshooting observed in Sema3A-null mice that are wild-type for the p75^{NTR} gene (bottom left). Scale bar, 1 mm. ***B–D***, Quantification of relative innervation of hind limbs (representative whole mount shown in ***A***) forelimbs (whole mount shown in S3) and trigeminal ganglia (whole mount shown in S4) using whole-mount anti-neurofilament immunohistochemistry. To quantify the axon growth capacity of each genotype, we measured the length of the longest axon. ***B***, ***C***, The length of the longest axon measured from the base of the limb and normalized to the length of the hind limb (***B***) or forelimb (***C***). ***D***, The length of the longest axon from the trigeminal ganglion normalized to the eye perimeter. Results represent the means ± SEM of seven wild-type embryos, 15 Sema3A mutants, four p75^{NTR} mutants and five Sema3A/p75^{NTR} double-mutant embryos from a total of eight litters.

Sema3A double-mutant embryos were significant [$p = 0.008$ (hind limb), $p = 0.008$ (forelimbs), and $p = 0.032$ (trigeminal ganglion), as determined by a two-tailed Mann–Whitney U test with four p75^{NTR} mutant and five Sema3A/p75^{NTR} double-mutant embryos]. The differences between Sema3A mutant embryos and p75^{NTR}/Sema3A double-mutant embryos were not significant [$p = 0.338$ (hind limb), $p = 0.672$ (forelimbs), and $p = 0.412$ (trigeminal ganglion), as determined by a two-tailed Mann–Whitney U test with 15 Sema3A mutants and five Sema3A/p75^{NTR} double-mutant embryos]. In addition to measuring the longest axon, we also calculated the total axon length per limb using stereological principles (data not shown) (Fig. 7*F*) (for details, see Materials and Methods). For the most part, these results were similar to the length-of-the-longest-axon data. However, the density of axons in the double-mutant mice (2.28-fold greater for hind limbs and 1.74-fold for forelimbs, compared with the wild-type) was an intermediate value between the wild-type and the Sema3A mutant (5.97-fold for hind limbs and 3.74-fold for forelimbs, compared with the wild-type). This intermediate phenotype for total axon length indicates that the Sema3A-null phenotype is not simply dominant over the p75^{NTR} mutant phenotype, but rather that Sema3A and p75^{NTR} interact genetically with regard to axon growth regulation. The lower density of axons observed in the double-mutant mice, compared with that observed in the Sema3A mutant, may reflect the massive decrease in the number of peripheral sensory neurons in the p75^{NTR} mutant (Lee et al., 1992; von Schack et al., 2001). However, it is possible that the differences between the axon density results and the longest growth measurements reflect Sema3A-related effects.

Growth inhibition of peripheral sensory projections in p75^{NTR} mutant mice is sensitive to changes in the level of Sema3A

Haplo-insufficiency in the semaphorin family has been described for Sema3B and Neuropilin2-null mice (Zou et al., 2000; Falk et al., 2005). In Sema3A mutant mice, mRNA levels in heterozygous mice are reduced by half and are so low as to be nearly undetectable in the homozygous mutant mice (Behar et al., 1996; Taniguchi et al., 1997). Because our results suggest that p75^{NTR} affects the level of Sema3A activity, we wanted to test whether reduction of Sema3A levels *in vivo* would suppress the inhibition of sensory axons in p75^{NTR} mutant mice. We, therefore, examined the effect of Sema3A levels on peripheral nerve growth in the absence of p75^{NTR}. Using whole-mount antineurofilament immunohistochemistry, we compared peripheral innervation in E12.5 p75^{NTR}−/− Sema3A+/− embryos and E12.5 p75^{NTR}−/− Sema3A+/+ embryos (Fig. 7). As shown in Figure 6, in p75^{NTR}−/− Sema3A+/+ mice, peripheral nerves at E12.5 were severely stunted. However, in p75^{NTR}−/− Sema3A+/− animals, E12.5 peripheral nerves grew better (Fig. 7, supplemental Fig. 5, available at www.jneurosci.org as supplemental material). This axon growth improvement in p75^{NTR}−/− Sema3A+/− animals is not simply a result of increased growth in Sema3A heterozygous embryos, because p75^{NTR}+/+ Sema3A+/− exhibited only slightly increased growth (1.19-fold for hind limbs, 1.25-fold for forelimbs and 1.19-fold trigeminal ganglion), compared with that of wild-type embryos (data not shown). In contrast, p75^{NTR}−/− Sema3A+/− showed a significantly greater increase in growth compared with p75^{NTR}−/− Sema3A+/+ (threefold for hind limbs, 2.7-fold for forelimbs, and 4.4-fold for trigeminal ganglion). The difference between p75^{NTR}−/− Sema3A+/+ and p75^{NTR}−/− Sema3A+/− mutant embryos is

