

# TrkA mediates developmental sympathetic neuron survival in vivo by silencing an ongoing p75NTR-mediated death signal

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**D**evelopmental sympathetic neuron death is determined by functional interactions between the TrkA/NGF receptor and the p75 neurotrophin receptor (p75NTR). A key question is whether p75NTR promotes apoptosis by directly inhibiting or modulating TrkA activity, or by stimulating cell death independently of TrkA. Here we provide evidence for the latter model. Specifically, experiments presented here demonstrate that the presence or absence of p75NTR does not alter Trk activity or NGF- and NT-3-mediated downstream survival signaling in primary

neurons. Crosses of p75NTR<sup>-/-</sup> and TrkA<sup>-/-</sup> mice indicate that the coincident absence of p75NTR substantially rescues TrkA<sup>-/-</sup> sympathetic neurons from developmental death in vivo. Thus, p75NTR induces death regardless of the presence or absence of TrkA expression. These data therefore support a model where developing sympathetic neurons are “destined to die” by an ongoing p75NTR-mediated apoptotic signal, and one of the major ways that TrkA promotes neuronal survival is by silencing this ongoing death signal.

## Introduction

The neurotrophic factor hypothesis, as originally formulated (Thoenen and Barde, 1980; Levi-Montalcini, 1987; Oppenheim, 1991), postulates that developing neurons are overproduced and they compete for limited quantities of target-derived growth factors such as NGF, which they need for survival. Recent studies of NGF-dependent sympathetic neurons have provided molecular insights into this process, and have shown that the TrkA/NGF receptor (Cordon-Cardo et al., 1991; Kaplan et al., 1991a,b; Klein et al., 1991) mediates the survival effects of NGF during development, but that the p75 neurotrophin receptor (p75NTR)\* (Johnson et al., 1986; Radeke et al., 1987) is also necessary for appropriate developmental sympathetic neuron death (reviewed in Kaplan and Miller, 2000). In particular, studies of the sympathetic superior cervical ganglia (SCG) in animals mutant in one of these two receptors have

shown that (a) TrkA<sup>-/-</sup> sympathetic neurons die during the late neonatal and early postnatal periods (Smeyne et al., 1994; Fagan et al., 1996); (b) in p75NTR<sup>-/-</sup> mice, sympathetic neuron number does not decrease during naturally occurring cell death, but instead there is a delayed loss of neurons between P21 and adulthood (Bamji et al., 1998); and (c) the apoptosis of cultured p75NTR<sup>-/-</sup> sympathetic neurons is delayed after NGF withdrawal (Bamji et al., 1998). Together these studies support a model wherein p75NTR has an essential role in ensuring rapid and appropriate apoptosis of sympathetic neurons that do not sequester adequate levels of target-derived NGF (Majdan and Miller, 1999).

Recent evidence suggests a second, related role for p75NTR during this same developmental period. Specifically, crosses of p75NTR<sup>-/-</sup> and NT-3<sup>-/-</sup> animals indicate that p75NTR is essential for sympathetic neurons to distinguish between “preferred” (NGF) and “nonpreferred” (NT-3) survival ligands (Brennan et al., 1999); sympathetic neurons only responded to NT-3 with survival in vivo when p75NTR was absent. Potential clues into the mechanism underlying this phenomena derive from biochemical studies of cultured sympathetic neurons, which demonstrated that NGF but not NT-3 supported neuronal survival at equivalent levels of TrkA activation (Belliveau et al., 1997). This latter finding suggests that p75NTR antagonizes NT-3-mediated survival downstream of TrkA.

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\*Abbreviations used in this paper: BDNF, brain-derived neurotrophic factor; P, postnatal day; p75NTR, p75 neurotrophin receptor; SCG, superior cervical ganglia; TUNEL, TdT-mediated dUTP-biotin nick end labeling.

Key words: neurotrophins; neuronal apoptosis; neurotrophin receptors; developmental cell death; Trk signaling

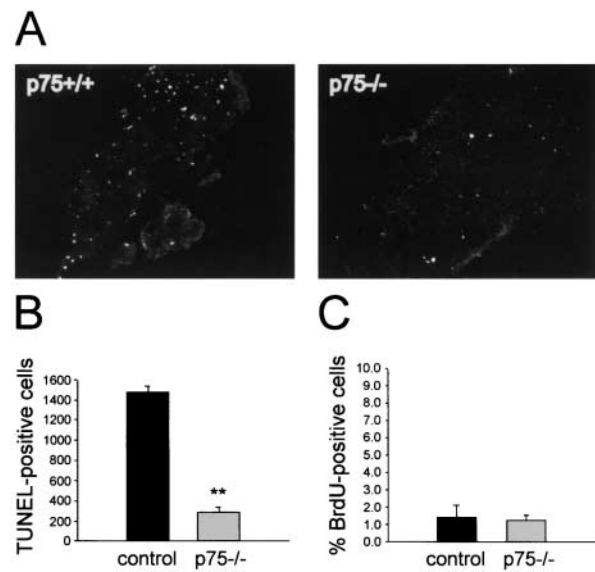
Together these findings indicate that developmental sympathetic neuron death is determined by a functionally antagonistic interplay between the TrkA and p75NTR receptors. However, defining this interplay has been difficult because in some cell types, p75NTR can directly interact with TrkA and modify its ability to bind neurotrophins (Hempstead et al., 1991; Benedetti et al., 1993; Ip et al., 1993; Bibel et al., 1999), whereas in other cells, including cultured sympathetic neurons, p75NTR can directly signal apoptosis in a Trk-independent fashion (Casaccia-Bonnet et al., 1996; Frade et al., 1996; Aloyz et al., 1998; Bamji et al., 1998; Davey and Davies, 1998; Soilu-Hanninen et al., 1999). Consideration of these findings has led to the proposal of two, not necessarily exclusive, models to explain the role of p75NTR during developmental neuron death. One model proposes that p75NTR mediates its proapoptotic effects indirectly by modulating TrkA function. In this model, p75NTR would rapidly eliminate neurons that fail to obtain sufficient NGF by “tuning-down” the suboptimal TrkA survival signals, and would also modulate the binding of neurotrophins to TrkA, so that only NGF (and not NT-3) would be used as a survival ligand. The second model derives from the hypothesis that all developing cells are “destined to die,” and survival factors ensure survival of only appropriately differentiated/connected cells (Raff, 1998). In this model, p75NTR would provide a direct death signal independent of Trk or Trk signaling, and sympathetic neurons would be rescued from death only if NGF activated TrkA to sufficient levels to silence this death signal. In this case, the ability of NT-3 to act as a survival ligand would be determined by the relative level of NT3-mediated activation of TrkA versus p75NTR (Belliveau et al., 1997).

In this paper, we have tested these two models biochemically and genetically, and provide evidence for the second model. Specifically, in these experiments, the presence or absence of p75NTR did not alter Trk activation or downstream signaling in primary sympathetic neurons, and crosses of p75NTR<sup>-/-</sup> and TrkA<sup>-/-</sup> mice demonstrated that the coincident absence of p75NTR substantially rescued the sympathetic neuron apoptosis observed in TrkA<sup>-/-</sup> animals. These data therefore indicate that p75NTR signals death in the presence or absence of TrkA, and support a model of naturally occurring cell death where p75NTR provides an ongoing death signal. Optimal TrkA activation can suppress this signal in neurons that successfully compete for adequate levels of target-derived trophic support.

