Molecular Mechanisms Regulating Survival of Peripheral Neurons During Development and Adulthood

Gregory S. Walsh

Neurology and Neurosurgery
McGill University
Montreal, Quebec

February, 2005

A thesis submitted to the McGill University Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Ph.D. in Science.

Your file Votre référence
ISBN: 978-0-494-21712-2
Our file Notre référence
ISBN: 978-0-494-21712-2

TABLE OF CONTENTS

ABSTRACT

Developmental 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 show 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 -/- and TrkA -/- mice indicate that the coincident absence of p75NTR substantially rescues TrkA -/- sympathetic neurons from developmental death in vivo. These data 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. We then examined a role for JNK-p53 apoptotic pathway in NGF-deprived neurons and in nerve injury-induced death. Specifically, inhibition of JNK by overexpression of JIP-1 was sufficient to rescue sympathetic neurons from NGF withdrawal-induced death. In addition, JNK is robustly activated in nerve-injured neonatal facial motoneurons and these neurons are rescued from nerve-injury-induced cell death in p53 null mice. We then investigated the intracellular mechanisms that underlie the relative invulnerability of adult versus developing DRG sensory neurons. In both adult and neonatal neurons, death stimuli induced the apoptotic JNK pathway, but JNK activation only caused death of neonatal neurons, indicating that adult neurons have a downstream block to apoptosis. An essential component of this "block" is the p53 family member, ΔNp73. Cultured adult p73+/- DRG neurons were more vulnerable to apoptotic stimuli than their p73+/+ counterparts, and invulnerability could be restored to the p73+/- neurons by increased expression of ΔNp73. Moreover, although DRG neuron development was normal in p73+/- animals in vivo, axotomy caused death of adult p73+/- but not p73+/+ DRG neurons. Thus, one way adult neurons become invulnerable is to enhance endogenous survival pathways, and one component of these adult pathways is p73.

RÉSUMÉ

La mort des neurones sympathiques au cours du développement est déterminée par les interactions fonctionnelles entre le récepteur du NGF, TrkA, et le récepteur des neurotrophines p75 (p75NTR). Une question centrale est de savoir si p75NTR induit l'apoptose via la régulation directe de l'activité de TrkA, ou si p75NTR agit de manière indépendante de TrkA. Dans cette étude, nous démontrons que la présence ou l'absence de p75NTR n'altère ni l'activité de TrkA, ni la survie induite par la signalisation du NGF ou du NT-3 dans des cultures primaires de neurones. Des croisements entre les souris p75NTR-/- et TrkA-/- indiquent que l'absence de p75NTR prévient considérablement la mort des neurones sympathiques TrkA-/- au cours du développement *in vivo*. Ces données supportent un modèle dans lequel les neurones sympathiques en développement sont « prédestinés à mourir », de par l'envoi d'un signal constant d'apoptose relayé par p75NTR et que l'un des mécanismes majeurs de promotion de la survie neuronale par TrkA est de réprimer ce constant signal de mort. Nous avons ensuite examiné la fonction du signal d'apoptose via JNK/p53 dans des neurones privés de NGF, ainsi que lors de la mort induite par blessure au nerf facial. Spécifiquement, nous démontrons que l'inhibition de JNK par la surexpression de JIP-1 est suffisante pour prévenir la mort des neurones sympathiques privés de NGF. De plus, JNK est fortement activé dans les motoneurones du nerf facial accidenté chez les animaux néonatals et l'absence de p53 permet la survie de ces neurones endommagés. Finalement, nous avons étudié les mécanismes intracellulaires responsables de l'invulnérabilité des neurones sensoriels de DRG adultes relativement aux neurones de DRG en cours de développement. Chez les neurones adultes comme chez les neurones néonatals, les stimuli apoptotiques conduisent à l'activation de JNK. Cependant, cette activation n'induit que la mort des neurones néonatals, suggérant que les neurones adultes sont capables d'inhiber le processus apoptotique en aval de JNK. Un composant essentiel du mécanisme d'inhibition de la mort chez les neurones adultes est ΔNp73, un membre de la famille de p53. Les neurones adultes de DRG p73+/- en culture sont plus vulnérables à des stimuli apoptotiques que leurs homologues p73+/+ et la surexpression de ΔNp73 permet aux neurones p73+/- de retrouver leur invulnérabilité. Bien que les neurones de DRG p73+/- présentent un

développement *in vivo* normal, une axotomie du nerf facial entraîne spécifiquement la mort de ces neurones chez l'adulte, tandis que leurs homologues p73+/+ ne sont pas affectés par un tel dommage. Ainsi, un mécanisme utilisé par les neurones adultes pour devenir invulnérables consiste à accentuer les signaux de survie endogènes dont un des composants est p73.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Freda Miller, for providing such a great environment in which to practice science. Freda has such incredible enthusiasm for her work and undying support for her students, which made it a pleasure to work with and learn from her. I would also like to thank the lab members, past and present, that I have worked with that have made the lab environment such a great place to be. In particular, I would like to thank Marta Majdan and Nina Orike, whom I collaborated with closely on several projects in this PhD. I would also like to thank Christine Pozniak and Jim Fawcett for their friendship, guidance, and support when I first began working in the lab. I would also like to acknowledge Anna Lee, whom I worked closely with in the latter parts of my PhD on the p73 project, doing experiments and tossing around ideas.

I would also like to thank David Kaplan for helpful advice throughout my studies. It is a rare and fortunate experience to be able to work closely with both Freda and David, who treat their lab members as part of their family.

I would also like to thank my family for their ever present love and support. Especially my mother, without whose friendship and support, I never would have gotten this far. And also my Bro and Sis, who had the patience to listen to me talk about science and pretend that they thought it was cool.

I would also like to thank Anne Aumont and Christine Laliberte, first for their friendship and second for helping make the lab run smoothly. I wouldn't have been able to find anything in the lab without their help, and will be forever greatful for their help. I would also like to acknowledge Yew Meng Heng, Julia Hwang, and Howard Rosenberg of the Electron Microscopy Laboratory, Hospital for Sick Children for their excellent technical assistance.

A special shoutout goes to my homeboy Ian McKenzie, longtime friend and roommate and best damn goalie to hit McGill's D League Hockey. Montreal would not have been the same without the beers, vids, shindigs, and late night visits to Lafleur's. Damn that apartment was sweet! A second shoutout to Karun Singh, the third member in the Montreal triumvirate, and the best student I ever got to work with. With friends like these you can't help but do well and have fun. Also to the Toronto additions, Brad Jacobs and Jeff Biernaskie, who participated in Friday pops, and who are never afraid to let the big dog eat on early Saturday mornings. And I would be remiss not to thank Fanie Barnane-Heider, who was like a sister to me in the lab, and was always bringing me gifts from the garden, organizing pool and volleyball. And last but not least, my love to Christine Paul, for all the little things, for keeping me sane, for making me laugh.

CONTRIBUTIONS OF AUTHORS

Chapter 2: TrkA mediates developmental sympathetic neuron survival in vivo by silencing an ongoing p75NTR-mediated death signal

I performed half the experiments, including sympathetic ganglion TUNEL labeling, sympathetic neuron cultures from p75+/+ and p75-/- mice, western blot, wheat germ agglutanin-pulldown, immunohistochemistry on SCG cryostat sections, neuron counting of immunopositive neurons, and whole ganglia protein extraction. I helped prepare the manuscript for publication.

Marta Majdan was responsible for BrdU studies, K252 studies with cultured sympathetic neurons, and counting of neuronal number in trkA-/-, p75-/-, and trkA-/-p75-/- double knockout animals.

Figure 1A: I did TUNEL-labelling of sections of sympathetic ganglia from p75+/+ and p75-/- mice.

Figure 2A: I extracted protein from whole SCG ganglia and did western blot anlaysis.
2B: I extracted protein from whole SCG ganglia, proteins were WGA precipitated and run on SDS-PAGE gels.
2D: I cultured SCG neurons from p75+/+ and p75-/- mice, made protein lysates, and analyzed the lysates by Western blot.
2E : I cultured SCG neurons from p75+/+ and p75-/- mice, made protein lysates, and analyzed the lysates by Western blot.
2F: I cultured SCG neurons from p75+/+ and p75-/- mice, made protein lysates, and analyzed the lysates by Western blot.

Figure 7: I immunostained sections of SCG taken from TrkA-/-,p75-/- double knockout mice

Majdan, M.*, **Walsh, G.S.***, Aloyz, R., Miller, F.D. (2001) TrkA mediates developmental sympathetic neuron survival in vivo by silencing an ongoing p75NTR-mediated death signal. *Journal of Cell Biology* 155:1275-85.

**co-first authors*

Chapter 3: A JNK-p53 apoptotic pathway is required for neuronal death after trophic factor withdrawal and following nerve injury.

I performed all experiments including generation of recombinant adenovirus, sympathetic neuron cultures, protein extraction from whole SCG ganglia, western blotting, facial nerve axotomy, counting of neuronal number, immunohistochemistry and I wrote the manuscript.

Chapter 4: The invulnerability of adult neurons: A critical role for p73.

I performed half the experiments including dorsal root ganglion neuron cultures, sympathetic neuron cultures, human embryonic kidney 293 cell cultures, western blot analysis, adenoviral infection, reverse-transcriptase chain reaction, sciatic nerve axotomy, immunocytochemistry, preparation of DRG tissues for electron microscopy. I helped write the manuscript.

Nina Orike was responsible for survival assays using cultured DRG sensory neurons from adult and neonatal mice, infection of DRG sensory neurons, western blot analysis of JNK activation in adult and neonatal neurons.

Figure 2C: I infected cultures of 293 cells and DRG neurons with MLK adenovirus, lysed the cells, and analyzed the lysates by Western blot.
2E: I cultured SCG neurons, infected with various adenoviruses, fixed and stained the cultures with Hoechst, and counted the number of apoptotic neurons.

Figure 3A: I extracted mRNA from DRG ganglia, and performed RT-PCR for p73 isoforms.

Figure 4A: I stained sections of DRG from p73+/+ and p73+/- mice for Nissl substance.
4B: I immunostained sections of DRG from p73+/+ and p73+/- mice for NF200, CGRP, and IB4.
4C: I prepared dorsal roots from p73+/+ and p73+/- mice for electron microscopy.
4D: I counted the number of myelinated and unmyelinated axons in cross sections of dorsal roots from p73+/+ and p73+/- mice.

Figure 5D: I cultured SCG neurons from p73+/+ and p73+/- mice, fixed and stained with Hoechst, and measured the percent apoptosis.

Figure 6A: I performed sciatic nerve axotomy on adult p73+/+ and p73+/- mice. I then prepared dorsal roots from nerve-injured mice for electron microscopy.
6B: I counted the number of myelinated and unmyelinated axons in cross sections of dorsal roots from p73+/+ and p73+/- mice following sciatic nerve axotomy.

Walsh, G.S.*, Orike, N.*, Kaplan, D.R., Miller, F.D. (2004) The invulnerability of adult neurons: A critical role for p73. *Journal of Neuroscience* 24:9638-47.

**co-first authors*

CHAPTER 1

INTRODUCTORY LITERATURE REVIEW

Nerve cells are among the most long-lived cell types in mammals, yet immature neurons die in large numbers during development. In fact, neuronal apoptosis is a normal feature of mammalian development, a process that helps sculpt the developing brain and has an important role in both acute nervous system injury and chronic neurodegenerative diseases. What are the molecular mechanisms that govern 'when' and 'how' nerve cells die?

Cellular apoptosis is crucial for the proper development of the nervous system and occurs during two distinct phases of neural development. The first phase occurs just prior to the onset of neurogenesis, when neural precursors undergo exponential proliferation. This phase serves to eliminate those progenitors that have not differentiated appropriately and to ensure that the correct number of cells is generated in structures such as the cerebral cortex (Blaschke et al., 1996; Gilmore et al., 2000). The existence of this period of developmental apoptosis has only recently been appreciated and the signals that dictate life versus death are not well understood (Kuan et al., 2000).

The second period of developmental apoptosis is the period of naturally-occurring neuronal death, and occurs after newly differentiated post-mitotic neurons have migrated to their final anatomic location, extended axons and attempted to establish appropriate target connections. These neurons then compete for limited quantities of target-derived trophic factors, with the prototype being nerve growth factor (NGF). Those neurons that do not receive optimal trophic support undergo apoptotic death due to an imbalance of proapoptotic signals over prosurvival signals. The cellular mechanisms regulating this process are arguably best understood in developing sympathetic neurons (Kaplan and Miller, 2000), and will be discussed in detail below. This period of developmental apoptosis is crucial to match neuronal numbers to the size of the target tissue, and results in the elimination of up to one half of the neurons originally present in any given population (Oppenheim, 1991).

In addition to the neuronal apoptosis that occurs developmentally, neuronal apoptosis also occurs as a pathological event following nervous system injury. Numerous

experimental and clinical investigations have determined that a significant proportion of the neuronal death that is observed in the hours to days after excitotoxic, ischemic or traumatic nervous system injury is apoptotic in nature (Wood and Youle, 1994; Beattie et al., 2000; Graham and Chen, 2001; Bengzon et al., 2002).

There is, however, still controversy about the importance of apoptosis in human neurodegenerative conditions (Yuan et al., 2003), an issue that is difficult to resolve given the necessity of using postmortem tissue from patients with long-term disease for these studies.

The molecular mechanisms that execute cell death in neurons have been extensively studied in the developing nervous system. Numerous studies indicate that the same molecules that regulate developmental neuron death also play a role in determining the survival of mature neurons following injury. Of particular importance in the nervous system is the intrinsic Bax/Apaf-1/caspase-9/caspase-3 mitochondrial death pathway (Green, 1998), as it has been shown to be critical in both developing and mature injured neurons. However, although extensive progress has been made in understanding how neurons die, we still do not understand how neurons integrate varied upstream apoptotic stimuli such as trophic withdrawal, DNA damage, hypoxia, and excitoxicity, and translate these death-inducing stimuli into the initiation of this common downstream mitochondrial apoptotic pathway. This thesis will describe my effort to elucidate the molecular mechanisms that regulate neuronal survival and death of peripheral neurons during development and into adulthood.

A Closer look at Naturally Occurring Neuron Death

A recurrent observation in the development of the nervous system is that neuronal populations are initially generated in surplus (on the order of two-or three-fold), followed by their large-scale elimination. Numerous studies over the past 50 years have contributed to the concept that the refinement in neuronal numbers in developing vertebrates is regulated by cellular interactions of developing neurons and the targets that they innervate (Purves and Lichtman, 1985). This phenomenon, referred to as naturally occurring cell death, occurs at a critical time for developing neurons - the period immediately following the arrival of their axons in the target fields. Experimental manipulation of the target

during this period can have profound effects on the survival of innervating neurons. For instance, removal of prospective targets early in development can enhance the degree of neuronal death (Hamburger and Levi-Montalcini, 1949), whereas implantation of a supernumary limbs in chick embryos increases the number of surviving neurons (Hollyday and Hamburger, 1976; Hollyday et al., 1977). Since the degree of neuronal survival is proportional to the amount of target available, it was proposed that the survival of developing neurons is directly related to the availablilty of their innervating targets. These and other studies helped establish the foundation for the *neurotrophic hypothesis*, which postulated that immature neurons compete for target-derived trophic factors that are in limited supply; only those neurons that are successful in establishing correct synaptic connections would obtain an adequate amount of trophic support to allow their survival. Regulation of neuronal numbers during development may provide a strategy though which vertebrates can correctly match numbers of neurons with the size of their targets (Purves, 1988).

Role of NGF in Neuronal Survival

It is now well established that target-neuron relationships are mediated by specific diffusible molecules (called neurotrophic factors) that are produced by target cells in limited amounts and are required for further survival of developing neurons. Perhaps one of the best studied family of neurotrophic factors are the Neurotrophins, which include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4).

Although NGF was discovered and characterized in the 1940's and 50's, the unambiguous demonstration of NGF as a neurotrophic protein is relatively recent. In his original paper describing the purification of NGF from the mouse submandibular gland, Stanley Cohen (1960) provided the best evidence that NGF is required during normal development: anti-NGF antibodies injected into newborn rodents specifically destroy the peripheral sympathetic nervous system. Similar experiments have revealed the near total loss of the sympathetic nervous system in newborn rats following systemic administration of anti-NGF antibodies during the period of naturally-occurring cell death (Levi-Montalcini and Angeletti, 1966; Levi-Montalcini and Booker, 1960; Ruit et al., 1990).

Furthermore, sympathetic nerve cells from ganglia of 8- to 11- day chick embryos fail to survive in culture, unless nanogram quantities of NGF are added daily to the culture medium. (Levi-Montalcini and Angeletti, 1963). Conversely exogenous administration of NGF during the period of naturally occurring cell death results in hypertrophy of sympathetic ganglia and an increase in the number of sympathetic neurons (Angeletti et al., 1971a; Ruit and Snider, 1991; Albers et al., 1994). Importantly, it has now been established that NGF and its mRNA are present, albeit at very low levels, in target tissues of the sympathetic and sensory nervous system. Taken together, these findings support the conclusion that NGF is a target-derived growth factor that controls neuronal survival at the time of target innervation.