significant [$p = 1.25 \times 10^{-4}$ (hind limb), $p = 1.25 \times 10^{-4}$ (forelimbs), and $p = 0.005$ (trigeminal ganglion), as determined by a one-tailed Mann–Whitney U test with four p75^{NTR}−/− mutant and seven Sema3A+/− p75^{NTR}−/− mutant embryos]. In fact, it appears that p75^{NTR}−/− Sema3A+/− embryos are not significantly different from those of wild-type mice [p75^{NTR}+/+ Sema3A+/+ $p = 0.579$ (hind limb), $p = 0.438$ (forelimbs), and $p = 0.931$ (trigeminal ganglion) as determined by a two-tailed Mann–Whitney U test with seven wild-type and seven Sema3A+/− p75^{NTR}−/− embryos].

Axon growth *in vitro* of p75^{NTR} heterozygous mutants showed an intermediate response for Sema3A (Fig. 1). It is therefore possible that p75^{NTR} heterozygous mutants will grow less also *in vivo*, compared with wild-type littermates. To address this question, we measured the longest axon in p75^{NTR} heterozygous mutants and wild-type controls. We found no significant difference between these two genotypes (data not shown). To investigate this possibility more carefully we also calculated the total axon length per limb using stereological principles (Fig. 7*F*) (for details, see Materials and Methods). We detected a reduction in axon growth according to the number of p75^{NTR} alleles (which likely corresponds to the levels of p75^{NTR} expression) for both the forelimbs and hind limbs. In fact, heterozygous mutants exhibited intermediate nerve coverage between wild-type and p75$^{NTR-/-}$. This trend in the nerve coverage of the p75^{NTR} genotypes was statistically significant (forelimbs, $p = 0.001$; hind limbs, $p = 0.01$, Jonckheere–Terpstra test). Consistent with the longest axon criteria, the density of axons in the Sema3A+/− p75^{NTR}−/− embryos was similar to the wild-type levels (1.07-fold greater for hind limbs and 1.47-fold for forelimbs, compared with the wild-type) (Fig. 7*F*), indicating that the absence of one Sema3A allele is sufficient to bring the growth capacity of p75^{NTR}−/− mutants to wild-type levels. As mentioned above, this is not the case with the Sema3A−/− p75^{NTR}−/− embryos. This difference in the relationship between the axon density and longest axon results in the two genotypes may reflect the multifunctional role of both genes in peripheral nerve patterning.