## Results

### The absence of p75NTR leads to reduced apoptosis of developing sympathetic neurons

We have previously reported (Bamji et al., 1998) that in p75NTR<sup>-/-</sup> mice, sympathetic neurons of the SCG do not decrease in number from postnatal day (P)1 to P21, the normal period of naturally occurring cell death, but instead undergo a delayed decrease in neuronal number by adulthood. A number of developmental processes could account for this perturbation: an increase in the proliferation of sympathetic neuroblasts, a decrease in the level of neuronal apoptosis, or an alteration in the cell fates adopted by precursor cells in

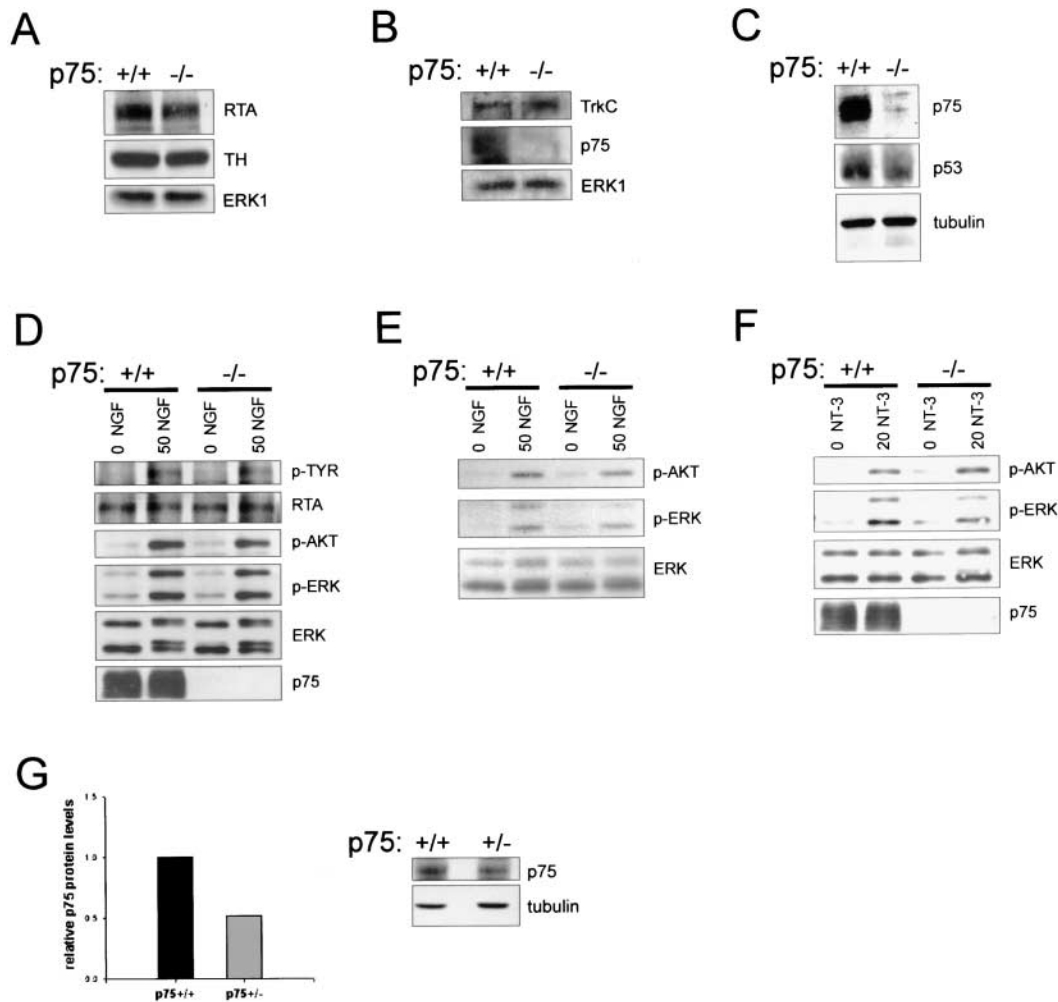


**Figure 1. The increase in sympathetic neuron number in the neonatal p75NTR<sup>-/-</sup> SCG is due to reduced apoptosis, not increased proliferation.** (A) Fluorescence photomicrographs of TUNEL analysis of representative sections through the P2 SCG of p75NTR<sup>+/+</sup> and p75NTR<sup>-/-</sup> animals. (B) Quantitation of TUNEL analysis similar to that seen in A. Numbers represent the total mean number of apoptotic nuclei in the SCG of p75NTR<sup>+/+</sup> (control) versus p75NTR<sup>-/-</sup> (p75<sup>-/-</sup>) animals. (\*\**P* < 0.0005, *n* = 3). (C) Percentage of BrdU-positive cells with neuronal morphology in the p75NTR<sup>+/+</sup> (control) versus p75NTR<sup>-/-</sup> (p75<sup>-/-</sup>) SCG at P3 and P4 (*P* = 0.4, *n* = 3 for each group). In both cases, error bars represent the standard error of the mean.

the ganglion. To distinguish between these possibilities, we assessed apoptosis and proliferation in p75NTR<sup>-/-</sup> versus wild-type SCG.

Initially, we performed TUNEL to measure the total number of apoptotic cells within the sympathetic SCG on P2. To perform this analysis, every fourth section was collected, TdT-mediated dUTP-biotin nick end labeling (TUNEL) was performed, the positive nuclei were counted, and the number of positive profiles were multiplied by four to determine the total number of apoptotic nuclei per ganglion. As predicted, many TUNEL-positive nuclei were detected within the wild-type SCG; 1,472 ± 60 per ganglion (*n* = 3). In contrast, the p75NTR<sup>-/-</sup> SCG contained only 290 ± 45 apoptotic profiles per ganglion (*n* = 3), a statistically significant decrease of ~80% (Fig. 1, A and B).

We next measured proliferation in the P3 and P4 p75NTR<sup>-/-</sup> versus p75NTR<sup>+/+</sup> ganglia. To examine the extent of ongoing cell division, p75NTR<sup>+/+</sup> and p75NTR<sup>-/-</sup> pups were injected twice with 50 mg/kg BrdU, which is incorporated into newly synthesized DNA during the S phase of the cell cycle. 2 d later, SCGs were removed and processed for anti-BrdU immunocytochemistry. Direct counts of fluorescently labeled cells with neuronal morphology demonstrated no change in the number of BrdU-positive neurons in p75NTR<sup>+/+</sup> and p75NTR<sup>-/-</sup> ganglia (1.43 ± 0.07%, *n* = 3 and 1.25 ± 0.03%, *n* = 3, respectively) (Fig. 1 C). Thus, in the absence of p75NTR, apoptotic sympathetic neuron death is greatly decreased, and neuroblast proliferation is unperturbed, resulting in a



**Figure 2. Levels of Trk receptors, Trk receptor activation, and downstream survival signaling in p75NTR<sup>-/-</sup> SCG neurons.** (A) Western blot analysis of equal amounts of protein from p75NTR<sup>-/-</sup> versus p75NTR<sup>+/+</sup> SCG at P7, probed for TrkA (RTA), tyrosine hydroxylase (TH) and ERK1. (B) Western blot analysis of lysates of P7 p75NTR<sup>-/-</sup> versus p75NTR<sup>+/+</sup> SCG that were precipitated with WGA and then probed with an antibody specific for TrkC or the intracellular region of p75NTR. Equal amounts of protein from the same lysates were also probed for ERK1. (C) Western blot analysis of equal amounts of protein from p75NTR<sup>-/-</sup> versus p75NTR<sup>+/+</sup> ganglia probed for p75NTR (p75), p53, or for total tubulin. (D) Western blot analysis of equal amounts of protein from cultured p75NTR<sup>-/-</sup> versus p75NTR<sup>+/+</sup> neonatal sympathetic neurons that were washed free of NGF, and then were induced with 0 or 50 ng/ml NGF for 10 min. Blots were probed with an antibody specific to phosphotyrosine to detect tyrosine phosphorylated Trk (p-TYR), or with antibodies for the activated phosphorylated forms of Akt (p-AKT) or the ERKs (p-ERK), and then reprobed for TrkA (RTA), total ERKs (ERK), or p75NTR (p75). Note that in the ERK reprobe, a mobility shift is evident in the lysates from NGF-treated neurons, consistent with the increased levels of phosphoERK observed. (E and F) Western blot analysis of equal amounts of protein from cultured p75NTR<sup>-/-</sup> versus p75NTR<sup>+/+</sup> neonatal sympathetic neurons that were washed free of NGF and induced either with 50 ng/ml NGF for 1 h (E) or 20 ng/ml NT-3 for 10 min (F). Blots were probed with antibodies specific to phosphorylated Akt (p-AKT) or phosphorylated ERKs (p-ERK) and then reprobed with antibodies for total ERKs or for p75NTR (p75). (G) Western blot analysis for p75NTR in equal amounts of protein from p75NTR<sup>+/+</sup> versus p75NTR<sup>+/-</sup> ganglia at P7. The blot was reprobed for tubulin, scanned, and the ratio of p75NTR to tubulin was plotted on the accompanying bar graph.

net increase in sympathetic neuron number relative to wild-type ganglia.