As sympathetic neurons mature into adulthood, they become less dependent upon target-derived NGF for survival. Continued dependence of sympathetic neurons on NGF has been studied *in vivo* in mature rodents either by prolonged treatment with large systemic doses of anti-NGF antibodies or by using an experimental autoimmune approach (Goedert et al., 1978; Otten et al., 1979; Gorin and Johnson, 1980; Ruit et al., 1990; Ruit and Snider, 1991). Whereas Gorin and Johnson reported ~35% reduction in neuron numbers in the SCG of adult rats immunized against mouse NGF, Otten and colleagues (1979), using a similar methodology, found only reversible effects on adult sympathetic neurons. Another study (Ruit and Snider, 1991) found 22-24% reduction in cell numbers in mature mice after 1 month of treatment with anti-NGF antibodies.

Although embryonic and newborn neurons are "programmed to die" and thus completely dependent on survival cues in the environment, those neurons that have successfully survived the selection period undergo a transition to become relatively invulnerable, a transition that allows them to survive the remainder of the animal's lifetime. This transition has been well characterized for peripheral neurons, and is best exemplified by two types of experiments. In one series of experiments, neutralizing NGF antibodies were used to sequester NGF in developing versus adult animals; in neonates, this led to the death of NGF-dependent sympathetic and sensory neurons, while in adult animals, the neurons hypotrophied, but did not die (Angeletti et al., 1971b; Bjerre et al., 1975; Goedert et al., 1978; Otten et al., 1979). The second type of experiment involved peripheral nerve injury; when neonatal peripheral nerves were axotomized, there was

rapid and extensive death of sympathetic, motor and sensory neurons (Hendry and Campbell, 1976; Yip et al., 1984; Himes and Tessler, 1989; Sendtner et al., 1990), while the same axotomy in adult animals caused only delayed and restricted neuronal loss (Tandrup et al, 2000; Ma et al., 2001). Taken together, loss of trophic support has a greater effect on peripheral (sympathetic, sensory, motor) neurons in neonates than in adult animals. Thus, adult neurons have either acquired intrinsic survival mechanisms that are not present in developing neurons, and/or they have suppressed apoptotic pathways.

The Neurotrophins

In mammals, there have been four neurotrophins identified, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4). All four neurotrophins share similar primary, secondary, tertiary and quartenary structure. They are initially synthesized as 31-35 kDa proproteins, that are then cleaved by a variety of processing enzymes to give rise to the mature processed neurotrophins (13-16 kDa). Once fully processed, neurotrophins exist as non-covalently linked homodimers that are secreted through either the constitutive secretory pathway (in the case of NGF and NT-3) or through a regulated activity-dependent pathway (in the case of BDNF) (Ghosh et al., 1994; Mowla et al., 1999). The other neurotrophins all share striking sequence homology with that of NGF (Leibrock et al, 1989; Maisonpierre et al., 1990; Hallböök et al., 1991). These neurotrophins, however, differ in their sites of synthesis, developmental time periods of expression, and in their ability to provide trophic support to distinct neuronal populations.

Trk Receptors

The neurotrophins have wide ranging actions that modulate many aspects of neuronal biology including neuronal survival, neuronal apoptosis, neuronal differentiation, axon growth and regeneration, dendrite morphology, and synaptic plasticity. Neurotrophins mediate their multiple functions by binding to two types of cell surface receptors, the Trk receptor tyrosine kinases and the p75 neurotrophin receptor (p75NTR). While the Trk receptors transmit the stereotypical "positive" effects of neurotrophins such as enhanced

growth and survival, p75NTR transmits both positive and negative signals, depending on the cell type and the cellular context. How neural cells respond to neurotrophins therefore depends on how the two receptors act together to integrate and coordinate intracellular signals. In some instances, the two receptors functionally antagonize the other's action, whereas in some cell contexts the receptors augment each others actions. First, I will discuss the function of the Trk receptors in transducing neurotrophin-mediated survival signals and will later detail the role of the p75NTR in neurotrophin biology.

The observation that NGF stimulates tyrosine kinase activity in PC12 cells (Maher, 1988) and that transcripts for the receptor tyrosine kinase protooncogene $p140^{trk}$ (Martin-Zanca et al., 1990) were found in tissues possessing NGF-responsive neurons helped lead to the discovery that tropomyosin-related kinase A (TrkA) is an NGF receptor. Kaplan and colleagues found that the trk receptor tyrosine kinase could bind NGF, and hence serve as a receptor for NGF (Kaplan et al., 1991a). It was subsequently demonstrated that addition of NGF to PC12 cells leads to efficient phosphorylation of tyrosine residues on a 140 kDa polypeptide, which could be immunoprecipitated by antisera against tyrosine protein kinase receptors (Kaplan et al., 1991b). Two other Trk receptors (TrkB, and TrkC) were discovered to act as receptors for the other neurotrophins. Thus, the Trk receptors are highly related transmembrane receptor tyrosine kinases that are expressed in discrete neuronal populations and which are bound by specific neurotrophins. TrkA binds preferentially to NGF (Kaplan et al., 1991a,b; Klein et al., 1991); TrkB binds preferentially to BDNF and NT-4 (Soppet et al., 1991); and TrkC binds preferentially to NT-3 (Lamballe et al., 1991). A small degree of redundancy exists for neurotrophin binding with the Trks; for instance, TrkA is able to bind NT-3, but with lower efficacy than its preferred ligand NGF (Cordon-Cardo et al., 1991; Belliveau et al., 1997). In addition to kinase active isoforms, *trkB* and *trkC* genes encode truncated isoforms, generated by alternative splicing, (Klein et al., 1990; Tsoulfas et al., 1993) which inhibit intracellular signaling when coexpressed with the full length Trk isoform (Eide et al., 1996). This observation has been extended to *in vivo* studies, as transgenic mice overexpressing the truncated TrkC receptor exhibit a phenotype similar to that observed with targeted deletion of kinase active TrkC or NT-3 (Palko et al., 1999). Activation of Trk receptors by their neurotrophic ligands elicits the "stereotypical"

positive signals normally associated with growth factors, including survival, growth, and differentiation.

Neurotrophin and Trk Receptor Knockout mice

The pattern of expression of neurotrophins and their Trk receptors in embryonic and adult tissues in rodents, in combination with *in vitro* and *in vivo* assays, have helped to predict the cells within peripheral ganglia and central nuclei likely to be the targets of neurotrophin action. Mice lacking individual members of the neurotrophin and *trk* receptor families have confirmed the physiological importance of these ligand/receptor partners in neuronal survival, where extensive loss of specific neuronal populations are observed (Snider, 1994). In particular, *NGF*- and *trkA*-deficient animals have similar neuronal deficits, hypoalgesia, and reductions in the number of small-diameter sensory and sympathetic neurons (Crowley et al., 1994; Smeyne et al., 1994). Neuroanatomical examination of *BDNF* and *trkB* null mice revealed neuronal deficiencies in the central and peripheral (trigeminal, vestibular, and dorsal root ganglia) sensory nervous systems, and most die within the first week after birth (Jones et al., 1994; Klein et al., 1993; Ernfors et al., 1994a). Although *BDNF* and *trkB* null mutant animals exhibit similar deficits, their analysis is complicated by an additional ligand for *trkB*, neurotrophin-4 (NT-4), as well as the persistent expression of truncated isoforms of the *trkB* receptors.

The survival and neuronal deficits in *NT-3* -/- mice and mice that lack only the kinase active isoforms of *trkC* (*trkC*-kinase -/-) are quite different even though both exhibit similar deficiencies in movement and loss of large diameter proprioceptive sensory neurons (Klein et al., 1994; Ernfors et al., 1994b; Farinas et al., 1994; Tessarollo et al., 1994; Tessarollo et al., 1997). The majority of the *NT-3*-deficient animals die just after birth, whereas the *trkC*-kinase -/- animals survive up to 3 weeks. Taken together, these results establish that neurotrophin/Trk receptor signaling is essential for the survival of specific neuronal populations during development.

One notable difference that warrants mention is the phenotype of sympathetic neurons in NT-3 and TrkC knockout mice. Similar to *NGF*-/- and *TrkA*-/- mice, *NT-3* null mice have 50% fewer sympathetic neurons (Ernfors et al., 1994b; Farinas et al., 1994; Francis et al., 1999) and display deficits in sympathetic target innervation that can be

rescued by exogenous NT-3 (El-Shamy et al., 1996). The sympathetic neuron phenotype in NT-3 null mice appears to be due to NT-3 action via the TrkA receptor, since no deficit in sympathetic neuron number is observed in *TrkC*-/- mice (Fagan et al., 1996; Tessarollo et al., 1997). In fact, at this time period in development, sympathetic neurons express high levels of TrkA and low or negligible levels of TrkC. Several lines of evidence suggest that the sympathetic neuron phenotype in NT-3 null mice is due to a deficit in NT-3/TrkA-mediated axon growth. First, NT-3 is highly expressed by blood vessels along which sympathetic axons extend towards their final targets (Francis et al., 1999). Second, NT-3 can activate the TrkA receptor in cultured sympathetic neurons and is as efficient as NGF at promoting neurite outgrowth (Belliveau et al., 1997; Kuruvilla et al., 2004). Similarly, NT-3 is required in vivo for sympathetic axon extension along intermediate targets, whereas both NT-3 and NGF are important for end-organ target innervation (Glebova and Ginty, 2004; Kuruvilla et al., 2004). Third, NT-3 is unable to support the survival of sympathetic neurons when added exclusively to distal axons in compartmented cultures (Kuruvilla et al., 2004), suggesting that NT-3, unlike NGF, is incapable of providing retrograde trophic support. Taken together, these finding indicate that NGF and NT-3 act through TrkA to coordinate distinct aspects of sympathetic neuron development. Thus, NT-3 mediates axon extension though intermediate targets and final target-derived NGF mediates both target innervation and retrograde survival and gene expression.

Trk receptor signaling

As mentioned above, the survival of developing neurons depends on the availability of neurotrophic factors. Neurotrophins generally activate and ligate the Trk receptors (TrkA, TrkB, and TrkC), which are cell surface receptors with intrinsic tyrosine kinase activity. Upon ligand binding, Trk receptors dimerize and autophosphorylate; the receptor phosphorylates several tyrosine residues within its own cytoplasmic tail. These phosphotyrosines in turn serve as docking sites for other molecules such as phospholipase-C and adaptor proteins such as Shc, and these and other signal transduction molecules coordinate the various functions of the Trk receptors including survival, differentiation, and growth.

Activation of the PI3K-Akt pathway plays a central role in Trk-mediated neuronal survival signaling. The involvement of PI3K in neurotrophin-mediated survival responses was first suggested by the observation that PI3K inhibitors block the survival of effects of NGF *in vitro* (Yao and Cooper, 1995). Numerous groups have now reported that PI3 kinase activity is responsible for the majority of neurotrophin-regulated cell survival in sympathetic, sensory, cortical, and cerebellar neurons (Crowder and Freeman, 1998; D'Mello et al., 1997; Vaillant et al., 1999; Klesse and Parada, 1998; Dolcet et al., 1999).

PI3 kinase is activated by Trk receptors by the combined actions of Ras, a small GTP-binding protein, and Gab-1, an adapter protein. Ras directly interacts with PI3 kinase and inhibition of Ras suppressed NGF-mediated PI3 kinase activity (Rodriguez-Viciana et al., 1994). Also, Ras-mediated survival can be blocked by the PI3K inhibitor LY294002 (Mazzoni et al., 1999; Vaillant et al., 1999). Similarly, Gab-1 physically binds and PI3 kinase and when overexpressed, promotes NGF-independent survival (Holgado-Madruga et al., 1997; Korhonen et al., 1999).

Active PI3K enzymes catalyse the formation of the lipid 3'-phosphorylated phosphoinositides that regulate the localization and activity of the Ser/Thr kinase Akt (Philpott et al., 1997). Overexpression of active Akt protein supports the survival of neurons whereas a dominant-negative mutant of Akt inhibits neuronal survival, even in the absence of survival factors (Dudek et al., 1997; Crowder and Freeman, 1998). Thus, Akt is a crucial component of survival pathways downstream of Trk receptor activation in neurotrophin-induced neuronal survival.

Active Akt (or protein kinase B) binds and phosphorylates those proteins containing the Akt consensus phosphorylation site RXRXXS/T (Datta et al., 1999). All of the proposed targets of Akt in neurons appear to regulate apoptosis, consistent with its role in keeping neurons alive. For example, Bad is a pro-apoptotic member of the Bcl-2 family, which in its unphohsphorylated form can bind to Bcl-x_L, and thus block survival (Yang et al., 1996). However, the activation of Akt induces the phosphorylation of Bad and promotes its interaction with the chaperone protein 14-3-3, which sequesters Bad in the cytoplasm and inhibits Bad's proapoptotic activity (Datta et al., 1997). While Akt-induced phosphorylation of Bad has been shown in growth factor-treated cerebellar neurons (Bonni et al., 1999), Bad knockout mice do not show alterations in neuronal

apoptosis, suggesting that Bad might not be important for neurotrophin-induced neuronal survival during development (Shindler et al., 1998). Another candidate target for active Akt in neurons is the transcription factor Forkhead 1 (FKHRL1). Akt has been shown to bind and phosphorylate FKHRL1 *in vitro*, and ectopic expression of mutant FKHRL1 lacking Akt phosphorylation sites (Thr32/Ser315) increases the apoptosis of cerebellar neurons (Brunet et al, 1999). Akt has also been reported to promote survival in many systems by inhibiting the activity of c-Jun N-terminal kinase (JNK, also called stress-activated protein kinase) (Cerezo et al., 1998; Kim et al., 2001; Okubo et al., 1998; Sarmiere and Freeman, 2001). Chao's group reported that Akt may inhibit JNK activity through its interaction with the JNK-interacting protein.1, a JNK-pathway scaffold. Direct association of Akt and JIP1 has been demonstrated in primary neurons, and this interaction inhibited JIP1-mediated potentiation of JNK activity by decreasing JIP1 binding to specific JNK pathway kinases (Kim et al., 2002). Thus, these findings indicate that Akt kinase promotes neuronal survival in part by negatively regulating death-promoting signals.

Neurotrophins may also stimulate survival by activation of a second signaling pathway consisting of Ras-MEK-MAPK. Activation of Trk receptors stimulates docking of the adaptor protein Shc, which triggers the activation of the small GTP-binding protein Ras and the downstream MAP kinase (MAPK) cascade, which includes the sequential phosphorylation of and activation of the kinases Raf, MAP kinase/Erk kinase (MEK) and the extracellular signal-regulated protein kinase (ERK). This pathway has many roles in neurons including synaptic plasticity, long-term potentiation, axonal sprouting, and survival (Grewal et al., 1999). The role of MEK/ERK in neuronal survival is controversial. Although NGF treatment induces a strong and sustained activation of ERK in sympathetic neurons and PC12 cells, inhibition of MEK has little to no effect on NGF-dependent neuronal survival (Mazzoni et al., 1999; Korhonen et al., 1999). However, activation of MEK/ERK has been shown to protect neurons from injury or toxicity-induced cell death. For instance, MEK/ERK protected sympathetic neurons from cytosine arabinoside (Anderson and Tolkovsky, 1999), cortical neurons from camptothecin (Hetman et al., 1999) cerebellar neurons from oxidative stress (Skaper et al., 1998) and retinal ganglion cells from death after axotomy (Shen et al., 1999). Thus,

MEK/ERK activity appears to play a role in regulating neuronal survival following injury or toxic insults, but may not be required for neurotrophin-mediated neuronal survival.

The effect of the MEK/ERK pathway on neuronal survival is mediated by stimulating the activity or expression of pro-survival proteins, including RSK, CREB, and Bcl-2. Activation of the pp90 ribosomal S6 kinase (RSK) may regulate neuronal survival in two ways. First, like Akt, RSK phosphorylates Bad and may act synergistically with Akt to inhibit Bad's pro-apoptotic effects. Second, RSKs are potent activators of the CREB transcription factor. Because CREB is known to activate transcription of the anti-apoptotic gene *bcl-2*, it can stimulate cell survival directly. Indeed, NGF stimulated increases in Bcl-2 levels is blocked when either MEK activity or CREB transactivation is inhibited (Liu et al., 1999; Riccio et al., 1999). Ginty and co-workers demonstrated that blocking the function of CREB using dominant inhibitory CREB mutants induced apoptosis in virtually all sympathetic neurons grown in NGF (Riccio et al., 1999). Analysis of CREB-null mice supported a role for CREB in peripheral neuron survival. Neuronal number in the DRG of CREB-/- mice, although similar to wildtype at E13.5, decreased over the period of developmental cell death for sensory neurons (Lonze et al., 2002). A decreased number of sympathetic neurons was also observed in CREB-/- mice, but it was not determined whether this neuronal loss was attributable to a role for CREB in neural crest migration, neural progenitor proliferation, or neuronal survival (Lonze et al., 2002).

It is noteworthy that neurotrophins are not the only factors that promote neuronal survival: electrical stimulation and depolarization at high KCl concentration have long been known to inhibit neuronal cell death (Koike et al., 1989). Recent studies indicate that membrane depolarization activates neuronal survival pathways by stimulating Ca^{2+} influx though L-type calcium channels, that in some neurons leads to the downstream activation of Ras/PI3 kinase (Vaillant et al., 1999). In this study, suboptimal levels of NGF and KCl synergistically stimulated maximal Akt activation and neuronal survival, indicating that Akt may be a convergence point for diverse upstream survival signals. During development, neurons exposed to suboptimal levels of neurotrophins but maintain their activity, may have a competitive advantage over those that are not active, due to increased amounts of Akt activation.