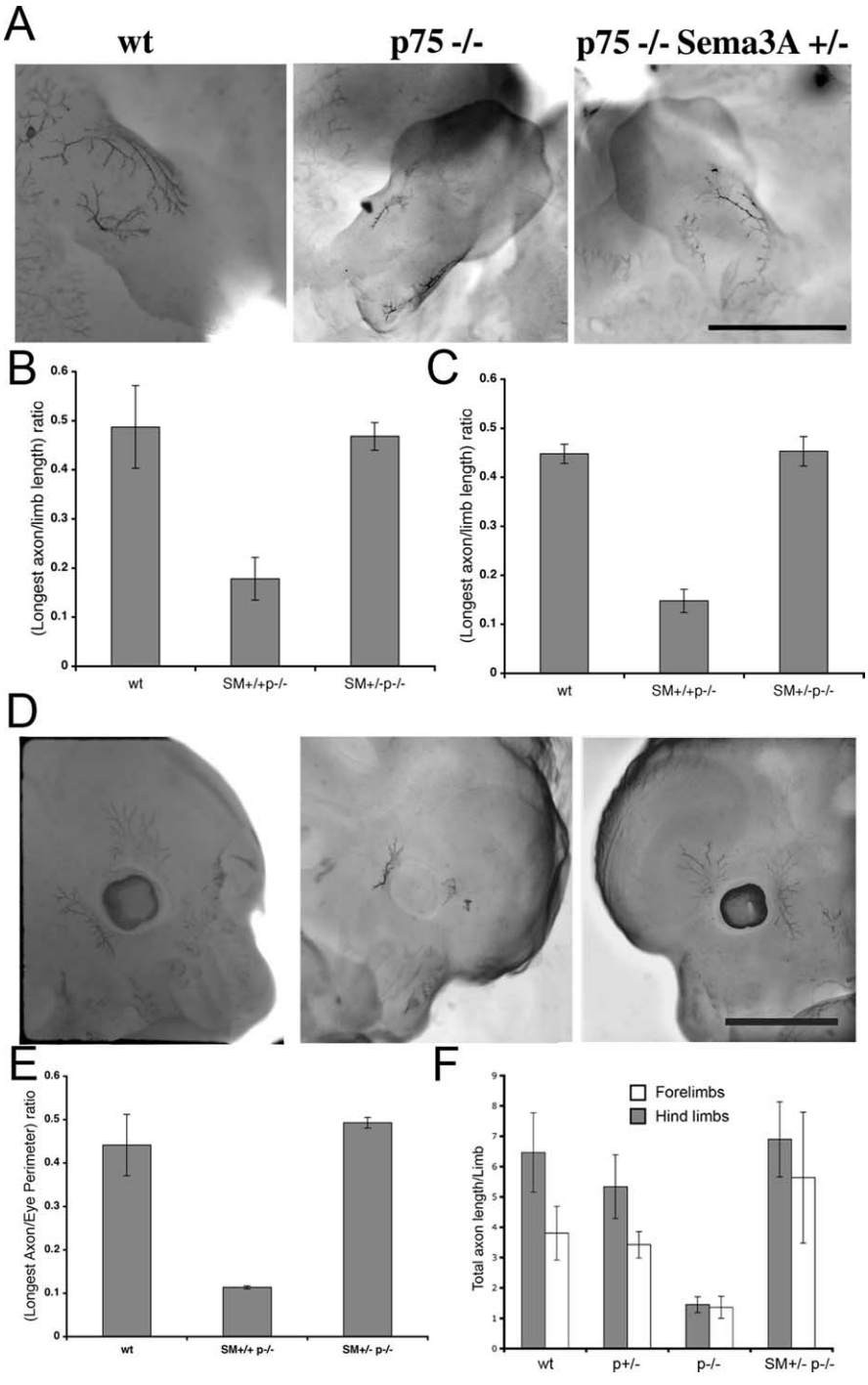

Figure 7. Growth inhibition of peripheral sensory projections in p75^{NTR} mutant mice is sensitive to changes in Sema3A levels. Whole-mount anti-neurofilament immunohistochemistry was performed on E12.5 embryos. ***A***, ***D***, Peripheral nerves in forelimbs (***A***) and trigeminal ganglia (***D***) are shown. Compared with those of the control wild-type littermates (left), peripheral nerves in p75^{NTR} mutant mice (middle) were severely stunted. In contrast, p75^{NTR}−/−, Sema3A+/− mutant mice (right) resemble their wild-type littermates. ***B***, ***C***, ***E***, Quantification of relative innervation of forelimbs (***A***), trigeminal ganglia (***D***) and hind limbs (S5) using whole-mount anti-neurofilament immunohistochemistry. We again used the longest axon length normalized to the length of the limb (***B***, ***C***) or the eye perimeter (***E***) to quantify the axon growth capacity of each genotype. Forelimbs (***B***), hind limbs (***C***), and trigeminal ganglion (***E***) are shown. ***F***, Quantification of total axon lengths in forelimbs (gray bars) and hind limbs (empty bars) was estimated using Buffon's needle problem equation (see Materials and Methods). Results represent the means ± SEM of seven wild-type, three p75^{NTR}+/−, four p75^{NTR}−/− mutant (wild type and p75^{NTR}−/− mutant are the same embryos used for Fig. 6), and seven Sema3A+/−, p75^{NTR}−/− embryos. Scale bar, 1 mm.

Discussion

Previous work has shown that p75^{NTR} is a necessary component of peripheral axon growth and patterning (Yamashita et al., 1999; Bentley and Lee, 2000). The mechanism by which p75^{NTR} is involved in this process is not entirely clear. Here, we investigated whether p75^{NTR} plays a role in Sema3A activity and whether this possible link might explain the effects of p75^{NTR} on axon growth.