### Trk receptor levels, activation, and downstream signaling in p75NTR<sup>-/-</sup> sympathetic neurons

Three potential explanations for the deficit in apoptosis observed in p75NTR<sup>-/-</sup> SCG are (1) Trk receptor levels, activation, and subsequent downstream survival signaling are increased in the absence of p75NTR; (2) the absence of p75NTR allows TrkA to respond more robustly to nonpreferred ligands such as NT-3 (Benedetti et al., 1993; Ip et al., 1993); and (3) p75NTR mediates a direct apoptotic signal-

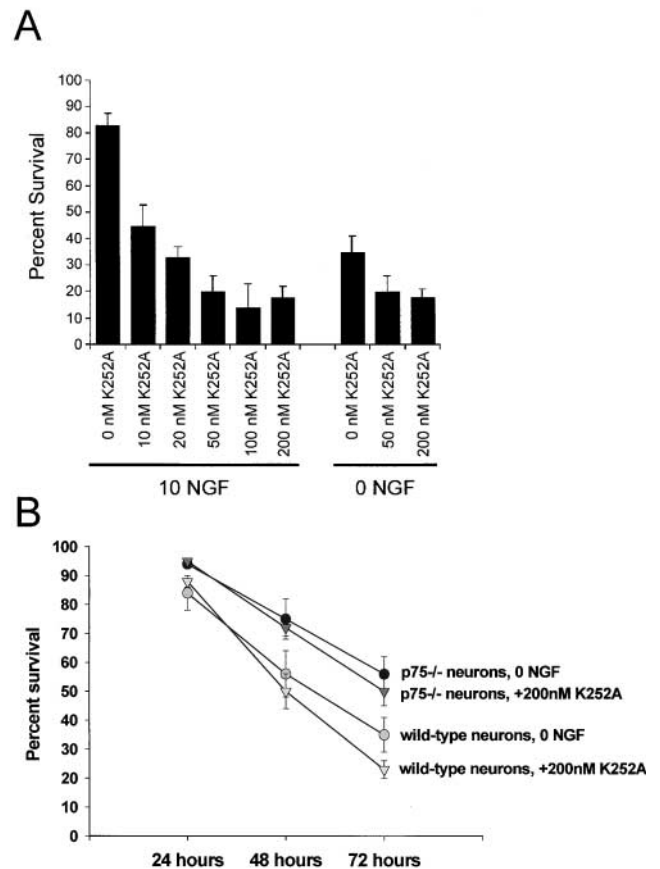
ing cascade that is eliminated in its absence (Aloyz et al., 1998). To examine the first two possibilities, we assayed Trk receptor levels, activation, and downstream survival signaling in p75NTR<sup>-/-</sup> ganglia and cultured p75NTR<sup>-/-</sup> neurons. Initially, we examined levels of TrkA and TrkC in p75NTR<sup>-/-</sup> sympathetic ganglia at P7. SCG lysates were run on SDS-PAGE, transferred to nitrocellulose, and probed with an antibody specific to TrkA (RTA) (Clary et al., 1994). Alternatively, lysates were precipitated with WGA, which precipitates glycosylated proteins, and analyzed on Western blots with an antibody specific to the full-length isoform of TrkC (Belliveau et al., 1997). This analy-

sis revealed that levels of TrkA were slightly but consistently decreased in the p75NTR<sup>-/-</sup> SCG (Fig. 2 A), whereas TrkC levels were constant (Fig. 2 B). In contrast, levels of ERK1 were unchanged (Figs. 2, A and B). Because full-length Trk receptors are only expressed on sympathetic neurons and not on nonneuronal cells in the ganglia, and neuronal number is increased in the absence of p75NTR, these data indicate that the decreased apoptosis in p75NTR<sup>-/-</sup> SCG is not due to an increase in Trk receptor levels.

We also compared a number of additional proteins in the p75NTR<sup>-/-</sup> and p75NTR<sup>+/+</sup> ganglia at P7. We first examined levels of p75NTR itself, using an antibody to the intracellular region that should recognize a splice variant still present in Schwann cells of the exon III p75NTR<sup>-/-</sup> animals examined in this study (Von Schack et al., 2001). No p75NTR protein corresponding to this smaller variant was detectable either in the p75NTR<sup>-/-</sup> ganglia or in cultured p75NTR<sup>-/-</sup> sympathetic neurons (Fig. 2, B–F), indicating that if this variant is expressed in developing sympathetic neurons, its levels are very low. We next examined levels of tyrosine hydroxylase and tubulin, two proteins associated with sympathetic neuron phenotype. Western blot analysis revealed that levels of both proteins were similar in p75NTR<sup>-/-</sup> and p75NTR<sup>+/+</sup> ganglia (Fig. 2, A and C). Finally, we examined levels of p53, an apoptotic protein in neurons (Slack et al., 1996) whose levels are increased by p75NTR signaling in sympathetic neurons (Aloyz et al., 1998). Western blot analysis revealed that, as would be predicted if p75NTR activation leads to increased levels of p53 during naturally occurring neuronal death, levels of p53 are somewhat decreased in the p75NTR<sup>-/-</sup> ganglia (Fig. 2 C).

Because examination of the p75NTR<sup>-/-</sup> ganglia indicated that the enhanced neuronal survival was not due simply to increased levels of TrkA or TrkC, we next determined whether Trk receptor activation and downstream survival signaling were increased in the absence of p75NTR. Previous work has demonstrated two survival pathways downstream of TrkA in sympathetic neurons, the PI 3-kinase–Akt pathway and the Mek–ERK pathway (for review see Kaplan and Miller, 2000). We therefore cultured sympathetic neurons from p75NTR<sup>-/-</sup> and p75NTR<sup>+/+</sup> SCG in 50 ng/ml NGF for 5 d, switched them into 0 or 50 ng/ml NGF, and then assayed activation of Trk and/or of these two survival pathways. Western blot analysis of sympathetic neurons induced for 10 min with 50 ng/ml NGF revealed that Trk receptor activation was similar in p75NTR<sup>+/+</sup> and p75NTR<sup>-/-</sup> neurons (Fig. 2 D). Moreover, Western blots probed with antibodies to the activated, phosphorylated forms of Akt or the ERKs indicated that activation of both of these pathways was similar in the presence or absence of p75NTR (Fig. 2 D). We performed similar studies after 1 h of stimulation with 50 ng/ml NGF to ask whether the kinetics of survival pathway activation were altered by the presence or absence of p75NTR. Western blot analysis revealed that even after 1 h, levels of activation of Akt and the ERKs were similar in p75NTR<sup>-/-</sup> and p75NTR<sup>+/+</sup> neurons (Fig. 2 E). Thus, TrkA activation and downstream survival signaling in response to NGF were not enhanced in the absence of p75NTR.