Although each member of the Trk receptor family exhibits very high conservation in their intracellular domains (eg., catalytic tyrosine kinase and Shc binding site), several differences in downstream signaling have been noted. For instance, as described above, TrkA signaling in sympathetic neurons relies predominantly on activation of PI3 kinase for survival. However, when TrkB is introduced into sympathetic neurons, BDNF-mediated activation of TrkB uses both PI3 kinase and ERK pathways to elicit neuronal survival (Atwal et al., 2000). This may reflect differences in the association/dissociation kinetics of adaptor protein binding or recruitment of unique target proteins.

An interesting feature of survival signaling in morphologically complex neurons is that NGF binding to TrkA on distal axons must signal survival to cell bodies that are often located millimeters, or more away. Several models have been proposed to explain the basis of long-range retrograde survival signaling. The "Signaling Endosome" model proposes that retrograde signals are carried by endosomes containing NGF and phosphorylated "activated" TrkA receptors that are transported to the cell bodies (Tsui-Pierchala and Ginty, 1999; Watson et al., 2001; Delcroix et al., 2003). Interestingly, internalization and subsequent transport of NGF is not required for NGF-mediated retrograde survival (MacInnis et al., 2002), whereas the movement of phosphorylated catalytically active TrkA from distal axon to cell body appears to be required for certain events including CREB phosphylation, gene expression, and neuronal survival (Riccio et al., 1999; Watson et al., 1999; Ye et al., 2003). Importantly, Senger and Campenot (1997) observed the appearance of phosphorylated TrkA in cell bodies after addition of NGF to distal axons much faster than would be predicted for retrograde vesicular transport. This finding led the authors to postulate that NGF binding to TrkA in nerve terminals initiates a "wave" of TrkA receptor phosphorylation that propagates from terminal to cell body independent of vesicular transport. Another possibility is that NGF binding to TrkA on distal axons stimulates the retrograde movement of downstream signaling proteins, such as cell body activation of the MAP kinase, ERK5 (Watson et al., 2001). Future studies will no doubt help to elucidate the molecular mechanism underlying retrograde transmission of survival signals in neurons.

Cell Death in the Nervous System

How do neurons die in the absence of trophic support? It was thought for quite a long time that neurons die simply of passive starvation in the absence of trophic factors. It is now known that this is not the case. Using cultured sympathetic neurons as a model system, Johnson and co-workers showed that inhibition of RNA and protein synthesis blocked sympathetic neuronal cell death (Martin et al., 1988) induced by NGF withdrawal, indicating that neuronal cell death is an active process that requires transcription and translation. It was subsequently discovered that neuronal cell death ultimately requires the participation of cysteine proteases, termed caspases, supporting the notion that trophic factor deprivation activates a cellular suicide program in nerve cells. What are the critical components of this cell death pathway in neurons and how is it activated in the absence of trophic support?

Key molecules in neuronal apoptosis

Neuronal apoptosis in vertebrates is regulated by the Bcl-2 family of proteins, the adaptor protein APAF-1 (for apoptotic protease-activating factor-1) and the cysteine protease caspase family, which are homologues of the *C. elegans* cell-death gene products CED-9, CED-4, and CED-3, respectively. Neurons share the same basic downstream apoptosis program with all other cell types, but neurons at different developmental stages express different combinations of Bcl-2 and caspase family members.

The Bcl-2 family of proteins plays a crucial role in intracellular apoptotic signal transduction. This gene family includes both anti-apoptotic and pro-apoptotic proteins that contain one or more Bcl-2 homology (BH) domains (Merry and Korsmeyer, 1997). The major anti-apoptotic family members include Bcl-2 and Bcl-x_L, which function subcellularly at the mitochondrial outer membrane. Bcl-2 is highly expressed in the CNS during development and is downregulated after birth, whereas the expression of Bcl-2 in the PNS is maintained thoughout life (Merry and Korsmeyer, 1997). Initial examination of Bcl-2 knockout mice revealed no phenotype; that is the development of nervous system is normal until birth. This result was surprising since transgenic expression of Bcl-2 in the nervous system was found to protect against neuronal cell death during development (Martinou et al., 1994). Similarly, ectopic expression of Bcl-2 can support

the survival of sympathetic neurons in the absence of NGF, supporting the idea that elevating the levels of Bcl-2 can override apoptotic signals that are activated in trophic-deprived neurons (Garcia et al., 1992). This lack of phenotype may be explained by redundancy in the Bcl-2 family, since Bcl-x_L is also expressed in the developing brain. Unlike Bcl-2 expression however, Bcl-x_L expression is maintained in the CNS into adulthood. Bcl-x_L-null mice die around embryonic day 13 with massive cell death in the developing nervous system (Motoyama et al., 1995). Closer examination of Bcl-2 knockout mice revealed that while development proceeded normally until birth, there is a slow progressive loss of sensory, motor and sympathetic neurons after birth. This finding suggests that Bcl-2 is crucial for the maintenance of neuronal survival for certain neuronal populations after the neonatal period.

Members of the Bcl-2 proteins function at a critical checkpoint in the cell death pathway to regulate mitochondrial permeability. Anti-apoptotic members (Bcl-2 and Bcl-x_L) prevent the release of mitochondrial apoptogens (including cytochrome c and Smac/Diablo). In contrast, pro-apoptotic members, including BAX, BAK, and BOK, which share BH1, BH2, and BH3 domains with Bcl-2, cause the release of cytochrome C from mitochondria (Jurgensmeier et al., 1998; Narita et al., 1998). BAX is widely expressed thoughout the nervous system. In BAX-deficient mice, superior cervical ganglia, dorsal root ganglia and Facial nuclei display increased neuron number. Furthermore, neonatal sympathetic neurons and facial motor neurons from BAX-/- mice are more resistant to cell death induced by NGF-withdrawal and axotomy, respectively. In fact, BAX-deficient sympathetic neurons can survive up to 23 days in the absence of NGF, albeit these neurons were atrophied with reduced elaboration of neurites (Deckwerth et al., 1996). In addition, translocation of BAX from cytosol to the mitochondria precedes cytochrome c release in NGF-deprived sympathetic neurons (Putcha et al., 1999). Thus, activation of BAX is a crucial event for neuronal cell death induced by trophic factor withdrawal as well as injury.

Recognized more recently within the Bcl-2 family is a subfamily of pro-apoptotic molecules that share homology only within the BH3 domain. These "BH3-only" proteins (eg., BAD, BID, BIM, HRK, PUMA and NOXA) transduce death signals to the mitochondrial checkpoint, culminating in BAX-, and/or BAK-dependent release of

mitochondrial apoptogens. The role of BH3-only proteins can vary in a stimulus-specific and cell-type specific manner, and apoptotic activity is modulated at multiple levels: transcriptional, post-transcriptional, and post-translational.

There is recent evidence to indicate that BIM plays an important role in inducing neuronal death in trophic factor-deprived neurons. Several reports have shown that trophic factor withdrawal caused not only induction of BIM_{EL} (mRNA and protein) but also concurrent phosphorylation of BIM_{EL} in sympathetic and cerebellar granule neurons (Harris and Johnson, 2001; Putcha et al., 2001; Whitfield et al., 2001; Putcha et al., 2003). Importanty, both the transcriptional control and post-translational phosphorylation of BIM_{EL} involves contributions from the mixed-lineage kinase (MLK)/JNK pathway, as evidenced by pharmacological inhibition of MLK, using CEP-1347, and JNK, using SP600125 (Harris and Johnson, 2001; Putcha et al., 2001; Whitfield et al., 2001; Putcha et al., 2003). The transcriptional control of BIM induction downstream of JNK activation appears to be mediated by the c-Jun phosphorylation because expression of dominant negative c-Jun reduces the levels of BIM induction following NGF withdrawal in sympathetic neurons (Whitfield et al., 2001). In addition, activation of JNKs in trophic factor-deprived neurons leads to the specific phosphorylation of BIM_{EL} at Ser65, enhancing its proapoptotic activity (Putcha et al., 2003). Thus, JNKs regulate the proapoptotic activity of BIM_{EL} both transcriptionally and posttranslationally. Finally, cell death caused by ectopic expression of BIM_{EL} requires BAX in cerebellar granule neurons (Harris and Johnson, 2001; Putcha et al., 2003). Taken together, these findings implicate BH3-only proteins, such as BIM_{EL}, as likely targets, which function genetically upstream of the BAX/Bcl-2 checkpoint to induce mitochondrial apoptogen release, caspase activation, and apoptosis.

Apoptotic protease-activating factor-1 (APAF-1) is a mammalian homologue of the *C. elegans* CED-4 cell death gene and transmits apoptotic signals from mitochondrial damage to activate caspases. Mitochondrial-released cytochrome-c forms a complex with APAF-1 and caspase-9 to mediate the activation of pro-caspase-9 (Zou et al., 1997). Activated caspase-9 in turn cleaves and activates caspase-3. APAF-1 knockout mice die during late embryonic development, exhibiting reduced apoptosis in the brain with a

marked enlargement of the periventricular proliferative zone (Cecconi et al., 1998). Thus, APAF-1 is indispensable in the apoptosis of neuronal progenitor cells.

The essential role that caspases play in executing apoptotic signaling in neurons is underscored by the ability of caspase inhibitors to block neuronal cell death induced by a variety of cytotoxic conditions, including trophic factor deprivation induced death. Determining the role of specific caspases has been difficult because mammals have at least 14 different caspases, and neurons can express several of them simultaneously. Caspases are expressed as catalytically inactive proenzymes composed of an amino-terminal pro-domain, a large subunit and a small subunit. Based on unique sequences in their pro-domain, caspases can be classified into two groups. Caspases that have a death effector domain, including caspase-8 and -10, are activated by interacting with the intracellular domains of death receptors, such as the CD95 (Apo-1/Fas) and tumor necrosis factor (TNF) receptors. Caspases with caspase-activating recruitment domains (CARDS), which include caspase-1, -2, -4, -5, -9, -11, and -12, are most probably activated though an intracellular activating complex exemplified by the cytochrome c/Apaf-1/caspase-9 complex (Li et al., 1997). Importantly, two major caspases involved in neuronal death are caspase-3 and caspase-9. Typically, caspase-3 is activated by caspase-9 in neuronal cells displaying evidence of cell death. Both caspase-3-null and caspase-9-null mice show severe and similar defects in developmental neuronal cell death (Kuida et al., 1996; Kuida et al., 1998). Both lines of mice exhibit marked exencephaly, with expansion of the periventricular zone, a phenotype very similar to that of Apaf-1-null mice. The defects in neuronal apoptosis seen in APAF-1-null, caspase-3-null, and caspase-9-null strongly suggest that this pathway is important in regulating neuronal cell death in the developing brain.

Inhibition of the damaging pro-apoptotic effects of caspases is mediated endogenously by the IAP (inhibitor of apoptosis) family of caspase inhibitors, which include X-linked inhibitor of apoptosis protein (XIAP), neuronal apoptosis inhibitory protein (NAIP), and human inhibitor of apoptosis protein (HIAP). Specifically, IAPs have been shown to directly inhibit activated caspase 3 and 7 (Deveraux et al., 1998). The caspases exist as inactive pro-caspases which require proteolytic activation; in this regard the IAPs have also been shown to inhbit the activation of pro-caspase-9 (Deveraux et al.,

1998). In chick sensory and sympathetic neurons, NGF upregulates levels of HIAP/ITA, and suppression of XIAP levels decreased NGF-induced survival (Wiese et al., 1999a). IAPs themselves are inhibited by the release of the mitochondrial apoptogen Smac/Diablo, thereby allowing caspases to be activated (Yuan and Yankner, 2000).

Taken togther, these findings suggest that activation of the intrinsic BAX/APAF-1/caspase-9/caspase-3 mitochondrial death pathway is essential for the execution of cell death signaling in both developing and mature injured neurons. However, although extensive progress has been made in understanding how neurons die, we still do not understand how neurons integrate varied upstream apoptotic stimuli such as trophic withdrawal, DNA damage, hypoxia, and excitoxicity, and translate these death-inducing stimuli into the initiation of this common downstream mitochondrial apoptotic pathway. Here, I will examine the contribution of the p53 tumor suppressor, c-Jun N-terminal kinase (JNK), and the p75 neurotrophin receptor in mediating neuronal cell death in developing and mature neurons.

Role of JNK in neuronal apoptosis

One apoptotic pathway that appears to be important in neurons includes the c-Jun N-terminal kinases (JNKs; also known as the stress activated protein kinases). The JNKs represent one subgroup of MAP kinases that is activated primarily by cytokines and exposure to environmental stress. JNKs are phosphorylated and activated in response to a variety of apoptotic stimuli, including tumor necrosis factor, DNA damage, ischemia-reperfusion, oxidative stress, hyperosmolarity, and loss of trophic support (Ip and Davis, 1998; Ham et al., 2000). Activation of the JNK pathway has been shown to occur following the removal of survival factors in a number of neuronal death paradigms, including PC12 cells (Xia et al., 1995), sympathetic neurons (Ham et al., 1995; Eilers et al., 1998), and embryonic motorneurons (Maroney et al., 1998). Activation of JNK has also been observed after neuronal injury in the adult brain (Herdegen et al., 1998).

The three genes that encode JNK (*Jnk1*, *Jnk2*, and *Jnk3*) have been disrupted by homologous recombination. These JNK-deficient mice are viable, but exhibit defects in apoptosis and immune responses. Compound mutations of the *Jnk1* and *Jnk2* genes

causes early embryonic death associated with exencephaly, decreased apoptosis in the hindbrain, and increased apoptosis in the forebrain (Kuan et al., 1999). A recent study using fibroblasts isolated from *Jnk1-/-Jnk2-/-* mice (*Jnk3* is not expressed in fibroblasts) demonstrated that these cells are resistant to DNA damage-induced apoptosis (Tournier et al., 2000). In contrast to *Jnk1* and *Jnk2*, which are expressed ubiquitously, the *Jnk3* gene exhibits a more restricted pattern of expression in the brain and heart. This has led some researchers to propose that JNK3 plays a prominent role in stress-induced apoptosis of neural cells. Consistent with this, *jnk3-/-* mice exhibit increased neuronal protection following kainate-induced seizures (Yang et al., 1997) and cerebral ischemia-hypoxia (Kuan et al., 2003).

Downstream of JNK in neuronal apoptosis

A major target of the JNK signaling pathway is the activation of the AP-1 (Activator protein-1) transcription factor that is mediated, in part, by the phosphorylation of c-Jun and related molecules. JNK phosphorylates c-Jun on two sites (Ser-63 and Ser-73) within the activation domain. Analysis of the *c-Jun-/-* mice has not been possible due to early embryonic lethality (Hilberg et al., 1993; Johnson et al., 1993). However, germline mutations in the murine *c-Jun* gene that replace the phosphorylation sites with a non-phosphorylated residue (Ala) have been reported (Behrens et al., 1999). These mice are viable and fertile, smaller than controls and resistant to epileptic seizures and neuronal apoptosis induced by the excitatory amino acid kainate (Behrens et al., 1999). An increase in both c-Jun mRNA and the activated, phosphorylated form of the protein have been observed in sympathetic neurons after NGF withdrawal (Estus et al., 1994; Ham et al., 1995). In addition, microinjection of antibodies to c-Jun (Estus et al., 1994), expression of dominant-negative c-Jun (Ham et al., 1995; Whitfield et al., 2001), or Cre recombinase-mediated *c-Jun* gene knockdown (Palmada et al., 2002) was demonstrated to protect sympathetic neurons from NGF withdrawal-induced apoptosis.

A new component of the JNK-dependent apoptotic signaling pathway in neurons has been described. NGF withdrawal induces the expression of Bim_{EL}, a BH3-only member of the Bcl-2 family, which mediates BAX-dependent cytochrome c release and apoptosis (Putcha et al., 2001). JNK appears to regulate Bim_{EL} apoptotic potential in two

ways: transcriptionally through activation of c-Jun (Whitfield et al., 2001) and post-translationally by direct phosphorylation of Bim at Ser65, potentiating its apoptotic activity (Putcha et al., 2003).

Another target of the JNK pathway may be the p53 tumor suppressor protein (see below for more information). JNKs are activated in response to DNA damage (Liu et al., 1996; Gibson et al., 1999) and can phosphorylate and stabilize p53 (Fuchs et al., 1998; Buschmann et al., 2001). Moreover, direct stimulation of JNK was sufficient to elevate p53 levels and induce sympathetic neuron apoptosis (Aloyz et al., 1998).

Upstream of JNK in neuronal apoptosis

Several upstream members of the JNK pathway have been defined in the context of trophic factor withdrawal induced apoptosis. The most distal of these are the Rho small GTPase family members Rac1 and Cdc42. Overexpression of constitutively active forms of Rac1 and Cdc42 leads to activation of the JNK pathway and to death of PC12 cells and sympathetic neurons (Bazenet et al., 1998). Conversely, overexpression of dominant negative mutants of Cdc42 and Rac1 in sympathetic neurons prevents the elevation of c-Jun and apoptosis evoked by NGF withdrawal (Bazenet et al., 1998).