P75^{NTR} expression reduces sensitivity to Sema3A repellent activity

Our results show that embryonic sensory neurons of homozygous p75^{NTR} mutant mice are significantly more sensitive to Sema3A activity than those of wild-type sensory neurons, as shown both in a collapse and in neurite outgrowth assays. This difference is shown most dramatically by the results of the growth assay. In this assay, we found that, at certain low concentrations of Sema3A, neurons of homozygous p75^{NTR} mutants almost stop growing whereas wild-type sensory neurons continue to grow. Under such conditions, the difference in total growth between the two genotypes is enormous, a result consistent with the growth differences observed in the different p75^{NTR} and Sema3A genotypes. Although this specific p75^{NTR} mouse mutant (lacking exon III) may still express a minor splice variant of p75^{NTR}, there are several reasons to believe that the increased sensitivity we detected in these neurons is probably attributable to their lack of p75^{NTR} activity. First, this residual splice variant (s-p75^{NTR}) was detected in Schwann cells (von Schack et al., 2001), but not in embryonic DRG growth cones (by immunohistochemistry) or embryonic brain tissue (examined using Western blots), indicating that s-p75^{NTR} is not present at the relevant stage and/or in the relevant neuronal population (Gehler et al., 2004). Second, s-p75^{NTR} does not bind neurotrophins (von Schack et al., 2001; Paul et al., 2004). Thirdly, DRG neurons from the p75^{ExonIII}-mutant animal respond differently than wild-type neurons to Nogo and MAG (Wang et al., 2002; Wong et al., 2002), suggesting that p75^{NTR} in these neurons is not functional. Furthermore, the inhibition of Sema3A collapse activity in the COS cell assay as a result of p75^{NTR} expression independently supports the idea that p75^{NTR} is a negative modulator of Sema3A repellent activity.

The mechanism of p75^{NTR} modulation of Sema3A activity

NGF and BDNF are both known to modulate Sema3A repellent activity (Tuttle and O'Leary, 1998; Dontchev and Letourneau, 2002). It is, therefore, possible that p75^{NTR} modulation of Sema3A repulsion is related to direct activation of p75^{NTR} by neurotrophins or indirect activation by increased activation of Trk receptors. Two lines of evidence indicate that p75^{NTR} modulation activity is at least partially neurotrophin-independent. First, NGF is able to reduce Sema3A activity equally well in both wild-type and p75^{NTR} mutant neurons. This result indicates that although NGF modulates Sema3A, this activity is not related to p75^{NTR}. Second, expression of p75^{NTR} in a COS cell contraction assay modulates Sema3A activity. If the effect of p75^{NTR} modulation is mostly independent of NGF, then how can this molecule affect Sema3A activity? One possible mechanism may involve RhoA activation. Sema3A-induced activity is mediated by activation of RhoA (Wu et al., 2005). p75^{NTR} activity is also related to RhoA. For instance, inhibition of axon growth by Nogo-66 or MAG via the Nogo-66 receptor/Lingo-1/p75^{NTR} complex is mediated by RhoA activation (Yamashita et al., 2002; Yamashita and Tohyama, 2003). Moreover, it has been shown that when p75^{NTR} is not bound to neurotrophins, it can activate RhoA (Yamashita et al., 1999; Gehler et al., 2004). However, it was also shown that levels of activated RhoA in growth cones and cerebellar cell extracts of p75^{NTR} mutant mice are lower than in wild-type neurons (Gehler et al., 2004). Because Sema3A activity requires activation of RhoA, it would be expected that neurons from p75^{NTR} mutant mice would be less sensitive to Sema3A-induced growth cone collapse. However, we found that p75^{NTR} neurons are actually more sensitive to Sema3A-induced collapse. Therefore, RhoA activation is probably not the cause of this effect.

How then does p75^{NTR} modulate Sema3A activity? The results of our immunostaining for p75^{NTR} and NP1 indicate that p75^{NTR} can be found in close proximity to the Sema3A receptor in growth cones and axons. This colocalization significantly increases in response to Sema3A, most prominently in Sema3A-induced NP1 clusters, which are believed to be involved with Sema3A receptor activation (Fournier et al., 2000). Moreover, the ability of p75^{NTR} to immunoprecipitate PlexinA4 and NP1 implies that p75^{NTR} may directly modulate the Sema3A receptor complex. Sema3A binds a complex receptor comprised of two essential components, plexin and NP1. The reduction in the ability of PlexinA4 to immunoprecipitate NP1 as a function of increasing p75^{NTR} concentration suggests that interaction between p75^{NTR} and either NP1 or PlexinA4 can inhibit the interaction between NP1 and PlexinA4. Moreover, the increase in the amount of p75^{NTR} immunoprecipitated with PlexinA4 as coimmunoprecipitation of NP1 decreases implies that p75^{NTR} can compete with NP1 in binding PlexinA4, thereby reducing levels of functional Sema3A receptor complex. However, because we were unable to detect this interaction in neurons, alternative explanations such as p75^{NTR} indirectly modulating Sema3A activity, cannot be ruled out.