We then examined Trk-mediated survival signaling in response to NT-3. p75NTR<sup>-/-</sup> sympathetic neurons have



**Figure 3. Cultured p75NTR<sup>-/-</sup> neurons show enhanced survival in the absence of all Trk signaling.** (A) Percentage survival of mouse sympathetic neurons switched for 72 h into varying concentrations of K252A in the presence or absence of 10 ng/ml NGF. Results are normalized so that the number of neurons at the time of NGF withdrawal is 100%. Each point represents the values pooled from two to four independent experiments, each repeated in triplicate. Error bars represent the standard error of the mean. (B) Percentage survival of p75NTR<sup>-/-</sup> versus p75NTR<sup>+/+</sup> (wild-type) neurons at various time points after a switch into 0 ng/ml NGF plus or minus 200 nM K252A. Results represent the mean  $\pm$  standard error of combined data from four separate experiments, each performed in triplicate.

previously been demonstrated to show enhanced survival in response to NT-3 both in culture (Lee et al., 1994b) and in vivo (Brennan et al., 1999). This increased NT-3-mediated survival could be due to either (a) enhanced TrkA activation and survival signaling in response to nonpreferred ligands such as NT-3 in the absence of p75NTR or (b) the absence of an antagonistic death signal resulting from NT-3 binding to p75NTR. To distinguish between these two possibilities, p75NTR<sup>-/-</sup> and p75NTR<sup>+/+</sup> neurons were cultured in NGF for 5 d, washed free of neurotrophin, and exposed to 20 ng/ml NT-3 for 10 min. We have previously demonstrated that 20 ng/ml NT-3 is sufficient to cause low levels of TrkA receptor activation in sympathetic neurons (Belliveau et al., 1997). Neurons were lysed and Western blots of the lysates were probed with antibodies to phospho-Akt or phospho-ERK, and reprobbed for total ERK protein. This analysis (Fig. 2 F) revealed that NT-3 caused a similar induction in levels of phospho-Akt and phospho-ERK in

p75NTR<sup>+/+</sup> and p75NTR<sup>-/-</sup> neurons, suggesting that the observed increase in NT-3-mediated survival is not likely due to enhanced Trk survival signaling, but is instead due to the absence of an antagonistic p75NTR-mediated death cascade in the knockout neurons.

### Cultured p75NTR<sup>-/-</sup> neurons show enhanced survival in the absence of all Trk signaling

Together, these data suggest that the enhanced survival observed in p75NTR<sup>-/-</sup> neurons is not due to enhanced Trk signaling. However, to formally rule out this possibility, we cultured p75NTR<sup>-/-</sup> neurons and asked whether they showed enhanced survival in the presence of K252a, a pharmacological agent that inhibits all Trk receptor signaling (Tapley et al., 1992).

Initially, we performed experiments to ensure that K252A was capable of blocking Trk-mediated survival in mouse neurons in the presence of exogenous NGF. We have previously performed similar studies with rat sympathetic neurons, and have demonstrated that 200 nM K252A was sufficient to completely eliminate NGF-mediated TrkA activation and block NGF-mediated survival (Vaillant et al., 1999). To perform these experiments, wild-type mouse sympathetic neurons were established in 50 ng/ml NGF for 5 d, and were then switched into 10 ng/ml NGF in the presence of 10–200 nM K252A. Fields of phase-bright neurons were then counted immediately after the switch, and at 24 h intervals thereafter. This analysis (Fig. 3 A) revealed that in the presence of 10 ng/ml NGF, K252A decreased neuronal survival in a concentration-dependent fashion over 72 h, with the maximal effect apparent at 50–200 nM K252A: in 10 nM K252A, 45 ± 8% of neurons were still alive at 72 h, in 20 nM, 33 ± 4% were alive, whereas with 100 nM, only 14 ± 9% were still alive. We then performed similar studies where neurons were withdrawn from NGF, and various concentrations of K252A were added for 72 h to ensure that all Trk-mediated survival signals were eliminated (Fig. 3 A). This analysis confirmed that the addition of 50 or 200 nM K252A eliminated any residual survival signaling that was due to small amounts of NGF present in the cultures after the washout period. Specifically, in 0 NGF 35 ± 6% of neurons were still alive, whereas in 50 and 200 nM K252A, 20 ± 6% and 18 ± 3% were still alive, respectively.

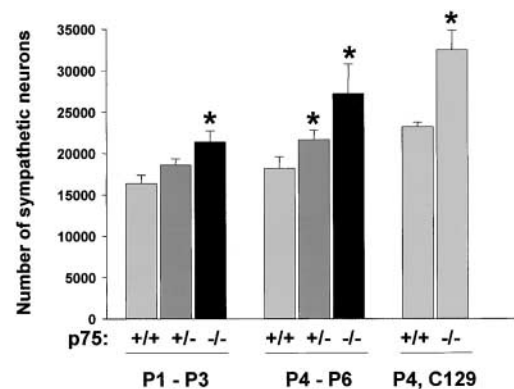
We then analyzed the survival of p75NTR<sup>+/+</sup> versus p75NTR<sup>-/-</sup> neurons after NGF withdrawal with and without 200 nM K252A to eliminate all Trk-mediated survival signaling. As previously reported (Bamji et al., 1998), combined results of four independent experiments (each performed in triplicate) revealed that p75NTR<sup>-/-</sup> neurons died significantly more slowly in the absence of NGF (Fig. 3 B). Specifically, 24 h after NGF withdrawal, 94 ± 1% of p75NTR<sup>-/-</sup> neurons were still alive, compared with 84 ± 6% of wild-type neurons ( $P = 0.07$ ). By 48 h, 75 ± 7% of p75NTR<sup>-/-</sup> neurons were still alive, compared with 56 ± 8% of control neurons ( $P = 0.06$ ). By 72 h, 56 ± 6% of p75NTR<sup>-/-</sup> neurons were alive, versus 35 ± 6% of controls ( $P < 0.05$ ). Interestingly, inhibition of Trk signaling with K252A significantly decreased the baseline survival observed with the wild-type neurons ( $P < 0.05$  at 72 h), whereas it

had no significant effect on the survival of p75NTR<sup>-/-</sup> neurons. Specifically, 24 h after NGF withdrawal, 95 ± 1% of p75NTR<sup>-/-</sup> neurons were still alive, compared with 88 ± 2% of controls ( $P < 0.05$ ), and by 48 h 72 ± 3% of p75NTR<sup>-/-</sup> neurons were alive, versus 50 ± 6% of the wild-type neurons ( $P < 0.05$ ). By 72 h, 50 ± 5% of the p75NTR<sup>-/-</sup> neurons were still alive, versus 23 ± 3% of the wild type ( $P < 0.005$ ). Thus, the absence of p75NTR prevents sympathetic neurons from undergoing appropriate apoptosis after NGF withdrawal, even when all Trk signaling is inhibited.

### The coincident absence of p75NTR significantly rescues TrkA<sup>-/-</sup> sympathetic neuron apoptosis in vivo

Although the data presented here on p75NTR<sup>-/-</sup> sympathetic neurons strongly support the hypothesis that p75NTR causes sympathetic neuron apoptosis via a Trk-independent mechanism, it is still possible that, in vivo, p75NTR might function by directly modulating TrkA function. To distinguish between a Trk-dependent versus Trk-independent action of p75NTR in vivo, we crossed the p75NTR<sup>-/-</sup> and TrkA<sup>-/-</sup> animals; sympathetic neurons undergo a rapid apoptotic death in TrkA<sup>-/-</sup> animals as a consequence of the lack of Trk-mediated survival signals (Smeyne et al., 1994; Fagan et al., 1996). If p75NTR mediates neuronal apoptosis by modulating TrkA and/or a TrkA-dependent signaling cascade, then the absence of p75NTR should have no effect on the TrkA<sup>-/-</sup> phenotype. If, in contrast, p75NTR mediates neuronal apoptosis in a Trk-independent fashion, then the severe neuronal loss seen in TrkA<sup>-/-</sup> SCG would be at least partially rescued in the absence of p75NTR.