The JNK signaling pathways are structurally organized like other MAP kinase signaling cascades, whereby JNKs are activated by dual specificity JNK kinases (JNKKs), which are themselves activated by a diverse group of JNK kinase kinases (JNKKKs). Two protein kinases that activate JNK have been identified as mitogen-activated protein kinases 4 and 7 (MKK4 and MKK7). Disruption of either *Mkk4* or *Mkk7* was found to cause partial defects in stress-stimulated JNK activation, whereas disruption of both genes prevented JNK activation by these stimuli (Tournier et al., 2001). Studies using dominant-interfering or constitutively activated mutants have indicated MKK4 and MKK7 lie downstream of Cdc42 and Rac1 and directly upstream of the JNKs (Xia et al., 1995; Holland et al., 1997; Foltz et al., 1998).

A diverse group of JNKKKs have been suggested as additional participants in the pathway that lies between Cdc42 and the downstream MKKs and JNKs in neuronal apoptosis. These include apoptosis signal-related kinase 1 (ASK-1), MEK kinases (MEKK1 and MEKK4), and members of the mixed-lineage kinase (MLK) family.

Overexpression of a constitutively active mutant of ASK-1 in SCG neurons activated JNK and induced apoptosis, whereas expression of a kinase inactive ASK-1 reduced both NGF withdrawal- and Cdc42-induced death (Kanamoto et al., 2000). Although overexpression of MEKK1 is sufficient to induce apoptosis in sympathetic neurons (Aloyz et al., 1998), loss of function studies have yet to show its requirement in NGF withdrawal induced death. Some members of the MLK family of JNKKKs (specifically MLK1, MLK2, MLK3, but not DLK) contain a Cdc42/Rac interactive binding (CRIB) domain, interact physically with GTP-Cdc42 and activate the JNK pathway (Nagata et al., 1998). Overexpression of MLKs effectively induces apoptotic cell death in sympathetic neurons, while expression of dominant negative forms of MLKs suppresses death evoked by NGF deprivation (Mota et al., 2001; Xu et al., 2001). Consistent with these results, treatment of sympathetic neurons with CEP-1347, a pharmacological inhibitor of MLK family members, protects these cells from NGF withdrawal-induced death (Maroney et al., 1999; Maroney et al., 2001). Taken together, these results suggest that a signaling pathway consisting of Cdc42/Rac-MLK-MKK-JNK is a major apoptotic pathway in trophic factor deprived neurons.

JNK scaffold proteins

The protein kinases that form the JNK signal transduction cascade may interact by being assembled into a JNK signaling module by specific scaffold proteins. Studies in yeast first established the concept that scaffold proteins can be critical components of MAP kinase pathways. There is evidence to support a role for scaffold proteins in the activation of JNK response to specific stimuli.

The JNK-interacting protein (JIP) group of potential scaffold proteins were initially identified as proteins that bind JNK (Dickens et al., 1997). JIP proteins interact with JNK through a JNK-binding domain (JBD), that is homologous to the JBD found in other JNK substrates like c-Jun (Dickens et al., 1997). It was subsequently found that JIP proteins also interact with members of the MLK family and MKK7 (Whitmarsh et al., 1998). The JIP family includes JIP1 (Dickens et al., 1997) and the related JIP2 (Yasuda et al., 1999) (also known as islet-brain1, IB1 and IB2). Studies of mice with targeted disruptions of the *Jip* genes have been described by two groups (Thompson et al., 2001;

Whitmarsh et al., 2001). One group found that the *Jip1* mutation caused death prior to blastocyst implantation, although the mechanism was not characterized (Thompson et al., 2001). The other group demonstrated that a null allele of the *Jip1* mutation did not cause embryonic death. Instead, these animals were viable and had a normal lifespan, but were defective in the regulation of JNK activation in hippocampal neurons *in vivo* following kainate-induced seizures (Whitmarsh et al., 2001). Thus, these findings support the hypothesis that JIP1 is a possible scaffold protein for the JNK signaling pathway.

JIP proteins may have other functions, as indicated by the observation that JIP proteins interact with numerous other proteins through an SH3 domain and a PTB domain. Ligands for the PTB domain include 190 Rho-GEF, the amyloid precursor protein, the LDL receptor-related family members ApoER2, Megalin, and LRP-1 (Meyer et al., 1999; Matsuda et al., 2001; Gotthardt et al., 2000; Stockinger et al., 2000). The carboxy terminal region of JIP proteins also interacts with microtubule motor protein kinesin-1, suggesting that JIP proteins may contribute to the localization of JNK signaling pathway components and other adaptor proteins (Verhey et al., 2001; Whitmarsh et al., 2001).

It has been suggested that scaffold proteins might act as molecular tethers to increase the local concentration of the components of a protein kinase module and thus increase signal transmission (Whitmarsh and Davis, 1998). However, overexpression of JIP scaffold proteins has been shown to effectively inhibit JNK signal transduction in two ways, 1) by blocking the association of JNK with downstream substrates and 2) by causing the cytoplasmic retention of JNK (Dickens et al., 1997).

It should be noted that other JNK scaffold proteins have now been described. These include CrkII, which may assemble a JNK module in response to Rac1 activation, filamin, which may be important for TNF-stimulated JNK activation, and the arrestin group of adapter proteins (β-arrestin-1, and β-arrestin-2). Recently, Greene and co-workers reported the identification of POSH (plenty of SH3s) as a scaffold for the JNK pathway in neuronal death (Xu et al., 2003). POSH was shown to bind GTP-Rac1, MLKs, MKKs 4 and 7, and with JNKs (Xu et al., 2003). POSH overexpression promotes apoptotic neuronal death, whereas a POSH antisense oligonucleotide and a POSH small interfering RNA (siRNA) suppressed c-Jun phosphorylation and neuronal apoptosis

induced by NGF withdrawal. Thus, POSH appears to function as a scaffold in a multiprotein complex that links activated Rac1 and downstream elements of the JNK apoptotic cascade.

p53 tumor suppressor family of proteins

Although extensive progress has been made in understanding how neurons die, we still do not understand how neurons integrate varied upstream apoptotic stimuli such as trophic withdrawal, DNA damage, hypoxia, and excitoxicity, and translate these death-inducing stimuli into the initiation of this common downstream mitochondrial apoptotic pathway. As will be discussed in detail below, emerging evidence indicates that, in mammalian neurons, the p53 family comprises an important component of this convergent upstream pathway.

The p53 phosphoprotein is a tumor suppressor protein that is mutated or inactivated in >50% of human cancers (Vogelstein et al., 2000). p53 appears to have evolved to sense diverse cellular stresses including DNA damage and hyperproliferative signals (Ko and Prives, 1996). Once stabilized and activated by genotoxic stress, p53 can either activate or repress a wide array of different gene targets, which in turn, can regulate proliferation by inducing cell cycle arrest and/or initiate apoptosis (Oren, 2003). Thus, p53 has been dubbed "the guardian of the genome", a function that is crucial in proliferating cells in order to protect the organism from the propagation of an aberrant genetic signal. Neurons, however, are a cell type that are forever "post-mitotic", and that must survive and maintain its genome over as long as a century in humans. Accumulating evidence suggests that the primary role for p53 in nerve cells is to regulate the apoptosis of neurons during development, after injury, and in the chronic degenerating nervous system.

For many years, p53 was thought to be "alone" in the genome, but in the late nineties, two new genes with high homology to p53 were discovered. These genes, p63 (Yang et al., 1998) and p73 (Jost et al., 1997; Kaghad et al., 1997), like p53, encoded transcription factors comprised of a transactivation domain, a DNA binding domain and an oligomerization domain. These full-length isoforms of p63 and p73 (termed TAp63 and TAp73) can, like p53, oligomerize, bind to DNA, transactivate at least some p53

target genes, and induce apoptosis (Jost et al., 1997; Yang et al., 1998). However, unlike p53, which in normal cells is present as a single protein isoform, many different isoforms are generated from the p73 and p63 genes. Most surprising was the finding that alternative promoter usage generates p73 and p63 N-terminal truncated isoforms that lack the transactivation domain, an omission that renders them incompetent to mediate transcription. This finding led to the idea that these truncated isoforms, termed ΔNp73 and ΔNp63, were naturally-occurring dominant-inhibitory p53 family members, an idea that proved to be true when it was shown that ΔNp73 was capable of inhibiting p53-mediated transcription (Yang et al., 2000; Fillippovich et al., 2001) and p53-mediated apoptosis (Pozniak et al., 2000). In the nervous system, it is the ΔNp73 isoforms that are most abundant, and these functionally antagonistic actions are known to be of key importance in neuronal survival, as discussed below.

While the ΔNp73 and ΔNp63 isoforms have received the most attention, alternative splicing also generates p73 and p63 isoforms that vary at the carboxyl-terminus; p63 exists as three C-terminal isoforms (α, β, γ) (Yang et al., 1998), while at least six different p73 isoforms (α, β, γ, δ, ε, ζ) have been identified (De Laurenzi et al., 1998; Casciano et al., 1999; De Laurenzi et al., 1999; Kong et al., 1999). These different isoforms are thought to differ in their ability to transactivate gene expression (Zhu et al., 1998; De Laurenzi et al., 1999; Lee and La Thangue, 1999; Shimada et al., 1999) and in this regard, the α-isoforms of p63 and p73 contain a sterile alpha-motif (SAM) domain, a putative protein-protein interaction region (Chi et al., 1999; Thanos and Bowie, 1999) that decreases transcriptional activity through intramolecular binding to the TA domain of other p53 family members (Serber et al., 2002). Whether these carboxyl-terminal differences are biologically important still remains to be determined.

What are the biological roles of p73 and p63? While the initial excitement centered around the possibility that these two new family members were, like p53, tumor suppressors, subsequent studies showed that they are only rarely mutated in tumors (Irwin and Kaelin, 2001). Moreover, unlike p53, targeted mutation of the p63 and p73 genes showed that both of these proteins are important during development. p73-/- mice (Yang et al., 2000) display neural deficits and immunological problems, and die within the first two to three postnatal weeks. p63-/- animals, which die at birth, display even more

striking developmental deficits and have absent or truncated forelimbs and absent hindlimbs (Mills et al., 1999; Yang et al., 1999), defects that are apparently secondary to the absence of the apical ectodermal ridge (AER), an embryological structure required for limb outgrowth. Other structures that require ectodermal-mesenchymal interactions during morphogenesis (hair follicles, teeth, mammary glands) are also absent, and the p63-/- mice display only a single layer of epidermal cells at birth (Mills et al., 1999; Yang et al., 1999). This epidermal phenotype is thought to be secondary to nonregenerative epidermal differentiation, which in zebrafish has been specifically related to a loss of ΔNp63 (Lee and Kimelman, 2002).

Thus, while these three family members share some similarities, it is clear that they play functionally distinct biological roles and, as discussed below, in the nervous system, even functionally antagonistic roles. Moreover, while p53 has a similar function in many cell types, the many isoforms generated from the p73 and p63 genes provide them with the molecular flexibility to play very different roles depending upon the cellular context.

Role of p53 in Developmental Neuron Cell Death

The initial analysis of animals with a targeted mutation in the p53 gene provided strong support for the hypothesis that p53 was a key tumor suppressor protein, since p53-/- mice exhibited a high rate of spontaneous tumor formation (Donehower et al., 1992). However, the apparent lack of a developmental phenotype led to the initial conclusion that p53 was dispensable for embryogenesis. It was not until a more detailed examination was carried out that it became evident that up to 23% of female p53-/- embryos died *in utero* as a result of mid-brain exencephaly, a neural tube malformation resulting from the overproduction of neural tissue and failure of neural tube closure (Armstrong et al., 1995; Sah et al., 1995). Interestingly, this exencephalic phenotype is very similar to that observed in animals with targeted mutations in other members of the intrinsic mitochondrial death pathway, including the proapoptotic proteins Apaf-1 (Cecconi et al., 1998; Yoshida et al., 1998), caspase-3 (Kuida et al., 1996), and caspase-9 (Kuida et al., 1998). In these animals, exencephaly results from an absence of cell death in the neuroepithelium of the developing neural tube. The similarity between these phenotypes

and the fact that all of these proteins, including p53, are components of the mitochondrial death pathway, suggested that the neural phenotype seen in the p53-/- embryos was the result of a deficit in progenitor cell apoptosis.

Is p53 important for naturally occurring cell death of neurons during development? A number of studies indicate that p53 plays a pro-apoptotic role for NGF withdrawal-induced death of sympathetic neurons. First, overexpression of p53 was sufficient to cause the death of sympathetic neurons (Slack et al., 1996). Second, p53-/- sympathetic neurons showed enhanced survival upon NGF withdrawal, although this deficit in apoptosis was much more pronounced in embryonic than in neonatal neurons (Vogel and Parada, 1998; Besirli et al., 2003). Third, trophic factor withdrawal-induced death of sympathetic neurons was rescued by ectopic expression of the viral E1B55K protein, which binds to and inhibits p53 (Aloyz et al., 1998). Finally, and most importantly, the rate of apoptosis and loss of neurons was decreased (albeit not eliminated) in sympathetic neurons *in vivo* during the first two postnatal weeks (Aloyz et al., 1998; Lee et al., 2004). These findings therefore strongly support the idea that p53 plays a proapoptotic role in the developmental cell death of sympathetic neurons.

To address the possibility that the newly-discovered p53 family members might collaborate with p53 during developmental sympathetic neuron death, Pozniak and others (2000), examined p73-/- mice. To their surprise, they found a decrease in sympathetic neuron number (~40%) in p73-/- SCGs, suggesting an unexpected anti-apoptotic role for the p73 gene (Pozniak et al., 2000). Further analysis revealed the molecular basis for this prosurvival role; the dominant isoforms of p73 in the developing brain and sympathetic ganglia were ΔNp73 variants (Pozniak et al., 2000; Yang et al., 2000), and basal levels of ΔNp73 protein decreased dramatically in NGF-deprived sympathetic neurons. Moreover, if this decrease in ΔNp73 was prevented by expression of exogenous ΔNp73, then sympathetic neurons were rescued from NGF withdrawal-induced death (Pozniak et al., 2000). Thus, ΔNp73 is an essential prosurvival protein in the nervous system, at least in developing sympathetic neurons.

How does ΔNp73 mediate its anti-apoptotic actions? A number of studies indicate that it does so partially by antagonizing the proapoptotic actions of p53. First, in non-neuronal cells, ectopic expression of ΔNp73 blocked p53-mediated transactivation of

reporter genes, and inhibited the ability of p53 to bind to DNA (Fillippovich et al., 2001; Zaika et al., 2002). Second, ΔNp73 inhibited sympathetic neuron apoptosis as mediated by exogenous expression of p53 (Pozniak et al., 2000). Third, analysis of sympathetic neurons in p73-/-, p53-/- mice revealed that the increased death of SCG neurons seen in p73-/- animals was partially rescued by the concomitant loss of p53 (Lee et al., 2004). However, while this latter study indicated that p73 partially mediated neuronal survival by antagonizing p53, it also provided genetic evidence that p73 promotes survival in a p53-independent fashion, potentially by acting upstream of the intrinsic mitochondrial death pathway (Lee et al., 2004). These p53-independent mechanisms remain to be elucidated.

p53 mediates neuronal apoptosis in response to DNA damage and injury

The p53 tumor suppressor provides a major survival checkpoint not just for developing neurons but also for adult neurons. Support for this idea derives from extensive literature documenting the importance of p53 as an injury-induced apoptotic signal in the mature nervous system (Miller et al., 2000; Morrison et al., 2000). Three major conclusions have come from this work. First, many studies showed that p53 levels were highly elevated following apoptotic insults to adult neurons. For example, seizure-induced excitotoxic damage was associated with p53 accumulation in neurons undergoing apoptosis following systemic administration of kainic acid (Sakhi et al., 1994; Sakhi et al., 1996), intastriatal infusions of *N*-methyl-D-aspartate (NMDA), or the NMDA agonist quinolinic acid (Qin et al., 1999; Wang et al., 1999), or lithium pilocarpine (Tan et al., 2002). Elevations in p53 expression have also been observed in the penumbra region surrounding the ischemic core following middle cerebral artery occlusion (Chopp et al., 1992; Watanabe et al., 1999), and following traumatic brain injury (Napieralski et al., 1999). Second, overexpression of p53 is sufficient to induce cell death in a variety of neuronal cell types, including sympathetic (Slack et al., 1996), hippocampal (Jordan et al., 1997), and cortical (Xiang et al., 1996) neurons. The third major conclusion from these studies was that this increase in p53 was essential for the neuronal apoptosis that ensues, a conclusion perhaps best-exemplified by the finding that kainate treatment caused death of p53+/+, but not p53-/-, neurons both *in vitro* and *in vivo* (Morrison et al., 1996; Xiang et al., 1996).

Similar studies have confirmed that p53 is also essential for neuronal apoptosis following ischemia (Crumrine et al., 1995), adrenalectomy (Sakhi et al., 1996), and hypoxia (Banasiak and Haddad, 1998). One potential explanation for the commonality of all of these findings is that excitotoxic damage leads to neuronal DNA damage (Didier et al., 1996), which in turn causes p53 upregulation and, when the damage is severe, p53-induced apoptosis.