Cross talk between p75^{NTR} and Sema3A activity is an important modulator of sensory innervation in the developing embryo

Previous studies have found that peripheral axons are severely stunted in p75^{NTR} mutant embryos (Yamashita et al., 1999; Bentley and Lee, 2000). *In vitro*, sensory neurons from the p75^{NTR} mutant mice grown on adult sciatic nerve cryosections showed a 12% reduction in growth capacity (Bentley and Lee, 2000). Based on this result, it was suggested that an intrinsic reduction in neurite elongation ability causes the dramatic reduction in innervation by sensory axons in p75^{NTR} mutant mice. Alternatively, p75^{NTR} mutant neurons could be more sensitive to a component of the sciatic nerve substratum, leading to reduced growth. Consistent with this idea, another study found that outgrowth of DRG neurites from p75^{NTR} mutants was indistinguishable from that of wild-type DRG neurites (Gehler et al., 2004). In this case, the reduction is a result of an extrinsic factor found in the sciatic nerve. [Interestingly, Sema3A expression has been detected in sciatic nerve cells after injury (Scarlato et al., 2003).] The result presented here regarding sensitivity to Sema3A repellent activity suggests that the dramatic reduction in axon elongation may be, at least in part, the result of extrinsic factors. This possibility is strongly supported by our *in vivo* results, which show that the Sema3A mutant is able to compensate for the p75^{NTR} mutant phenotype with regard to the degree of axon growth. Because p75^{NTR}/Sema3A double-mutant axons grow almost as well as Sema3A mutant axons, it appears that the lack of axon growth in p75^{NTR} mutants is the result of the extrinsic levels of Sema3A expression. Careful analysis of the Sema3A+/− p75^{NTR}+/+ embryos revealed a slight increase in axon growth of ~20%, compared with that of wild-type embryos. However, Sema3A+/− p75^{NTR}−/− embryos showed an increase of ~240% in axon growth (as evaluated by the longest axon measurement) compared with Sema3A+/+ p75^{NTR}−/− embryos, indicating that this phenotype is very similar to that of

the wild-type in terms of axon growth. This finding supports the possibility of an interaction between the p75^{NTR} and Sema3A genes.

In summary, the results of our colocalization experiments and the biochemical data are consistent with a model in which p75^{NTR} acts as a competitive inhibitor for the Sema3A receptor complex, modulating the activity of this complex. Nevertheless, alternative explanations cannot be ruled out. However, considering the genetic and the cell biology results, it is clear that p75^{NTR} is a functional modulator of Sema3A activity and, in its absence, sensory neurons are overly influenced by Sema3A, resulting in severe growth redundancy.

p75^{NTR} in nerve regeneration: a double-edged sword?

The inability of adult mammalian CNS neurons to regenerate after injury is due, at least in part, to axonal repulsion signals from the environment (Schwab and Bartholdi, 1996). Important inhibitory molecules include the myelin-associated molecules, MAG, OMgP, and Nogo-66. All three of these proteins exert their inhibitory activities by binding to a neuronal receptor complex containing the Nogo-66 receptor, Lingo-1, and p75^{NTR} (Wang et al., 2002; Wong et al., 2002). (p75^{NTR} can be replaced in this complex by another member of the TNF receptor superfamily, Troy.) Therefore, inhibition of p75^{NTR} has been proposed as a target for efforts to improve regeneration of CNS neurons by blocking myelin inhibitory activity. Sema3A expression is induced at the site of CNS injury and accumulating evidence suggests that it is also involved in inhibition of axon regeneration (Pasterkamp et al., 1999; De Winter et al., 2002; Kikuchi et al., 2003). In this study, we show that p75^{NTR} is a negative modulator of the Sema3A repulsion complex. In its absence, the sensitivity of neurons to Sema3A activity increases. Therefore, strategies for inhibiting p75^{NTR} to inhibit myelin-associated molecules will strengthen Sema3A activity, but the net result may not improve regeneration capacity. We, therefore, suggest that other components of the myelin-associated receptor complex may be more appropriate targets for efforts to improve neuronal regeneration capacity.

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