To perform these studies, we initially confirmed the p75NTR<sup>-/-</sup> phenotype in the mixed C129/C57BL6 background that resulted from these crosses. TrkA<sup>+/+</sup> and



**Figure 4. Sympathetic neuron number is increased in the developing C129/C57BL6 SCG as an inverse function of p75NTR gene dosage.** Sympathetic neuron number in the SCG of p75NTR<sup>+/+</sup>, p75NTR<sup>+/-</sup>, and p75NTR<sup>-/-</sup> animals at ages P1–P3 and P4–P6. Results represent mean ± standard error ( $n = 3–7$  for each genotype). At P1–P3, p75NTR<sup>+/+</sup> and p75NTR<sup>-/-</sup> SCG numbers are significantly different ( $*P < 0.05$ ), and at P4–P6, p75NTR<sup>+/+</sup> neuron counts are significantly different from the p75NTR<sup>+/-</sup> and p75NTR<sup>-/-</sup> counts ( $*P < 0.05$  for both groups). For comparison, neuronal numbers from the P4 SCG of p75NTR<sup>+/+</sup> and p75NTR<sup>-/-</sup> C129 animals are shown (Bamji et al., 1998).

p75NTR<sup>-/-</sup> animals were crossed, and then their TrkA<sup>+/-</sup>, p75NTR<sup>+/-</sup> progeny were bred to produce animals for analysis. The SCGs were analyzed from the resultant TrkA<sup>+/+</sup>, p75NTR<sup>+/+</sup>, p75NTR<sup>+/-</sup>, and p75NTR<sup>-/-</sup> animals; ganglia were grouped by age, P1–P3 and P4–P6. This analysis revealed an increase in the numbers of neurons in the p75NTR<sup>-/-</sup> versus p75NTR<sup>+/+</sup> SCG at both ages (P1–P3: 21,425 ± 1,324, *n* = 3 versus 16,390 ± 1,003, *n* = 5, *P* < 0.05; P4–P6: 27,221 ± 3,570, *n* = 6 versus 18,211 ± 1,401, *n* = 4, *P* < 0.05) (Fig. 4), a phenotype similar to that observed in the C129 background (Bamji et al., 1998).

Interestingly, an intermediate neuron number was observed in the p75NTR<sup>+/-</sup> SCG at both P1–P3 (18,606 ± 787, *n* = 3) and P4–P6 (21,684 ± 1,114, *n* = 7) (Fig. 4), suggesting that p75NTR levels are a key determinant of sympathetic neuron survival. To confirm that p75NTR levels were actually regulated as a function of gene dosage, as these results suggest, we performed Western blot analysis in the SCG of p75NTR<sup>+/+</sup> and p75NTR<sup>+/-</sup> animals at P7 (Fig. 2 G). This analysis revealed that p75NTR levels were lower in the p75NTR<sup>+/-</sup> animals, correlating with the observed increase in neuronal survival.

We then characterized neuronal numbers in the SCG of the p75NTR<sup>+/+</sup>, TrkA<sup>+/+</sup>, TrkA<sup>+/-</sup>, and TrkA<sup>-/-</sup> animals over this same time frame. This analysis revealed that, as pre-

viously reported (Fagan et al., 1996), there was a dramatic decrease in the number of SCG neurons at P1–P3 in the TrkA<sup>-/-</sup> animals relative to their TrkA<sup>+/+</sup> littermates (6,108 ± 411, *n* = 3 versus 16,390 ± 1,003, *n* = 5). By P4–P6, the neuronal loss in the TrkA<sup>-/-</sup> SCGs was even more substantial (3,557 ± 724, *n* = 3 versus 18,211 ± 1,401, *n* = 4) (Fig. 5 A). These numbers represent a 63 and 80% decrease in neuronal number in the TrkA<sup>-/-</sup> ganglia at P1–P3 and P4–P6, respectively.

Interestingly, quantitation of sympathetic neurons in the TrkA<sup>+/-</sup> SCG revealed no significant difference between numbers in TrkA<sup>+/+</sup> and TrkA<sup>+/-</sup> ganglia at these two time points (P1–P3: 15,176 ± 796, *n* = 6; P4–P6: 19,777 ± 1880, *n* = 3 for the TrkA<sup>+/-</sup> SCG) (Fig. 5 A), although Western blot analysis indicated that levels of TrkA were reduced in the TrkA heterozygotes (Fig. 5 B). This same analysis revealed that levels of TrkC were similar in the TrkA<sup>+/+</sup> and TrkA<sup>+/-</sup> SCG (Fig. 5 B), indicating that TrkC was not compensating for the lower levels of TrkA in these ganglia. Thus, somewhat surprisingly, levels of TrkA are not rate-limiting for survival at this age, whereas relatively small (i.e., twofold) differences in levels of p75NTR significantly affect the level of neuronal survival.

Finally, we determined whether the absence of p75NTR was able to rescue the dramatic loss of sympathetic neurons observed in the TrkA<sup>-/-</sup> SCG. At P1–P3, the concomitant absence of p75NTR almost completely rescued sympathetic neuron numbers in the TrkA<sup>-/-</sup>, p75NTR<sup>-/-</sup> SCG; 13,665 ± 730 neurons (*n* = 3) versus 16,390 ± 1,003 neurons (*n* = 5) in the TrkA<sup>+/+</sup>, p75NTR<sup>+/+</sup> SCG (Fig. 6 A). Moreover, at this age, even the loss of one p75NTR allele was enough to significantly increase neuronal survival; the TrkA<sup>-/-</sup>, p75NTR<sup>+/-</sup> SCG contained 11,000 neurons, whereas the TrkA<sup>-/-</sup>, p75NTR<sup>+/+</sup> ganglia contained only 6,108 ± 411 neurons. A rescue was also observed at P4–P6. The magnitude was, however, lower than that seen at P1–P3; 9,861 ± 622 neurons (*n* = 5) versus 3,557 ± 724 neurons (*n* = 3) in the TrkA<sup>-/-</sup>, p75NTR<sup>+/+</sup> SCG (Fig. 6 A). Similarly, a rescue was still observed in the TrkA<sup>-/-</sup>, p75NTR<sup>+/-</sup> ganglia at P4–P6, although this was of a lesser magnitude than that observed in the p75NTR<sup>-/-</sup> SCG. Thus, independent signaling via p75NTR represents a major default death pathway for developing sympathetic neurons.

In spite of the increase in neuron number, double mutant animals were not healthier than TrkA<sup>-/-</sup> animals, and most died within the first three postnatal days. Moreover, cresyl violet-stained sections of P4 wild-type and TrkA<sup>-/-</sup>, p75NTR<sup>-/-</sup> SCGs showed that the rescued cells were much smaller than their wild-type counterparts, a phenotype similar to that previously reported for sympathetic neurons lacking other components of the cell death machinery, such as Bax<sup>-/-</sup> cells (Deckwerth et al., 1996). To ensure that these smaller cells were in fact neurons, we immunostained sections from P2 TrkA<sup>-/-</sup>, p75NTR<sup>-/-</sup> SCGs with an antibody for neuron-specific βIII-tubulin, and then Nissl-stained the alternate sections (Fig. 7). This analysis revealed that all of the smaller neuronal cells expressed βIII-tubulin, and that the numbers of neurons obtained by counting the immunostained versus Nissl-