How does p53 induce apoptosis?

p53 is a transcription factor and, as such, is thought to mediate apoptosis by transactivation of genes that regulate the intrinsic mitochondrial death pathway. The first of these is BAX, a proapoptotic Bcl2 family member that is essential for the developmental death of most peripheral neurons, as described above (Deckwerth et al., 1996; White et al., 1998). Several studies have also demonstrated that BAX is required for p53-dependent apoptosis in neurons. For instance, overexpression of p53 in cerebellar granule cells induces apoptosis in wildtype, but not BAX-/- neurons (Cregan et al., 1999). In addition, BAX-/- cortical and hippocampal neurons are resistant to excitotoxicity- and radiation-induced cell death, respectively, (Johnson et al., 1998; Xiang et al., 1998), two apoptotic stimuli known to be dependent on p53 function (Xiang et al., 1996).

During neuronal apoptosis, BAX redistributes from the cytosol to the mitochondria, where it participates in the release of cytochrome c and activation of the intrinsic mitochondrial death pathway (Scorrano and Korsmeyer, 2003). A second relevant p53 target may be Peg3/Pw1 (Deng and Wu, 2000), which has been reported to mediate p53-dependent BAX translocation to the mitochondria following DNA damage in neurons (Johnson et al., 2002). Ectopic expression of p53 in neurons has also been shown to transactivate the pro-apoptotic protein SIVA, which interacts with anti-apoptotic Bcl-2 family proteins (Fortin et al., 2004), and the BH3-only proteins, NOXA and PUMA (Cregan et al., 2004). Lastly, p53 may direct the transcription of APAF1 (Moroni et al., 2001), a component of the apoptosome that is essential for DNA damage-induced neuronal death (Fortin et al., 2001). Thus, p53 transactivates several genes that directly promote neuronal apoptosis via BAX-dependent activation of the intrinsic mitochondrial death pathway, although the extent and level of transactivation of each

gene target is dependent on the cell type and cellular stress. p53 may also promote apoptosis via transcriptional repression of the anti-apoptotic Bcl-2 and inhibitor of apoptosis proteins (IAPs) (Johnstone et al., 2002).

Importantly, accumulating evidence also suggests that p53 can mediate apoptosis in a transcription-independent manner via direct actions at the mitochondrion (Schuler et al., 2000; Mihara et al., 2003). However, Slack and colleagues have reported that the first transactivation domain of p53 is indispensable for the induction of neuronal cell death (Cregan et al., 2004), supporting the notion that the transcriptional abilities of p53 are required for neuronal death.

Upstream of p53 during Neuronal Death

The studies described above strongly argue that the p53 family provides a major apoptotic checkpoint in neurons. Activation of p53 is generally the result of posttranslation modifications such as phosphorylation or acetylation, which affect protein stability and/or function (Vogelstein et al., 2000; Oren, 2003). As described above, p53 is stabilized in neurons in response to a variety of apoptotic stimuli, including trophic factor withdrawal, DNA damage, ischemia-reperfusion, oxidative stress, excitotoxic injury (Miller et al., 2000).

What are the molecular signals that activate p53 in trophic factor deprived neurons? Evidence indicates that NGF withdrawal activates a JNK-p53-BAX pathway. JNK has also been shown to bind, phosphorylate, and stabilize the cellular levels of the tumor suppressor protein p53 (Fuchs et al., 1998; Buschmann et al., 2001). Direct stimulation of JNK was sufficient to elevate p53 levels and induce sympathetic neuron apoptosis (Aloyz et al., 1998). Moreover, expression of the viral p53 inhibitor (E1B55K) protects cultured sympathetic neurons from cell death induced either by NGF withdrawal or MEKK1-induced activation of JNK (Aloyz et al., 1998), indicating that JNK acts upstream of p53 in NGF withdrawal induced neuronal death.

Acute cell death of adult neurons resulting from seizure, ischemia, and hypoxia are all thought to involve oxidative damage associated with the generation of free radicals (Beal, 1996), and the production of free radicals is known to damage cellular components, including DNA (Didier et al., 1996). In this regard, numerous studies have shown that

direct DNA damage with agents such as camptothecin cause the apoptosis of both PNS and CNS neurons, and that DNA damage-induced neuron death is dependent on p53 (Park et al., 1998; Xiang et al., 1998; Anderson and Tolkovsky, 1999; Morris et al., 2001).

How does DNA damage cause p53 activation? A large body of work indicates that multiple signaling pathways are recruited following DNA damage to stabilize and activate p53 (Vogelstein et al., 2000). One such pathway in neurons involves ATM (the ataxia telangiectasia protein). For example, ionizing radiation, which directly damages DNA, elicits upregulation of p53 and induces apoptosis in ATM+/+, but not ATM-/- mice (Herzog et al., 1998). A similar resistance to ionizing radiation was observed in both p53 -/- and Bax-/- mice (Herzog et al., 1998; Chong et al., 2000), supporting the existence of an ATM-p53-Bax pathway. A recent report indicates that DNA damage-induced apoptosis is inhibited in JNK1-/-, JNK2-/- cells (Tournier et al., 2000), suggesting that there are two pathways to p53 following DNA damage. Consistent with this, similar to p53-/- mice (Morrison et al., 1996), JNK3-/- mice show a reduction in seizure-induced neuronal apoptosis (Yang et al., 1997).

Together, these findings strongly support the hypothesis that the p53 family provides a fundamental survival checkpoint in neurons that lies upstream of the mitochondrion, in a key location to act as a sensor responsible for integrating multiple proapoptotic cues.

It is clear from the above discussion that we are starting to understand the mechanisms that lie upstream of p53 and potentially of the proapoptotic forms of p73 and p63 during neuronal apoptosis. However, we know virtually nothing of the mechanisms that regulate the levels and/or activity of the prosurvival family members, such as ΔNp73, except that in developing neurons, growth factors such as NGF are necessary to maintain their expression (Pozniak et al., 2000). Elucidating these upstream pathways, as well as their potential protein partners will be essential if we are to understand the important role these ΔN family members play in the nervous system.

The p75 neurotrophin receptor

Although p75NTR was the first isolated neurotrophin receptor, our understanding of its physiological role has lagged considerably behind our understanding of the Trk neurotrophin receptors. It has become clear that activation of the p75NTR can elicit a variety of responses, depending on the cell type, the cellular context, and the degree of coexpression with Trk receptors. Biological roles for the p75NTR include induction of apoptosis, promotion of cell survival, peripheral nerve myelination, and regulation of neurite extension.

The p75 neurotrophin receptor (p75NTR) is a multifunctional protein with numerous ligands and downstream functions. The receptor binds all neurotrophins, including NGF, BDNF, NT-3, and NT-4, with similar affinity (Huang and Reichardt, 2001). p75NTR was the first reported member of the TNF receptor superfamily that now contains approximately 25 receptors including TNFR1, Fas, Rank, and CD-40 (Baker and Reddy, 1998). Although p75NTR shares some structural homology with other members of the TNFR superfamily, including cysteine-rich modules in the extracellular domain and an intracellular death domain (DD), it is clear that p75NTR is an atypical member of this protein family. First, p75 binds dimeric ligands, unlike other TNFR ligands that are trimeric (Banfield et al., 2001; Robertson et al., 2001). Secondly, the death domain (DD) of p75NTR is different from the DD of other TNFRs in its tertiary structure and as a consequence does not bind similar adaptor proteins that link other TNFRs to Caspase-8 activation (reviewed in Wallach et al., 1996). Thus, while the apoptotic and inflammatory signaling pathways downstream of most TNFRs are well characterized, the physiological role and signaling downstream of p75NTR have remained elusive for many years.

Our understanding of the physiological role of the p75NTR receptor has lagged behind that of our understanding of the role Trk receptors play in neurotrophin biology. This is perhaps due to the fact that unlike Trks, p75NTR does not contain a tyrosine kinase domain. Historically, it was unclear whether p75NTR could mediate signal transduction alone through its intracellular domain. Since it was known that p75 could bind all of the neurotrophins, early *in vitro* studies focused on whether p75NTR modulated neurotrophin-Trk receptor interactions and signaling. Expressed on their own, both Trk and p75NTR receptors bind neurotrophins with similar affinity, having a Kd of

about 1-10 nM. When both receptor components are coexpressed in the same cell, however, p75NTR increases the affinity of Trk receptors to bind neurotrophins, creating so-called "high-affinity binding sites" (Hempstead et al., 1991; Hantzopoulos et al., 1994; Mahadeo et al., 1994; Chao and Hempstead, 1995). Secondly, in response to low concentrations of neurotrophins, Trk activation is enhanced by the co-expression of p75NTR (Hantzopoulos et al., 1994; Barker et al., 1994; Verdi et al., 1994). Because the concentration of NGF in the target field is limiting, high affinity binding sites were suggested to play an important role in increasing neuronal responsiveness to target-derived neurotrophins (Barde, 1989).

How does p75NTR mediate the increased Trk responsiveness to NGF? A widely shared view is that high affinity NGF binding sites are the result of a functional receptor complex consisting of p75NTR and TrkA. Although it is unclear whether p75NTR and TrkA bind directly, there is evidence that complexes do form, possibly in the absence of ligand (Huber and Chao, 1995; Ross et al., 1998; Bibel et al., 1999; Yano and Chao, 2000). Receptor deletion studies have shown that the transmembrane and ICD of p75NTR are required for the generation of "high affinity" neurotrophin binding sites (Esposito et al., 2001). However, the stoichiometry, and even the actual presence under physiological conditions remain in doubt. Nevertheless, it has been demonstrated that neonatal DRG or SCG neurons cultured from p75-deficient mice displayed a decreased sensitivity to NGF (Davies et al., 1993; Lee et al., 1994a). It is difficult to reconcile these findings with the analysis of p75NTR mutant mice (Lee et al., 1992). If p75NTR was required to increase the affinity and sensitivity of Trk receptors, a lack of p75 expression would be predicted to result in an excessive loss of peripheral neurons. However, sensory neurons are the only population of neurons to show a modest reduction in adult neuronal number in p75NTR-/- mice, with DRG and trigeminal ganglia exhibiting a 50% and 30% loss, respectively (Lee et al., 1992; Bergmann et al., 1997; Stucky and Koltzenburg, 1997; Walsh et al., 1999), phenotypes that are less severe than those seen in *NGF* and *TrkA* knockout mice (Crowley et al., 1994; Smeyne et al., 1994). Moreover, it remains unclear from these studies whether this reduction in neuronal number is due to the lack of p75NTR expression in sensory neurons, Schwann cells, or target tissues.

Other lines of evidence propose that physical associations of p75NTR with Trk receptors subserves a second function, that is p75NTR can alter the ligand specificity of Trk receptors. For instance, in cells coexpressing TrkB and p75NTR, only BDNF stimulates a functional response, whereas, BDNF, NT-3, and NT-4 can all bind TrkB in the absence of p75NTR (Bibel et al., 1999). Similarly, both NGF and NT-3 bind trkA, but p75NTR expression modulates NT-3's ability to activate TrkA (Benedetti et al., 1993; Mischel et al., 2001). Genetic evidence has suggested that p75NTR may modulate the responsiveness of sympathetic neurons to NT-3 in vivo (Brennan et al., 1999). Recently, Ginty and colleagues reported that NT-3 is more effective at promoting TrkA-mediated axon growth from p75NTR-deficient sympathetic neurons (Kuruvilla et al., 2004), suggesting that axonal sensitivity to NT-3 is modulated by p75NTR. Moreover, these authors report that the absence of p75 does not affect the rate of axon extension in response to NGF, indicating that modulation of neurite growth by p75 is specific to NT-3 (Kuruvilla et al., 2004). It is interesting to note that levels of p75 are very low in developing sympathetic neurons as they extend along NT-3 expressing intermediate targets, and rise sharply upon innervation of final target fields (Wyatt and Davies, 1995). In addition, NGF, but not NT-3, can induce expression of p75 in mass cultures of sympathetic neurons (Belliveau et al., 1997). Taken together, Ginty and co-workers proposed a model wherein NT-3 promotes axon extension of developing sympathetic neurons along vasculature at times when p75NTR are low. As axons begin to approach final target organs and obtain NGF, the resulting NGF/TrkA retrograde signal not only promotes survival and final target innervation,but also p75 induction. The increase in p75, in turn, diminishes axonal responsiveness to NT-3, allowing sympathetic axons to leave intermediate targets and properly invade final peripheral targets (Kuruvilla et al., 2004).

p75NTR as an apoptotic receptor

The properties of p75NTR as an independent signaling receptor were long overshadowed by the rapid progress made in elucidating the necessity of Trk receptors in mediating many of the classic trophic effects of neurotrophins. As discussed above, p75NTR was thought to subserve Trk family members by either acting as a neurotrophin resevoir for passing ligand to Trk receptors (Jing et al., 1992) or in the modulation of Trk receptor

function (Barker et al., 1994; Hantzopoulos et al., 1994). However, the homology of p75NTR to other members of the TNFR superfamily led to an investigation of the ability of p75NTR to induce cell death in a similar manner as other TNFRs. A number of studies indicate that cell death by p75 occurs quite differently than TNF and Fas receptors. p75NTR does not interact with death domain containing proteins, such as FADD, and does not activate caspase-8, key components of apoptotic signaling downstream of TNF and Fas receptors (Coulson et al., 1999; Gu et al., 1999).

The original finding that p75NTR could mediate neuronal apoptosis came from a study showing that overexpression of p75NTR in a neural cell line was sufficient to induce cell death (Rabizadeh et al., 1993). It has subsequently been reported that p75NTR overexpression induces cell death in many, although not all, cell types (for review, see Roux et al., 2002). Interestingly, the cytoplasmic death domain of p75NTR does not appear to be necessary for cell death in some cells, such as sensory neurons. Instead, a 29 amino-acid segment of the p75-juxtamembrane region was found to be responsible for apoptosis (Coulson et al., 2000). Lastly, nervous system specific overexpression of the whole intracellular domain of p75NTR in transgenic mice leads to considerable cell losses in both peripheral and central neuronal populations, revealing that this receptor has a substantial death potential *in vivo* (Majdan et al., 1997).

Over the past several years, p75NTR loss-of-function studies have supported the conclusion that p75NTR is essential for apoptosis following trophic factor withdrawal. For example, cultured sensory neurons that normally apoptose rapidly when deprived of neurotrophin show reduced rates of neurotrophin withdrawal-induced death if p75NTR levels are reduced (Barrett and Bartlett, 1994). Similarly, the rate of apoptosis of p75NTR-/- sympathetic neurons is greatly delayed following NGF withdrawal (Bamji et al., 1998). This finding was recently extended to p75NTR expressing non-neuronal cells; p75NTR-/- Schwann cells showed enhanced survival in the absence of survival factors (Soliu-Hanninen et al., 1999).

Analysis of the p75NTR-/- mouse supports the idea that p75NTR contributes to cell death in the developing nervous system. Mice carrying a deletion in either the NGF or p75NTR gene show reduced cell death in the retina and spinal cord during development (Frade and Barde, 1999), indicating that a significant proportion of the early

cell death in these tissues is mediated by NGF acting through p75NTR. In addition, eliminating p75NTR in mice leads to a marked attenuation of apoptosis during the programmed cell death period of the trigeminal ganglion sensory neurons (Agerman et al., 2000). Analysis of p75NTR-deficient mice has revealed a role for p75NTR in regulating the survival of basal forebrain cholinergic neurons (which express TrkA and p75NTR). An increase in forebrain cholinergic neurons has been reported in two different strains of p75-deficient mice ($p75^{EXONIII}$-/-, Yeo et al., 1997; $p75^{EXONIV}$-/-, Naumann et al., 2002). These results are controversial, however, since Van Der Zee et al. (1999) retracted their study that reported similar findings, and Gage's group reported a cholinergic neuron loss in p75-/- animals (Peterson et al., 1999). These reported discrepancies may reflect differences between genetic strains.

p75NTR also regulates neuronal number of sympathetic neurons during the period of naturally occurring neuron death. Initial analysis of p75 knockout mice revealed a loss of sympathetic innervation in some targets without an apparent loss of sympathetic neurons (Lee et al., 1992; Lee et al., 1994b). A more detailed analysis of p75NTR-deficient mice revealed that sympathetic neuron number is increased compared to wildtype littermates between postanatal day 1 (P1) and P23 (Bamji et al., 1998). Unlike TrkA-/- mice which display a near total loss of the sympathetic nervous system and die several days after birth, the normal period of naturally occurring cell death for sympathetic neurons does not occur in p75NTR-/- mice (Bamji et al., 1998). Sympathetic neuron number in p75NTR-/- animals does decline later in adulthood (Bamji et al., 1998; Brennan et al., 1999), similar to the *in vitro* observation that the rate of apoptosis is delayed in p75NTR-/- sympathetic neurons following removal of NGF (Bamji et al., 1998). This latter observation is consistent with the idea that the deficit in sympathetic neuron apoptosis is due to a lack of p75NTR in sympathetic neurons. Taken together, these findings suggest that elimination of sympathetic neurons during naturally occurring cell death occurs in part due to p75NTR action.