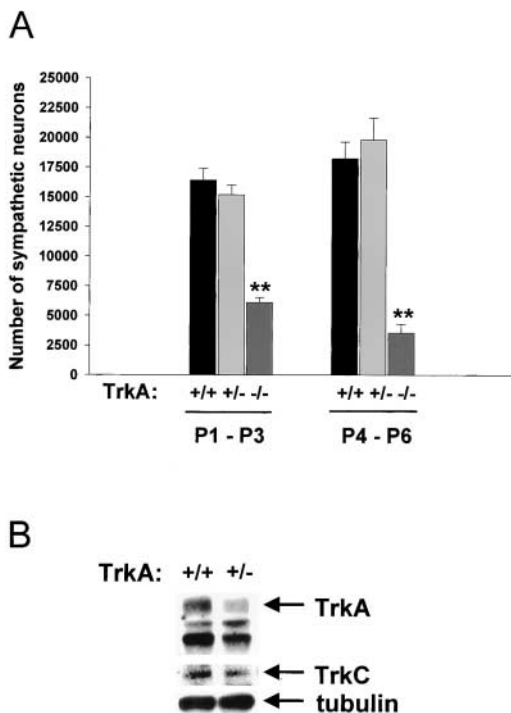
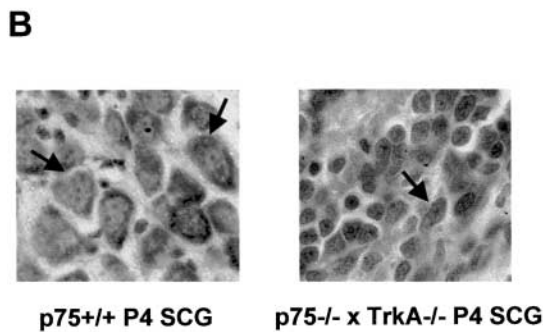
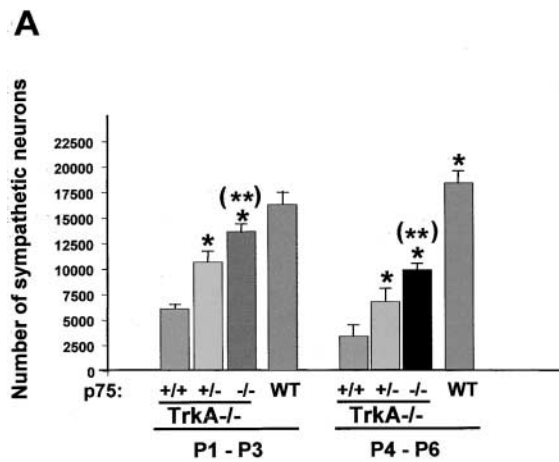
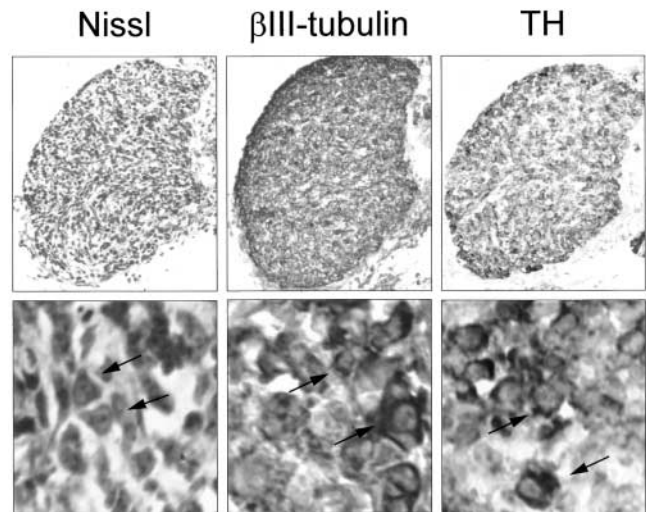


Figure 5. Analysis of sympathetic neuron number in TrkA<sup>+/+</sup>, TrkA<sup>+/-</sup>, and TrkA<sup>-/-</sup> SCG. (A) Sympathetic neuron number in the SCG of TrkA<sup>+/+</sup>, TrkA<sup>+/-</sup>, and TrkA<sup>-/-</sup> animals at ages P1–P3 and P4–P6 in the C129/C57BL6 background. Results represent mean ± standard error (*n* = 3–5 for each genotype). At both ages, TrkA<sup>-/-</sup> neuron number is greatly decreased (\*\**P* < 0.005), but there is no significant difference in the TrkA<sup>+/+</sup> ganglia. (B) Western blot analysis of equal amounts of protein from the TrkA<sup>+/+</sup> and TrkA<sup>+/-</sup> SCG at P10. Blots were probed for TrkA, TrkC, and tubulin.



**Figure 6. The absence of p75NTR rescues the sympathetic neuron apoptosis observed in the neonatal TrkA<sup>-/-</sup> SCG.** (A) Sympathetic neuron number in TrkA<sup>-/-</sup>, p75NTR<sup>+/+</sup>, p75NTR<sup>+/-</sup>, and p75NTR<sup>-/-</sup> SCG at P1–P3 and P4–P6. For comparison, TrkA<sup>+/+</sup>, p75NTR<sup>+/+</sup> (WT) SCG counts are also shown. Results represent mean  $\pm$  standard error ( $n = 3-5$  for each genotype). In the absence of TrkA, the number of neurons is increased in the p75NTR<sup>-/-</sup> versus p75NTR<sup>+/+</sup> SCG at both P1–P3 (\*\* $P < 0.0005$ ) and P4–P6 (\*\* $P < 0.001$ ). In addition, at both developmental ages, the number of neurons in p75NTR<sup>+/-</sup> SCG is significantly greater than in p75NTR<sup>+/+</sup> SCG (\* $P < 0.05$  for both groups). (B) Photomicrographs of cresyl violet-stained sections showing the morphology of SCG sympathetic neurons in p75NTR<sup>+/+</sup>, TrkA<sup>+/+</sup> (left) and p75NTR<sup>-/-</sup>, TrkA<sup>-/-</sup> (right) mice at P4. Arrows indicate sympathetic neurons.

stained sections were similar: in two different p75NTR<sup>-/-</sup>, TrkA<sup>-/-</sup> SCGs, 790 and 1032 cresyl violet-stained neurons were present in representative sections, and 930 and 808  $\beta$ III tubulin-positive cells were present in the adjacent sections, respectively. We also immunostained alternate sections from the same animals for tyrosine hydroxylase (Fig. 7). Whereas there was significantly more variability in tyrosine hydroxylase levels from cell to cell, likely as a consequence of the fact that tyrosine hydroxylase levels are highly upregulated by Trk signaling, this analysis confirmed that the small,  $\beta$ III-tubulin-positive cells were sympathetic neurons (Fig. 7). Thus, although the absence of p75NTR significantly rescued and/or delayed the cell death that occurs in the absence of TrkA signaling, it was unable to rescue other TrkA-dependent phenotypes, such as cell body hypertrophy and, presumably, target innervation.



**Figure 7. p75NTR<sup>-/-</sup>, TrkA<sup>-/-</sup> sympathetic neurons express neuron-specific  $\beta$ III-tubulin and tyrosine hydroxylase.** Photomicrographs of alternate sections taken from a p75NTR<sup>-/-</sup>, TrkA<sup>-/-</sup> ganglia and then either Nissl-stained or immunostained for the neuron-specific protein  $\beta$ III-tubulin or for tyrosine hydroxylase (TH). The upper panels are low-power micrographs of entire sections, and the bottom panels are high-power micrographs showing stained neurons (arrows).

## Discussion

The results described in this paper indicate that p75NTR provides an apoptotic signal for developing sympathetic neurons in the presence or absence of TrkA, and one of the major ways that TrkA supports neuronal survival is by suppressing this death signal. Specifically, these results support five major conclusions. First, studies on the p75NTR<sup>-/-</sup> SCG indicate that the increased sympathetic neuron number previously reported (Bamji et al., 1998) is due to a dramatic deficit in neuronal apoptosis, and not to an increase in neuroblast proliferation. Second, biochemical studies demonstrate that there is no increase in TrkA or TrkC levels or in Trk receptor activation in response to NGF in p75NTR<sup>-/-</sup> sympathetic neurons. Third, downstream NGF or NT-3-mediated survival signaling is similar in p75NTR<sup>+/+</sup> and p75NTR<sup>-/-</sup> sympathetic neurons. Fourth, pharmacological studies demonstrate that p75NTR<sup>-/-</sup> sympathetic neurons are deficient in apoptosis after NGF withdrawal, even when all Trk signaling is inhibited. Fifth, crosses of the p75NTR<sup>-/-</sup> and TrkA<sup>-/-</sup> animals demonstrate that the loss of sympathetic neurons seen in neonatal TrkA<sup>-/-</sup> animals is substantially rescued by the coincident absence of p75NTR.

Together, these data support a model of naturally occurring neuronal death where p75NTR provides an ongoing apoptotic signal that is suppressed by optimal TrkA activation in those neurons that successfully compete for NGF to survive. These data also strongly argue that p75NTR does not mediate its proapoptotic effects by modulating TrkA and/or TrkA signaling cascades. Instead, these findings indicate that p75NTR directly signals apoptosis in sympathetic neurons in a TrkA-independent fashion. What is the biological rationale for such a mechanism? It is likely that p75NTR provides a molecular mechanism for ensuring

rapid and active apoptosis when a neuron is unsuccessful in competing for adequate amounts of the appropriate neurotrophin. If a sympathetic neuron reaches the appropriate target and sequesters NGF, TrkA is robustly activated and this activation silences any ongoing apoptotic signal deriving from p75NTR (Bamji et al., 1998; Yoon et al., 1998; Mazzoni et al., 1999). Conversely, when a neuron is late arriving and/or reaches an inappropriate target, TrkA is only weakly (if at all) activated as a consequence of the lack of NGF, and an ongoing p75NTR-mediated death signal would cause the rapid apoptotic elimination of that neuron, thereby ensuring that the subsequent period of target innervation occurs appropriately. The importance of this rapid neuronal elimination is emphasized by the finding that sympathetic neuron target innervation is highly aberrant in p75NTR<sup>-/-</sup> animals (Lee et al., 1994a).

What if a developing sympathetic neuron encounters a neurotrophin such as NT-3, which has the capacity to weakly activate TrkA (Belliveau et al., 1997)? Recent evidence indicates that the absence of p75NTR enhances the ability of NT-3 to function as a sympathetic neuron survival factor both in culture (Lee et al., 1994b) and, importantly, in vivo (Brennan et al., 1999). How does p75NTR mediate this activity? As shown here, NT-3 activates downstream survival signaling in p75NTR<sup>+/+</sup> and p75NTR<sup>-/-</sup> neurons to a similar extent, and coincident p75NTR activation does not affect the levels of sympathetic neuron TrkA activation (Aloyz et al., 1998; Bamji et al., 1998). Thus, it is likely that p75NTR “selects” survival ligands by antagonistically signaling neuronal apoptosis. Thus, a weak TrkA survival signal deriving from NT-3 would normally be overridden by a strong apoptotic signal deriving from p75NTR, but in the absence of p75NTR, this weak TrkA signal would be sufficient for survival. It is still possible that NT-3 binding to p75NTR might, in some cellular contexts, dampen downstream TrkA signaling, as has been recently observed in *Xenopus* oocyte experiments (Mischel et al., 2001). However, the data presented here argue that this is not the major mechanism responsible for the enhanced survival of p75NTR<sup>-/-</sup> sympathetic neurons in response to NT-3 (Lee et al., 1994b; Brennan et al., 1999).

Data presented here also support the conclusion that p75NTR plays a major role in sympathetic neuron apoptosis after NGF withdrawal both in culture and in vivo: p75NTR<sup>-/-</sup> sympathetic neurons are delayed in their apoptosis in the absence of all Trk receptor signaling, and the absence of p75NTR<sup>-/-</sup> substantially rescues neonatal TrkA<sup>-/-</sup> sympathetic neurons in vivo. Although the deficit in apoptosis observed in vivo could be due to the absence of p75NTR on ganglionic satellite cells, Schwann cells, and/or peripheral targets, the deficit in apoptosis observed in culture must be intrinsic to sympathetic neurons themselves, making it more likely that the in vivo alterations are also cell autonomous. These findings also predict that p75NTR signaling constitutes a major component of the apoptotic signaling pathways activated after NGF withdrawal. Support for this idea derives from our previous work on the p53 tumor suppressor protein during sympathetic neuron apoptosis (Miller et al., 2000). These studies showed that (a) p53 is essential for sympathetic neuron apoptosis after both NGF withdrawal

and p75NTR activation (Aloyz et al., 1998); (b) when Trk-mediated activation of the Ras pathway is inhibited, sympathetic neurons die via a p53-mediated pathway (Mazzoni et al., 1999); and (c) in the p53<sup>-/-</sup> SCG, sympathetic neurons show reduced apoptosis (Aloyz et al., 1998), although this deficit is not of the same magnitude as that reported here for the p75NTR<sup>-/-</sup> SCG. Together these studies support a model where p75NTR leads to the activation of a p53-mediated apoptotic pathway, and TrkA signaling silences this apoptotic pathway via activation of Ras (Kaplan and Miller, 2000). Thus, when NGF is withdrawn, the p75NTR-mediated death pathway is “unmasked,” a process that substantially contributes to the subsequent neuronal apoptosis. Interestingly, we show here that levels of p53 are decreased in the developing p75NTR<sup>-/-</sup> SCG, suggesting that p75NTR may contribute to the activation of this same apoptotic pathway in vivo.

Previous work on p75NTR<sup>-/-</sup> sympathetic neurons in culture have reached conclusions somewhat different from those reported here. Davies et al. (1993), studying embryonic sympathetic neurons, reported that NGF supported survival of p75NTR<sup>-/-</sup> and p75NTR<sup>+/+</sup> neurons equally well. In contrast, Lee et al. (1994b) reported that acutely dissociated sympathetic neurons from p75NTR<sup>-/-</sup> P3 or P4 animals required slightly higher concentrations of NGF to maintain full survival. These two studies differ from the current study in two respects. First, the neurons cultured here were derived from P1 animals. Second, and likely of more importance, is the fact that both of the previous studies examined acutely dissociated sympathetic neurons. In the experiments reported here, we established sympathetic neurons for 5 d in NGF before NGF withdrawal to ensure that we were studying healthy neurons that had not been recently axotomized. In this regard, we have directly compared biochemical parameters in acutely dissociated versus established sympathetic neuron cultures, and have demonstrated that these two neuron populations differ significantly with respect to p75NTR levels and stress pathway induction (unpublished data). We therefore feel it is likely that any differences from these previous studies are likely due to differences in culture models.

What are the ligands for p75NTR during developmental sympathetic neuron death or in culture after NGF withdrawal? In vivo, p75NTR is likely robustly activated by non-TrkA-binding neurotrophins, such as brain-derived neurotrophic factor (BDNF; Leibrock et al., 1989), that are encountered in the target environment (Kohn et al., 1999) and/or that are made by sympathetic neurons themselves (Causing et al., 1997). In this regard, sympathetic neuron number is increased in BDNF<sup>-/-</sup> animals (Bamji et al., 1998), supporting the idea that BDNF is one endogenous apoptotic ligand for p75NTR. Similarly, BDNF (Causing et al., 1997) and NT-4 (unpublished data) are both made by cultured sympathetic neurons and may contribute to an autocrine p75NTR-driven apoptotic loop after NGF withdrawal. However, it is also formally possible that, as previously proposed (Bredesen et al., 1998), p75NTR may signal in an unliganded fashion in certain situations, or that it might bind to an as yet unidentified autocrine ligand to provide an ongoing receptor-mediated apoptotic signal.