The fact that p75NTR is a transmembrane receptor that binds multiple neurotrophins sparked numerous labs to determine whether ligand-binding mediates p75-receptor dependent cell death, both in culture and *in vivo*. In neurons that express both p75NTR and a Trk receptor, the neurotrophin that mediates p75-induced cell death is not

the preferred ligand for that specific Trk receptor. For instance, BDNF activation of p75NTR causes death of cultured TrkA-expressing sympathetic neurons (Bamji et al., 1998). NGF induces p75NTR-dependent death of trkB-expressing sensory neurons (Davey and Davies, 1998), and embryonic motorneurons (Wiese et al., 1999). In cells that express only p75NTR and no kinase active Trk receptors, NGF appears to be the preferred ligand for p75-mediated dell death. For example, NGF binding to p75NTR induces cell death of cultured oligodendrocytes (Cassacia-Bonnefil et al., 1996; Yoon et al., 1998) and Schwann cells (Soliu-Hanninen et al., 1999).

The observation that p75NTR mediates ligand-dependent neuronal cell death has been extended *in vivo*. The addition of NGF to developing chick optic nuclei or NGF (attached to glass beads) applied to developing chick retinas leads to increased cell death (Von Bartheld et al., 1994; Frade and Barde, 1998), whereas the addition of blocking antibodies to either NGF or p75NTR decreases naturally occurring chick retinal cell death (Frade et al., 1996). Eliminating p75NTR or neurotrophin 4 (NT4) in mice leads to a marked attenuation of apoptosis during the programmed cell death period of the trigeminal ganglion neurons, suggesting that NT4 can induce the death of these neurons through the p75NTR (Agerman et al., 2000). Interestingly, BDNF -/- mice show an increase in sympathetic neuron number relative to BDNF +/+ littermates, consistent with the idea that BDNF may be a physiological ligand for p75-mediated naturally occurring sympathetic neuron death (Bamji et al., 1998).

Several important features about p75NTR-mediated cell death have emerged from these studies that should be considered. First, the signaling capacity and biological function of p75NTR is a function of cellular Trk activation status. That is, p75NTR can only induce apoptosis when Trk receptors are either inactive or are sub-optimally activated. For example, robust activation of Trk receptors (as would occur when neurons are cultured in high concentrations of preferred neurotrophin) blocked p75NTR-mediated death of sympathetic neurons (Bamji et al., 1998) and trigeminal mesencephalic neurons (Davey and Davies, 1998). Dose response experiments with cultured sympathetic neurons indicated that BDNF could induce a p75NTR-dependent apoptotic response when neurons were maintained in low quantities of NGF (<10 ng/mL) (Bamji et al., 1998). In addition, ectopic expression of TrkA in oligodendrocytes (which normally express

p75NTR, but not Trk), inhibited NGF-induced apoptosis (Yoon et al., 1998). Taken together, these studies suggest that Trk activation silences p75NTR-mediated apoptotic signaling. As a corollary to this idea, p75NTR can mediate apoptosis in Trk receptor-expressing cells if they are maintained in Trk-independent survival signals. For example, p75NTR activation caused apoptosis when sympathetic neurons were maintained in KCl (Aloyz et al, 1998; Vaillant et al., 1999). Second, sensory neurons maintained in CNTF are susceptible to NGF/p75NTR-mediated apoptosis (Davey and Davies, 1998). Thus, the outcome of neurotrophin-mediated p75NTR-dependent cell death depends on the expression of Trk receptors.

Pro-neurotrophins as ligands for p75NTR

Thus, the developmental defects observed in the p75NTR knockout mice demonstrate that this receptor is functionally potent. The fact that similar phenotypes can be observed in some of the neurotrophin knockout mice (eg., *BDNF-/-* and *NT-4-/-*) supports the notion that neurotrophins can act as bona fide ligands for p75NTR-dependent cellular responses. However, for most cell culture experiments, the cellular response of p75NTR to purified neurotrophins are generally weak and are typically observed only at neurotrophin concentrations that are far greater than those known to occur *in vivo*.

A recent study provides a possible resolution to this dilemma. It has been known for decades that all of the neurotrophins are initially produced as 30-35kDa precursor proteins. The calcium dependent serine protease furin and other members of the prohormone convertase family cleave each of the neurotrophins at a dibasic cleavage site in the middle of the precursor protein, releasing the biologically active 12-14 kDa C-terminal product (Seidah et al., 1996). However, Lee et al. (2001) noticed that unprocessed proneurotrophins were more abundant in whole tissue protein extracts than predicted, and could represent a large proportion of secreted neurotrophin. Surprisingly, they found that proNGF bound to p75NTR with a higher affinity than mature NGF (Lee et al., 2001). Moreover, proNGF bound TrkA much less strongly than mature NGF, and was unable to activate Trk receptors. The greater affinity of proNGF for p75NTR suggested that proNGF was a better agonist for p75-mediated cellular responses. Consistent with this, Lee et al. (2001) showed that proNGF induced apoptosis of smooth

muscle cells at much lower concentrations than had been previously reported for mature NGF. Even more significant was the finding that proNGF binding to p75NTR induces apoptosis of sympathetic neurons, which express both TrkA and p75NTR. The authors suggest that proNGF, unlike mature NGF, can activate p75NTR without activating TrkA.

These findings have now been extended *in vivo* in two paradigms. In the injured spinal cord, oligodendrocytes undergo p75NTR-dependent cell death (Beattie et al., 2002). ProNGF is likely responsible for oligodendrocyte death in this injury paradigm, since proNGF and not mature NGF was induced in mice after spinal cord injury. In addition, immunodepletion of proNGF from injured spinal cord extracts eliminates its proapoptotic actions on cultured oligodendrocytes (Beattie et al 2002). In order for pro-neurotrophins to be true physiological ligands for p75, endogenous pro-neurotrophin must be secreted and then bind and activate p75 *in vivo*. A second study examined this issue in the p75NTR-dependent cell death of corticospinal neurons (CSN) following proximal axotomy at the level of the internal capsule (Harrington et al., 2004). These authors show that proNGF is elevated in the cerebral spinal fluid following CSN lesion, indicating that endogenous active proNGF is secreted in the injured central nervous system. Secondly, the authors show that proNGF in the injured tissue co-precipitates with p75NTR, indicating thet proNGF binds to p75NTR in vivo. Lastly, the authors show that CSN loss is reduced either following administration of antibodies directed specifically against proNGF or in mice containing only one allele of NGF (NGF+/-) (Harrington et al., 2004), thus providing direct evidence for the participation of proNGF in p75NTR-dependent cell death *in vivo*.

These results help clarify why p75NTR loss of function studies clearly demonstrate the apoptotic potential of this receptor, yet receptor activation studies required the use of high concentrations of neurotrophins to elicit effects (Bamji et al., 1998; Yoon et al., 1998). These findings have new implications for the regulation of neuron number during the period of naturally occurring cell death. The balance between life or death of a neuron expressing both TrkA and p75NTR receptors may be determined by the relative ratio of these two receptors and by the relative ratio of proNGF and mature NGF secreted by target cells. Although it remains to be formally demonstrated that pro-neurotrophins play a similar role during development as after injury in the adult nervous

system, these findings suggest that the ultimate survival of a neuron is dependent on the set of growth factors encountered, be they positive (mature neurotrophins) or negative (pro-neurotrophins). In the event that the appropriate set of trophic factors is not encountered, neurons may be eliminated though p75NTR action.

It may be predicted then that the proteolytic processing of pro-neurotrophins may represent a critical decision making step to ensure neuronal survival during development. In that regard, Lee et al. (2001) reported that proteases like plasmin and specific matrix metalloproteinases can efficiently convert proNGF and proBDNF to a form resembling the mature product (Lee et al., 2001). Matrix metalloproteinases are suitably positioned in the extracellular space and as transmembrane proteins to modify proneurotrophins as they are secreted. It will be of interest to determine what role these proteases play in regulating the availability of proneurotrophins to developing neurons during the period of naturally occurring neuron death.

How does the addition of the pro-domain allow pro-neurotrophins to bind p75NTR with higher affinity? New evidence indicates that p75NTR does not act alone as a receptor for pro-neurotrophins, but rather p75NTR acts as a co-receptor in conjunction with another transmembrane protein to properly bind and respond to pro-neurotrophins. Nykjaer et al. (2004) identified the transmembrane protein Sortilin as a p75NTR co-receptor that is necessary for proNGF-induced cell death. Sortilin is a type I transmembrane protein expressed in a wide variety of tissues but is most abundant in the central nervous system during development and in adults. Surprisingly, the NGF pro-domain directly binds the extracellular domain of Sortilin, even in the absence of p75NTR, and crosslinking studies suggested that p75NTR and Sortilin form a receptor complex that binds proNGF at the cell surface (Nykjaer et al., 2004). Both receptors appear to be required to transduce the apoptotic effects of proNGF. Blocking the interaction of proNGF with Sortilin inhibits proNGF-mediated apoptosis, whereas expression of exogenous Sortilin in Schwann cells, which normally express only p75NTR, renders these cells sensitive to the apoptotic effect of proNGF (Nykjaer et al., 2004). Further loss-of-function studies either using knockout mice or RNAi will be valuable in elucidating the role of Sortilin in trophic factor deprivation-induced neuron death.

It should be noted that p75NTR-dependent cell death may occur in a ligand-independent manner (Bredesen et al., 1998). Although little is known regarding the mechanism of the ligand-independent pathway, p75NTR expression has been correlated with elevated ceramide and increased caspase activity in neuroblastoma cells (Lievremont et al., 1999), may involve Bcl-2 in sensory neurons (Coulson et al., 1999), and TRAF2 interactions (Ye et al., 1999).

p75NTR mediates apoptosis following injury

A number of studies have recently indicated that p75NTR signaling plays a physiological role after stress or injury. The upregulation of p75 receptors in many cell types is observed under pathological, or inflammatory conditions. Following a crush injury or transection of hypoglossal, facial, or sciatic nerves, p75NTR is transiently and highly upregulated in the corresponding motor neurons that do not express TrkA (Hayes et al., 1992). Application of NGF to transected facial nerves of newborn rats resulted in a dramatic loss in neuronal number, suggesting that p75NTR may induce apoptosis in this paradigm (Sendtner et al., 1992). Further, the survival of motor neurons in adult or newborn murine facial nucleus following axotomy was significantly higher in p75NTR-deficient mice (Ferri et al., 1998). In the adult CNS, cortical spinal neurons upregulate p75NTR after proximal injury and undergo p75-dependent cell death, since CSN loss is reduced either by intracerebral infusion of p75-blocking antibodies or by genetic deletion of p75 (p75NTR-/- mice)(Harrington et al., 2004). Similarly, an increase in p75NTR expression has been reported in Purkinje cells following traumatic lesion, in striatal cholinergic neurons after ischemic injury (Kokaia et al, 1998), and in hippocampal neurons after ischemia (Lee et al., 1995) or pilocarpine-induced seizure (Roux et al., 1999). In the brains of rats with pilocarpine-induced seizures, the upregulation of p75NTR is prominent in cortical neurons that do not normally express this receptor, and the induction of p75NTR occurred in those cells undergoing apoptosis (Roux et al., 1999).

Interestingly, under normal physiological conditions, oligodendrocytes do not express p75NTR. However, oligodendrocytes present in multiple sclerosis plaques exhibit strong p75NTR expression (Dowling et al., 1999). Following spinal cord injury,

oligodendrocytes near the lesion epicenter undergo apoptosis (Brandoli et al., 2001; Casha et al., 2001) and these cells were also found to upregulate p75NTR (Casha et al., 2001; Beattie et al., 2002). Importantly, apoptosis of oligodendrocytes was reduced after spinal cord injury in p75NTR-deficient mice (Beattie et al., 2002).

p75NTR expression is also induced in Schwann cells after peripheral nerve injury and has been shown to play a death-inducing role in neonatal Schwann cells following axotomy (Ferri and Bisby, 1999; Syroid et al., 2000). However, p75NTR signaling has been described to subserve other functions in Schwann cells, such as migration (Bentley and Lee, 2000), and cell survival (Gentry et al., 2000), depending on the cellular context.

p75NTR apoptotic signal transduction

How does p75NTR negatively regulate neuronal survival? Formally there are several possiblilties. p75NTR may mediate its apoptotic effects by antagonizing TrkA survival signaling, perhaps by a direct action on the TrkA receptor itself. Such a mechanism might involve alterations in p75NTR-TrkA interactions to modify a high affinity signaling complex (Hempstead et al., 1991;Wolf et al., 1995) and/or a direct effect of p75NTR signaling on the TrkA receptor. In fact, p75NTR has been shown cause the serine/threonine phosphorylation of TrkA by ceramide-activated kinases (MacPhee and Barker, 1997). It remains unclear, however, what role this phosphorylation has on Trk receptor function. There is a great deal of evidence to suggest that p75NTR may mediate its apoptotic effects by autonomous activation of an apoptotic signaling cascade that is independent of Trk receptor signaling.

Historically, it has been generally assumed that the mechanism of p75-mediated cell death is similar to apoptotic signaling by other TNF receptor family members. A number of studies indicate that cell death by p75 occurs quite differently than TNF and Fas receptors. p75NTR does not interact with death domain containing proteins, such as FADD, and does not activate caspase-8, key components of apoptotic signaling downstream of TNF and Fas receptors (Coulson et al., 1999; Gu et al., 1999). In fact, the death domain of p75NTR is not necessary for sensory neuronal cell death. Instead, a 29 amino-acid segment of the p75-juxtamembrane region was found to be responsible for apoptosis (Coulson et al., 2000).

The original finding that the p75NTR is capable of autonomous signaling was first demonstrated in p75NTR-expressing cell lines and glial cells, where neurotrophin binding to p75NTR led to increased sphingomyelinase activity and accumulation of the potent lipid second messenger ceramide (Dobrowsky et al., 1994; Dobrowsky et al., 1995; Cassacia-Bonnefil et al., 1996). Other early studies showed that NGF binding to p75NTR on Schwann cells causes activation of nuclear factor (NF)-κB (Carter et al., 1996), whereas NGF treatment of oligodendrocytes causes activation of c-jun N-terminal kinase (JNK) (Cassacia-Bonnefil et al., 1996; Yoon et al., 1998). Both ceramide accumulation and JNK activation have been correlated with apoptotic stimuli in a number of systems, whereas NF-κB translocation in some cells may underlie p75NTR-mediated survival signaling (see below).

Of these signaling pathways, activation of the JNK pathway appears to play a key role in p75NTR-dependent apoptosis. The finding that neurotrophin binding to p75NTR activates JNK in oligodendrocytes (Cassacia-Bonnefil et al., 1996; Yoon et al., 1998), has also been shown in sympathetic neurons (Aloyz et al., 1999), and hippocampal neurons (Friedman, 2000). Furthermore, blocking the activation of JNK with a pharmacological inhibitor (Yoon et al., 1998) or a dominant-negative JNK (Harrington et al., 2002), prevented p75NTR-mediated apoptosis of oligodendrocytes. How does p75NTR activate JNK? One candidate upstream is the Rac GTPase, since NGF binding to p75NTR leads to the prolonged activation of Rac in oligodendrocytes (Harrington et al., 2002). Furthermore, inhibition of Rac activity using N17Rac blocks NGF-dependent JNK activation and subsequent apoptosis (Harrington et al., 2002).

What is downstream of JNK in p75NTR-mediated apoptosis? In sympathetic neurons, similar to NGF withdrawal, BDNF binding to p75NTR has been shown to increase phosphorylation of c-Jun (Bamji et al., 1998). However, BDNF-mediated p75NTR activation leads to apoptosis even in sympathetic neurons that express no c-Jun (as a consequence of Cre recombinase-mediated gene deletion) (Palmada et al., 2002), suggesting that c-Jun is not essential for p75NTR-mediated cell death. Nevertheless, cycloheximide treatment prevented BDNF-mediated apoptosis of sympathetic neurons, indicating that p75NTR-activation induced apoptosis is a transcription-dependent event (Palmada et al., 2002). In that regard, other JNK substrates have been implicated in the

p75NTR death pathway, including the tumor suppressor p53 (Aloyz et al., 1998). In sympathetic neurons, ectopic expression of the upstream kinase MEKK1, which activates JNK, is sufficient to elevate the cellular levels of p53 and cause apoptosis (Aloyz et al., 1998). p53 was suggested to function as a downstream mediator of p75NTR signaling since ectopic expression of the viral p53 inhibitor, EIB55K, blocked BDNF/p75NTR-activation induced death (Aloyz et al., 1998). Taken together, these findings suggest that p75NTR-mediated cell death occurs through the activation of a JNK-p53 pathway.

How does stimulation of p75NTR with neurotrophin lead to the activation of downstream apoptotic signals, such as JNK? A number of proteins have recently been shown to interact with the intracellular domain of p75NTR that may transduce the apoptotic effects of this receptor. Four proteins have been proposed to play a role in p75NTR-mediated apoptosis, including NRIF (Casademunt et al., 1999), NADE (Mukai et al., 2000), NRAGE (Salehi et al., 2000), and TRAF6 (Khursigara et al., 1999).

The NADE protein was identified by its ability to bind to the death domain of p75NTR. Co-expression of NADE and p75NTR was necessary for p75-mediated caspase activation and cell death in 293 cells (Mukai et al., 2000). NRAGE, is a member of the MAGE family, that can be co-immunoprecipitated with p75NTR (Salehi et al., 2000), and ectopic expression of NRAGE can induce JNK-dependent caspase activation and cell death (Salehi et al., 2002). Another p75NTR-dependent apoptotic pathway involves the recently reported neurotrophin receptor-interacting factor (NRIF). Analysis of NRIF-/- mice revealed a deficit in apoptosis in the developing retina, a phenotype similar to that seen in p75NTR-/- mice, raising the possibility that p75NTR might signal apoptosis in some cells via NRIF (Casademunt et al., 1999). TRAF6 was originally shown to bind p75NTR in a ligand dependent manner (Khursigara et al., 1999). A recent study has shown that BDNF-mediated activation of p75NTR causes JNK phosphorylation in *traf6*+/+, but not *traf6*-/-, sympathetic neurons (Yeiser et al., 2004).