Although all of these data support the idea that p75NTR plays a major role in regulating developmental sympathetic neuron apoptosis, several observations reported here indicate that p75NTR-independent pathways are also very important. In particular, our work shows that sympathetic neuron rescue is substantial but not complete in the p75NTR<sup>-/-</sup>, TrkA<sup>-/-</sup> mice at birth, and sympathetic neuron number decreases in these double knockout animals between birth and P4–P6, suggesting that death is still ongoing in vivo, but at a reduced rate. Moreover, p75NTR<sup>-/-</sup> sympathetic neurons still die in culture, albeit more slowly, when NGF is withdrawn or when Trk function is pharmacologically inhibited. What might these p75NTR-independent pathways be? Previous work indicates that, after NGF withdrawal, sympathetic neurons activate a number of components of the cell cycle (Park et al., 1996, 1997), an activation that contributes to neuronal apoptosis. This cell cycle pathway may well represent a p75NTR-independent pathway that is responsible for the delayed apoptosis of p75NTR<sup>-/-</sup> sympathetic neurons. Such a model implies that TrkA would suppress this pathway independent of its effects on p75NTR; TrkA is known to lock PC12 cells out of the cell cycle (Burstein and Greene, 1982), and a number of Trk family members are thought to play key roles in regulating the progenitor to postmitotic neuron transition (Verdi and Anderson, 1994; Ghosh and Greenberg, 1995). Interestingly, cell cycle deregulation can lead to p53 activation (Sherr and Weber, 2000), and it is therefore possible that p53 and/or other p53 family members such as p63 (Yang et al., 1998) or p73 (Jost et al., 1997; Kaghad et al., 1997; Pozniak et al., 2000) may play a key role in integrating both p75NTR-dependent and -independent apoptotic pathways in developing sympathetic neurons.

Together, the data reported here support a model of naturally occurring neuronal death where an ongoing, receptor-mediated apoptotic signal destines cells to die, and where one of the major roles of exogenous survival ligands is to silence this ongoing apoptotic signal. In the case of sympathetic neurons, p75NTR provides the death signal and TrkA the survival signal. The emerging evidence of a similar interplay between death and survival receptors in other developing neurons (Raoul et al., 1999; Agerman et al., 2000) argues that such a mechanism may prove to be the rule rather than the exception.

## Materials and methods

### Analysis of C129/C57BL6 mice

Mice homozygous for a targeted mutation in the p75NTR gene (Lee et al., 1992; genetic background C129) and heterozygous for a targeted mutation in the TrkA gene (Smeyne et al., 1994; genetic background C57BL6) were obtained from Jackson ImmunoResearch Laboratories. Initially, p75NTR<sup>-/-</sup> animals were crossed with TrkA<sup>+/-</sup> animals, their progeny were genotyped using PCR, and the p75NTR<sup>+/-</sup>, TrkA<sup>+/-</sup> animals (now in a mixed background) were bred. The progeny of these crosses were analyzed and/or used for breeding to generate second generation progeny for analysis. No differences were noted in phenotype or sympathetic neuron number in animals that were first or second generation in the mixed C129/C57BL6 background. For morphometric analyses, SCGs were removed and immersion fixed in 4% paraformaldehyde in phosphate buffer for 1 h at 4°C. Ganglia were cryoprotected in graded sucrose solutions, 7- $\mu$ m-thick sections were serially cut on a cryostat, and every section was collected on chromium/aluminum/gelatin-coated slides. Slides were stained with cresyl violet, and

morphometric analyses were performed with the Northern Eclipse computer-based image analysis software (Empix Inc.) using a Sony XC-75CE CCD video camera, as we have previously described (Aloyz et al., 1998; Bamji et al., 1998; Pozniak et al., 2000). Neuronal numbers were determined by counting all neuronal profiles with nucleoli on every fourth section and multiplying the obtained number by four, as per Coggeshall (1984). This method does not correct for split nucleoli. Statistical results were expressed as the mean  $\pm$  the standard error of the mean and were tested for significance by a one-tailed Student's *t* test.

Alternatively, alternate sections were immunostained for neuron-specific  $\beta$ III-tubulin or tyrosine hydroxylase. Sections were initially treated in 0.3% hydrogen peroxide in PBS, pH 7.4, for 30 min. They were then incubated in 10% normal goat serum and 0.25% Triton X-100 in PBS for 30 min, and incubated for 24 h at room temperature in antibodies either for neuron-specific  $\beta$ III-tubulin (1:2,000; RDI) or tyrosine hydroxylase (1:1,000; Chemicon). Primary antibodies were diluted in PBS containing 3% normal goat serum and 0.25% Triton X-100. After a rinse in PBS, sections were incubated in the same solution containing biotinylated goat anti-rabbit IgG (1:200; Jackson ImmunoResearch Laboratories) for 1 h at room temperature. They were then rinsed, incubated in avidin-biotin complex (Vector Laboratories) for 1 h at room temperature, and then rinsed again. Sections were reacted with a solution containing 0.05% DAB tetrachloride, 0.04% nickel chloride, and 0.015% hydrogen peroxide in 0.1 M PBS. After the DAB reaction, sections were rinsed, dehydrated through a graded series of ethanols, coverslipped, and viewed under brightfield optics.

For TUNEL, ganglia were fixed for 30 min in 4% paraformaldehyde, cryoprotected, sectioned, and collected as above. TUNEL was performed immediately on every fourth section (in situ cell detection kit; Boehringer) as per the manufacturer's instructions, and as we have previously described (Aloyz et al., 1998). For BrdU incorporation assays, P3 and P4 pups were injected intraperitoneally on two consecutive days with 50 mg/kg BrdU. Sympathetic ganglia were processed as above, and anti-BrdU immunocytochemistry was performed. The number of BrdU-positive cells was determined by direct counts of labeled cells with neuronal morphology.

### Primary neuronal cultures

Mass cultures from the SCG of P1 mice were cultured by a modification of the method used for rat neurons. Specifically, ganglia were dissected and triturated as for rat ganglia (Belliveau et al., 1997; Vaillant et al., 1999), except that neurons were dissociated in the presence of Ultraculture media (Biowhitaker, Inc.) instead of saline solution. Neurons were then plated on collagen-coated 96-well culture dishes (Falcon Plastics) in Ultraculture media containing 2 mM glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 3% FBS (Life Technologies), and 50 ng/ml mouse 2.5 S NGF prepared from mouse salivary gland (Cedarlane Labs, Ltd.). 3 d after plating, neurons were fed with the same media containing 0.5% cytosine arabinoside (Sigma-Aldrich).

For survival assays, after 5 d in culture, neurons were washed three times with neurotrophin-free media for 2.5 h total. After the washout, neurons were cultured with or without 10 ng/ml NGF plus or minus various concentrations of K252A (Calbiochem-Novabiochem). The number of phase-bright neurons with neurites in randomly selected, 5.3-mm<sup>2</sup> fields was counted immediately after the NGF washout in all conditions, and then recounted at 24-h intervals for 4 d. In every experiment, each condition was repeated in triplicate. In three of the four experiments analyzed, p75NTR<sup>-/-</sup> and p75NTR<sup>+/+</sup> neurons were cultured at the same time in the same 96-well plates to eliminate variability.

### Western blot analysis

For Western blot analysis of SCGs and cultured neurons, ganglia or neurons were lysed and analyzed as previously described (Aloyz et al., 1998; Vaillant et al., 1999). Immunoprecipitations of total Trk protein and WGA precipitations of TrkC were also performed as previously described (Belliveau et al., 1997; Bamji et al., 1998). The antibodies used for these analyses were anti-phosphotyrosine 4G10 (1:5,000; Upstate Biotechnology), anti-pan Trk 203B (1:2,000; gift of D. Kaplan, Montreal Neurological Institute, McGill University, Montreal, Canada), anti-TrkA RTA (1:2,000; gift of L. Reichardt, University of California San Francisco, San Francisco, CA), anti-p75NTR (1:2,000; Promega), anti-erk 1 (1:5,000; Santa Cruz Biotechnology, Inc.), anti-tubulin (1:5,000; Oncogene Research Products), anti-TrkC (gift of D. Kaplan), anti-phospho-MAPK (p-ERK, 1:5,000; Promega), anti-phospho-Akt (1:1000; Cell Signaling Technology), and anti-tyrosine hydroxylase (1:1000; Chemicon). Secondary antibodies were incubated for 1.5 h at room temperature, and were used at a 1:10,000 for both the goat anti-mouse HRP antibody and the goat anti-rabbit HRP antibody

(both from Boehringer). Detection was performed using ECL (Amersham Pharmacia Biotech) and XAR x-ray film (Eastman Kodak Co.)

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