What is the difference between cell death induced by NGF withdrawal and p75NTR activation? As described above, cdc42/Rac1, Ask1, MLK, JNK, c-Jun, and p53 have been shown to act in a signaling pathway regulating NGF withdrawal induced apoptosis. On the other hand, MEKK and JNK function upstream of p53 in p75NTR-mediated cell death. The presence of apoptotic proteins common to both NGF withdrawal

and p75NTR activation-induced cell death, and the observation that *p75NTR*-/- sympathetic neurons are greatly delayed in their death following NGF withdrawal, suggests that a major component of NGF withdrawal induced apoptosis involves p75NTR-driven activation of a JNK-p53-Bax pathway.

Interestingly, as no exogenous p75NTR ligand is present in cultures following NGF withdrawal, it is unclear whether p75NTR is acting in a ligand-dependent manner or ligand-independent manner. However, sympathetic neurons themselves make endogenous p75NTR ligands (BDNF and NT-4), suggesting the possibility of an autocrine p75NTR-driven apoptosis loop (Causing et al., 1997). The presence of an autocrine cell death loop may imply that neurons are destined to die unless they obtain adequate amounts of survival factors to silence the cell death pathway. Consistent with this idea, TrkA activation in sympathetic neurons utilizes Ras to block p75NTR-mediated activation of a JNK-p53 pathway (Mazzoni et al., 1999). Similarly, ectopic expression of TrkA silences JNK activation coincident with its repression of p75NTR-mediated apoptosis (Yoon et al., 1998).

p75NTR survival signaling

In contrast to its role in cell death, p75NTR may signal survival in some cells under certain circumstances. Several reports have indicated that p75NTR can promote survival in sensory neurons deprived of trophic support (Longo et al., 1997), hippocampal neurons treated with glutamate (Bui et al., 2002), and subplate neurons during development of the rodent cortex (Defreitas et al., 2001; McQuillen et al., 2002). p75NTR activation of the transcription factor NF-κB has been implicated in the pro-survival response (Hamanoue et al., 1999). p75NTR-mediated activation of NF-κB has now been shown in several cell types, including Schwann cells (Carter et al., 1996; Hamanoue et al., 1999), oligodendrocytes (Ladiwala et al., 1998), and sensory neurons (Kimpinski et al., 1999). How does p75NTR activate NF-κB? TRAF6 has been suggested to mediate NF-κB activation based on dominant negative studies (Khursigara et al., 1999; Foehr et al., 2000) and that *traf6*-/- Schwann cells are defective in p75-mediated activation of NF-κB (Yeiser et al., 2004). Another p75NTR-interacting protein RIP2 (receptor-interacting protein-2)

has been implicated since ectopic expression of RIP2 is sufficient to reconstitute NGF activation of NF-κB (Khursigara et al., 1999).

Statement of Projects and Objectives

I pursued three distinct projects in my PhD research. Firstly, I examined the molecular mechanism through which p75NTR promotes naturally occurring cell death of sympathetic neurons. Previous evidence had suggested that in the absence of p75NTR expression, sympathetic neuron number does not decrease appropriately during the period of developmental neuron death (Bamji et al., 1998). This phenotype appears to be due to a loss of p75NTR specifically in sympathetic neurons since the rate of death of sympathetic neurons cultured from p75-deficient mice is slower than their wildtype counterparts in response to NGF withdrawal (Bamji et al., 1998). Given these data, we hypothesized that p75NTR promotes apoptosis by directly stimulating cell death independently of the TrkA. We were aware of the alternative hypothesis that p75NTR promotes apoptosis indirectly by inhibiting or modulating TrkA survival activity. Using sympathetic neurons cultured from wildtype and p75NTR knockout mice, my specific aim was to determine whether Trk receptor levels and/or TrkA receptor activity was altered (i.e., increased) in the absence of p75NTR expression. In collaboration with Marta Majdan, using *trkA/p75NTR* double knockout mice, our aim was to determine whether the coincident absence of p75NTR could rescue sympathetic neuron cell death observed in *trkA*-/- mice.

My second project was to examine the role of c-Jun N-terminal kinase (JNK) and the tumor suppressor protein p53 in mediating neuronal apoptosis in two neuron cell death paradigms. Two pieces of evidence suggested that a JNK-p53 signaling pathway is essential for trophic factor withdrawal induced death of sympathetic neurons. First, p53-/- mice display an increase in sympathetic neuron number during the period of developmental cell death (Aloyz et al., 1998). Second, activation of JNK by ectopic expression of the upstream MEKK1 induces p53 protein levels and is sufficient to induce sympathetic neuron death (Aloyz et al., 1998). Given these data, I hypothesized that a JNK-p53 signaling pathway was essential for neuronal apoptosis following both trophic factor withdrawal and neonatal nerve injury. In order to inhibit JNK signal transduction, I made use of an adenovirus expressing JNK-interacting protein 1 (JIP1), a scaffold protein

which when overexpressed had been shown to inhibit JNK activity (Dickens et al., 1997). My specific aim was to determine whether NGF withdrawl induced death was JNK dependent. Second, using p53 knockout mice, my aim was to determine whether the absence of p53 protected facial motor neurons from neonatal axotomy-induced cell death.

My third project was to examine the intracellular mechanisms that underlie the relative invulnerability of adult versus developing sensory neurons. Previous studies had suggested that adult peripheral neurons were resistant to apoptotic stimuli (such as trophic factor withdrawal and nerve injury) that normally cause death of developing neurons (Lindsay, 1988; Snider et al., 1992). Second, whereas trophic factor withdrawal activates the same initial apoptotic signaling events in developing neurons and neurons that have been aged "3 weeks" in culture, aged neurons are protected from this insult, suggesting a cell intrinsic mechanism that confers apoptotic resistance on the older neurons. We had previously shown that ΔNp73, a member of the p53 tumor suppressor family, is an essential pro-survival protein during the developmental neuron cell death period for sympathetic neurons (Pozniak et al., 2000). Given this finding, we hypothesized that the apoptotic resistance displayed by adult neurons was due, in part, to an enhancement in intrinsic survival pathways that might "block" apoptosis and that one critical component of these survival pathways is p73. My specific aim was to determine the expression of p73 isoforms in peripheral ganglia using RT-PCR techniques. In collaboration with Nina Orike, our aim was to determine whether the resistance of adult DRG neurons to apoptotic stimuli was altered in the absence of one allele of p73. And lastly, using p73+/- mice, my aim was first to ensure that sensory neuron development was normal in hypomorphic animals, and then to determine whether adult sensory neuron survival was perturbed in p73+/- mice following sciatic nerve injury.

CHAPTER 2

TRKA MEDIATES DEVELOPMENTAL SYMPATHETIC NEURON SURVIVAL IN VIVO BY SILENCING AN ONGOING p75NTR-MEDIATED DEATH SIGNAL

Marta Majdan[2], Gregory S. Walsh[1,2], Raquel Aloyz[2], Freda D. Miller[1].

[1]Developmental Biology, Hospital for Sick Children, University of Toronto

[2]Center for Neuronal Survival and Brain Tumor Research Centre, Montreal Neurological Institute, McGill University

ABSTRACT

Developmental 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 -/- and TrkA -/- mice indicate that the coincident absence of p75NTR substantially rescues TrkA -/- 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 -/- sympathetic neurons die during the late neonatal and early postnatal periods (Smeyne et al., 1994; Fagan et al., 1996); (b) in p75NTR -/- 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 -/- 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-/- and NT-3 -/- 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.

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-Bonnefil 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 -/- and TrkA -/- mice demonstrated that the coincident absence of p75NTR substantially rescued the sympathetic neuron apoptosis observed in TrkA -/- 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.

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 -/- animals were crossed with TrkA +/- animals, their progeny were genotyped using PCR, and the p75NTR +/- , TrkA +/- 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-μm–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 ± 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 ßIII-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 ßIII-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 (Biowhittaker, 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 μg/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). The number of phase-bright neurons with neurites in randomly selected, 5.3-mm 2fields

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 -/- and p75NTR +/+ 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.)

RESULTS

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

We have previously reported (Bamji et al., 1998) that in p75NTR -/- 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 the ganglion. To distinguish between these possibilities, we assessed apoptosis and proliferation in p75NTR -/- 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 ganglia. As predicted, many TUNEL-positive nuclei were detected within the wild-type SCG; 1,472 ± 60 per ganglion (n= 3). In contrast, the p75NTR -/- 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 -/- versus p75NTR +/+ ganglia. To examine the extent of ongoing cell division, p75NTR +/+ and p75NTR -/- 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 +/+ and p75NTR -/- ganglia (1.43 ± 0.7%, n= 3 and 1.25 ± 0.3%, 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 net increase in sympathetic neuron number relative to wild-type ganglia.

Trk receptor levels, activation, and downstream signaling in p75NTR-/- sympathetic neurons

Three potential explanations for the deficit in apoptosis observed in p75NTR-/- 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 signaling 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 -/- ganglia and cultured p75NTR -/- neurons. Initially, we examined levels of TrkA and TrkC in p75NTR -/- 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 analysis revealed that levels of TrkA were slightly but consistently decreased in the p75NTR -/- 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 -/- SCG is not due to an increase in Trk receptor levels.

We also compared a number of additional proteins in the p75NTR -/- and p75NTR +/+ 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 -/- animals examined in this study (Von Schack et al., 2001). No p75NTR protein corresponding to this smaller variant was detectable either in the p75NTR -/- ganglia or in cultured p75NTR -/- 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 -/- and p75NTR +/+ ganglia (Fig. 2, A and C).

Next, 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 -/- ganglia (Fig. 2 C). Finally, since caspase-3 has been shown to be required for sympathetic developmental cell death, we examined the extent of caspase-3 cleavage in p75+/+ and p75-/- ganglia. Western blot analysis revealed that the extent of caspase-3 cleavage was markedly reduced on postnatal day 2 (P2) in p75NTR -/- ganglia as compared to p75NTR +/+ gangli (Fig. 2 H).

Because examination of the p75NTR -/- 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 -/- and p75NTR +/+ 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 +/+ and p75NTR -/- 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 -/- and p75NTR +/+ 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 -/- sympathetic neurons have 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 -/- and p75NTR +/+ 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 reprobed 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 +/+ and p75NTR -/- 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-/- neurons show enhanced survival in the absence of all Trk signaling

Together, these data suggest that the enhanced survival observed in p75NTR -/- neurons is not due to enhanced Trk signaling. However, to formally rule out this possibility, we cultured p75NTR -/- 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 +/+ versus p75NTR -/- 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 -/- neurons died significantly more slowly in the absence of NGF (Fig. 3 B). Specifically, 24 h after NGF withdrawal, 94 ± 1% of p75NTR -/- neurons were still alive, compared with 84 ± 6% of wild-type neurons (P= 0.07). By 48 h, 75 ± 7% of p75NTR -/- neurons were still alive, compared with 56 ± 8% of control neurons (P= 0.06). By 72 h, 56 ± 6% of p75NTR -/- 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 -/- neurons. Specifically, 24 h after NGF withdrawal, 95 ± 1% of p75NTR -/- neurons were still alive, compared with 88 ± 2% of controls (P< 0.05), and by 48 h 72 ± 3% of p75NTR -/- neurons were alive, versus 50 ± 6% of the wild-type neurons (P< 0.05). By 72 h, 50 ± 5% of the p75NTR -/- 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-/- sympathetic neuron apoptosis in vivo

Although the data presented here on p75NTR -/- 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 -/- and TrkA -/- animals; sympathetic neurons undergo a rapid apoptotic death in TrkA -/- 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 -/- phenotype. If, in contrast, p75NTR mediates neuronal apoptosis in a Trk-independent fashion, then the severe neuronal loss seen in TrkA -/- SCG would be at least partially rescued in the absence of p75NTR.

To perform these studies, we initially confirmed the p75NTR -/- phenotype in the mixed C129/C57BL6 background that resulted from these crosses. TrkA +/- and p75NTR -/- animals were crossed, and then their TrkA +/-, p75NTR +/- progeny were bred to produce animals for analysis. The SCGs were analyzed from the resultant TrkA +/+, p75NTR +/+ , p75NTR +/-, and p75NTR -/- animals; ganglia were grouped by age, P1–P3 and P4–P6. This analysis revealed an increase in the numbers of neurons in the p75NTR -/- versus p75NTR +/+ 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 +/- 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 +/+ and p75NTR +/- animals at P7 (Fig. 2 G). This analysis revealed that p75NTR levels were lower in the p75NTR +/- animals, correlating with the observed increase in neuronal survival.

We then characterized neuronal numbers in the SCG of the p75NTR +/+,TrkA +/+, TrkA +/-, and TrkA -/- animals over this same time frame. This analysis revealed that, as previously reported (Fagan et al., 1996), there was a dramatic decrease in the number of SCG neurons at P1–P3 in the TrkA -/- animals relative to their TrkA +/+ littermates (6,108 ± 411, *n*= 3 versus 16,390 ± 1,003, *n*= 5). By P4–P6, the neuronal loss in the TrkA -/- 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 -/- ganglia at P1–P3 and P4–P6, respectively.

Interestingly, quantitation of sympathetic neurons in the TrkA +/- SCG revealed no significant difference between numbers in TrkA +/+ and TrkA +/- ganglia at these two time points (P1–P3: 15,176 ± 796, *n*= 6; P4–P6: 19,777 ± 1880, *n*=3 for the TrkA +/- 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 +/+ and TrkA +/- 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 -/- SCG. At P1–P3, the concomitant absence of p75NTR almost completely rescued sympathetic neuron numbers in the TrkA -/- , p75NTR -/- SCG; 13,665 ± 730 neurons (*n*= 3) versus 16,390 ± 1,003 neurons (*n*= 5) in the TrkA +/+ ,p75NTR +/+ SCG (Fig. 6 A). Moreover, at this age, even the loss of one p75NTR allele was enough to significantly increase neuronal survival; the TrkA -/-, p75NTR +/- SCG contained 11,000 neurons, whereas the TrkA -/- , p75NTR +/+ 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 -/-, p75NTR +/+ SCG (Fig. 6 A). Similarly, a rescue was still observed in the TrkA -/-, p75NTR +/- ganglia at P4–P6, although this was of a lesser magnitude than that observed in the p75NTR -/- 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 -/- animals, and most died within the first three postnatal days. Moreover, cresyl violet–stained sections of P4 wild-type and TrkA -/-, p75NTR -/- 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 -/- cells (Deckwerth et al., 1996). To ensure that these smaller cells were in fact neurons, we immunostained sections from P2 TrkA -/-, p75NTR -/- SCGs with an antibody for neuron-specific ßIII-tubulin, and then Nissl-stained the alternate sections (Fig. 7). This analysis revealed that all of the smaller neuronal cells expressed ßIII-tubulin, and that the numbers of neurons obtained by counting the immunostained versus Nissl-stained sections were similar: in two different p75NTR -/-, TrkA -/- SCGs, 790 and 1032 cresyl violet–stained neurons were present in representative sections, and 930 and 808 ßIII 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, ßIII-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.

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 -/- 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 -/- sympathetic neurons. Third, downstream NGF or NT-3–mediated survival signaling is similar in p75NTR +/+ and p75NTR -/- sympathetic neurons. Fourth, pharmacological studies demonstrate that p75NTR -/- sympathetic neurons are deficient in apoptosis after NGF withdrawal, even when all Trk signaling is inhibited. Fifth, crosses of the p75NTR -/- and TrkA -/- animals demonstrate that the loss of sympathetic neurons seen in neonatal TrkA -/- 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 -/- animals (Lee et al., 1994b).

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., 1994a) 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 +/+ and p75NTR -/- 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 -/- sympathetic neurons in response to NT-3 (Lee et al., 1994a; 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 -/- sympathetic neurons are delayed in their apoptosis in the absence of all Trk receptor signaling, and the absence of p75NTR -/- substantially rescues neonatal TrkA -/- 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 -/- 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 -/- 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 -/- SCG, suggesting that p75NTR may contribute to the activation of this same apoptotic pathway in vivo.

Previous work on p75NTR -/- 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 -/- and p75NTR +/+ neurons equally well. In contrast, Lee et al. (1994a) reported that acutely dissociated sympathetic neurons from p75NTR -/- 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. 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 -/- 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 -/-, TrkA -/- 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 -/- 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 -/- 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.

Figure 1. ***The increase in sympathetic neuron number in the neonatal p75NTR -/-SCG is due to reduced apoptosis, not increased proliferation.*** (A) Fluorescence photomicrographs of TUNEL analysis of representative sections through the P2 SCG of p75NTR +/+ and p75NTR -/- 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 +/+ (control) versus p75NTR -/- (p75 -/-) animals. (** $P< 0.0005$, $n= 3$). (C) Percentage of BrdU-positive cells with neuronal morphology in the p75NTR +/+ (control) versus p75NTR -/- (p75 -/-) 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.

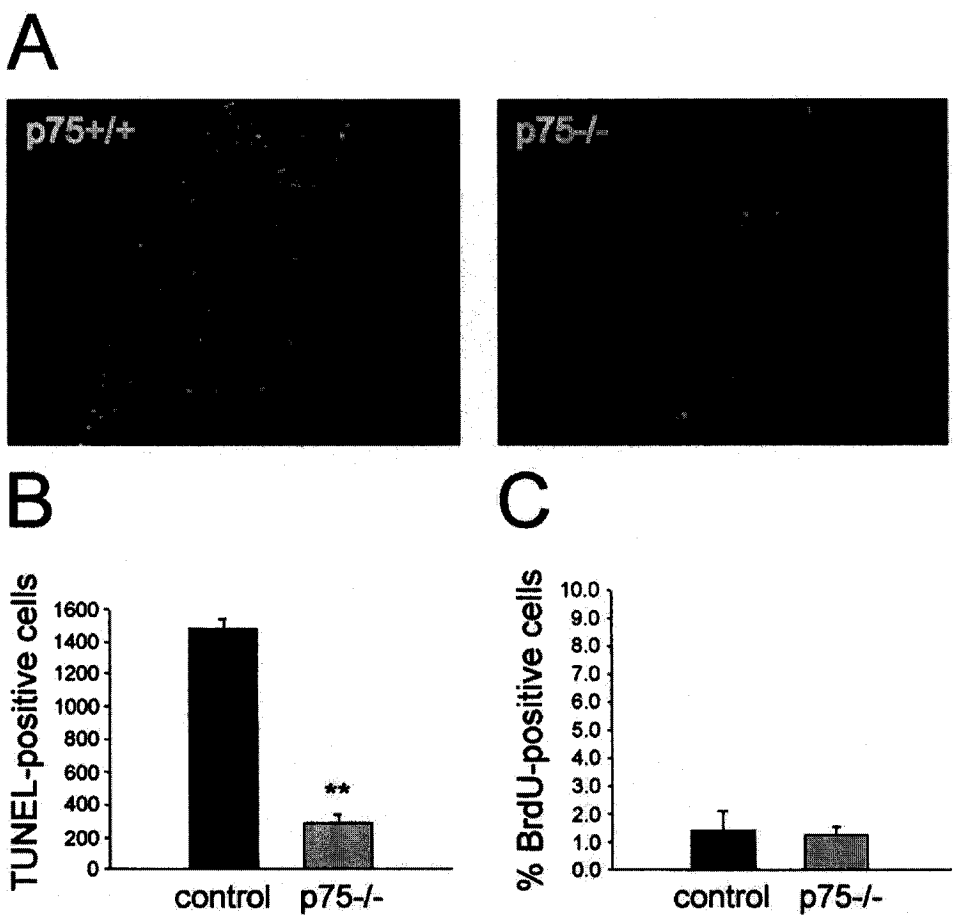

A
p75+/+
p75-/-
B
TUNEL-positive cells
1600
1400
1200
1000
800
600
400
200
0
**
control
p75-/-
C
% BrdU-positive cells
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
control
p75-/-

Figure 2. ***Levels of Trk receptors, Trk receptor activation, and downstream survival signaling in p75NTR -/- SCG neurons.*** (A) Western blot analysis of equal amounts of protein from p75NTR -/- versus p75NTR +/+ SCG at P7, probed for TrkA (RTA), tyrosine hydroxylase (TH) and ERK1. (B) Western blot analysis of lysates of P7 p75NTR -/- versus p75NTR +/+ 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 -/- versus p75NTR +/+ ganglia probed for p75NTR (p75), p53, or for total tubulin. (D) Western blot analysis of equal amounts of protein from cultured p75NTR -/- versus p75NTR +/+ 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 -/- versus p75NTR +/+ 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 +/+ versus p75NTR +/- ganglia at P7. The blot was reprobed for tubulin, scanned, and the ratio of p75NTR to tubulin was plotted on the accompanying bar graph. (H) Western blot analysis of equal amounts of protein from p75NTR -/- versus p75NTR +/+ ganglia probed for p75NTR (p75), and cleaved caspase-3. All blots are representative examples of at least three separate experiments.

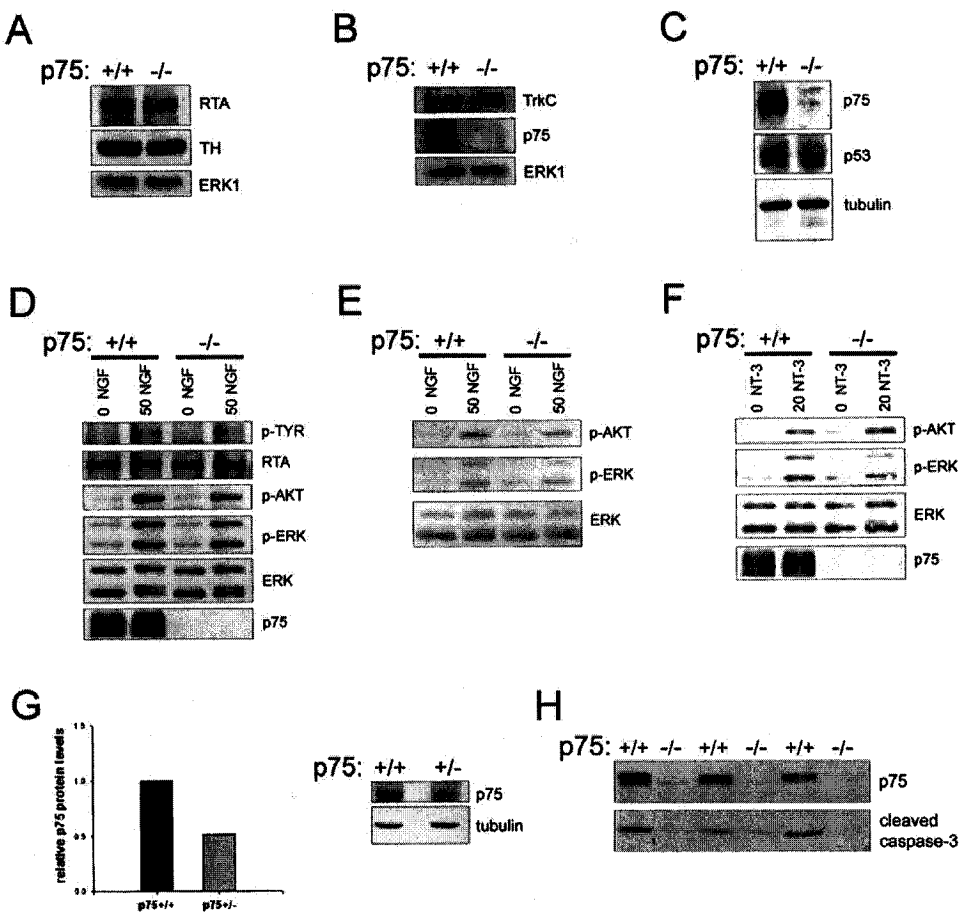
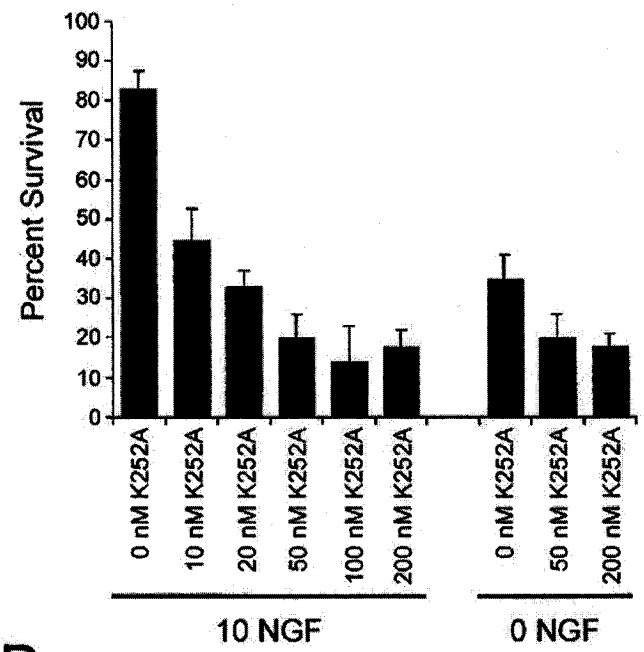

A
p75: +/+ -/-
RTA
TH
ERK1
B
p75: +/+ -/-
TrkC
p75
ERK1
C
p75: +/+ -/-
p75
p53
tubulin
D
p75: +/+ -/-
0 NGF
50 NGF
0 NGF
50 NGF
p-TYR
RTA
p-AKT
p-ERK
ERK
p75
E
p75: +/+ -/-
0 NGF
50 NGF
0 NGF
50 NGF
p-AKT
p-ERK
ERK
F
p75: +/+ -/-
0 NT-3
20 NT-3
0 NT-3
20 NT-3
p-AKT
p-ERK
ERK
p75
G
relative p75 protein levels
p75+/+
p75+/-
p75: +/+ +/-
p75
tubulin
H
p75: +/+ -/- +/+ -/- +/+ -/-
p75
cleaved caspase-3

Figure 3. ***Cultured p75NTR -/- 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 -/- versus p75NTR +/+ (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 ± standard error of combined data from four separate experiments, each performed in triplicate.

A


Percent Survival
100
90
80
70
60
50
40
30
20
10
0
0 nM K252A
10 nM K252A
20 nM K252A
50 nM K252A
100 nM K252A
200 nM K252A
0 nM K252A
50 nM K252A
200 nM K252A
10 NGF
0 NGF

B

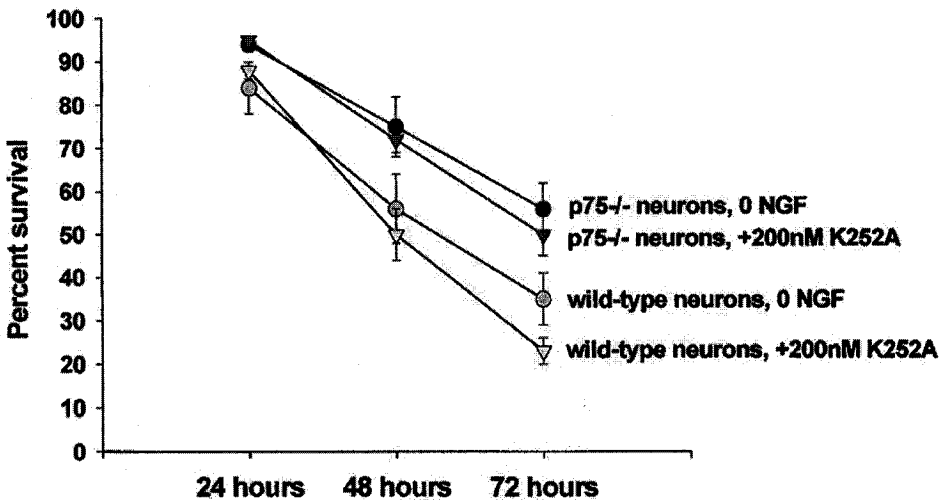

Percent survival
100
90
80
70
60
50
40
30
20
10
0
24 hours
48 hours
72 hours
p75-/- neurons, 0 NGF
p75-/- neurons, +200nM K252A
wild-type neurons, 0 NGF
wild-type neurons, +200nM K252A

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 +/+, p75NTR +/-, and p75NTR -/- animals at ages P1–P3 and P4–P6. Results represent mean ± standard error (n= 3–7 for each genotype). At P1–P3, p75NTR +/+ and p75NTR -/- SCG numbers are significantly different (* $P< 0.05$), and at P4–P6, p75NTR +/+ neuron counts are significantly different from the p75NTR +/- and p75NTR -/- counts (*$P< 0.05$ for both groups). For comparison, neuronal numbers from the P4 SCG of p75NTR +/+ and p75NTR -/- C129 animals are shown (Bamji et al., 1998).

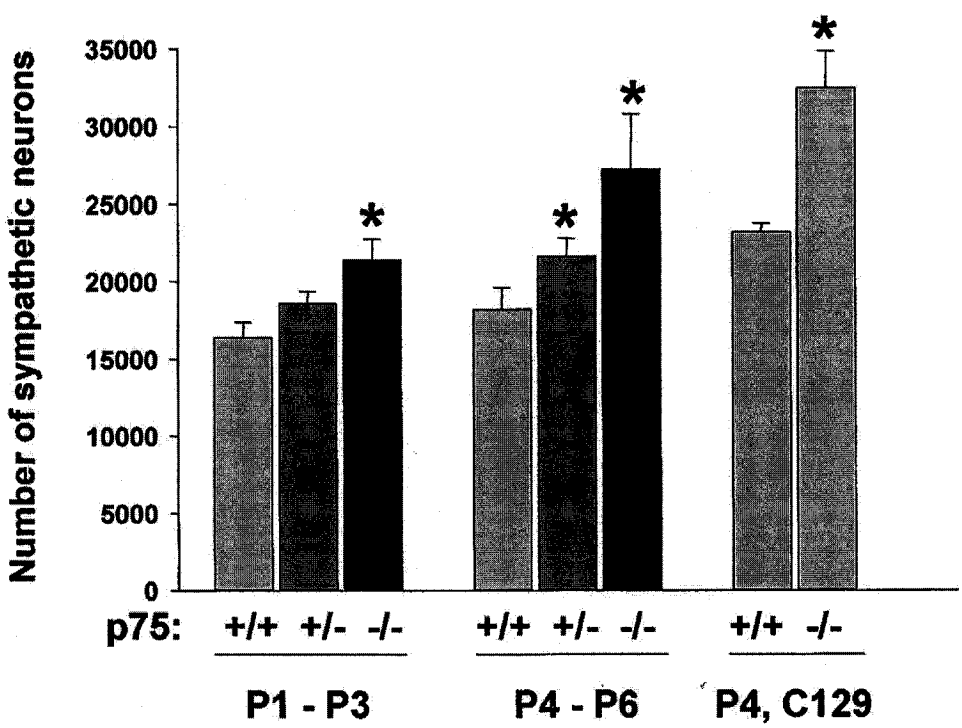
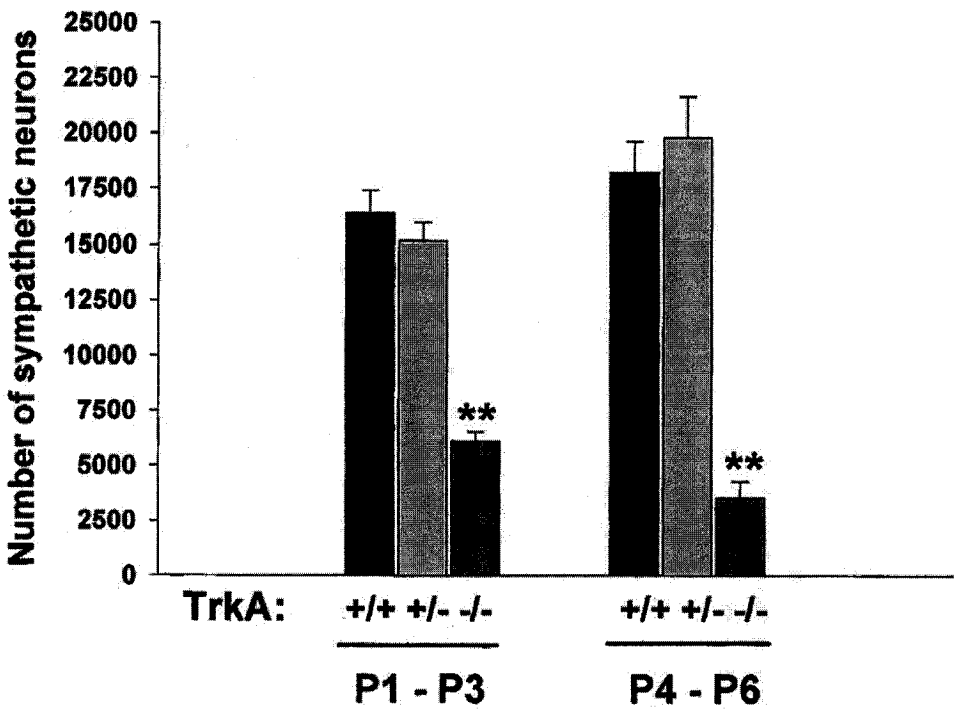

Number of sympathetic neurons
35000
30000
25000
20000
15000
10000
5000
0
p75: +/+ +/- -/- +/+ +/- -/- +/+ -/-
P1 - P3
P4 - P6
P4, C129
*
*
*
*

Figure 5. ***Analysis of sympathetic neuron number in TrkA +/+ , TrkA +/- , and TrkA -/- SCG.*** (A) Sympathetic neuron number in the SCG of TrkA +/+ , TrkA +/- , and TrkA -/- 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 -/- neuron number is greatly decreased (** P< 0.005), but there is no significant difference in the TrkA +/- ganglia. (B) Western blot analysis of equal amounts of protein from the TrkA +/+ and TrkA +/- SCG at P10. Blots were probed for TrkA, TrkC, and tubulin.

A


Number of sympathetic neurons
25000
22500
20000
17500
15000
12500
10000
7500
5000
2500
0
**
**
TrkA:
+/+ +/- -/-
+/+ +/- -/-
P1 - P3
P4 - P6

B

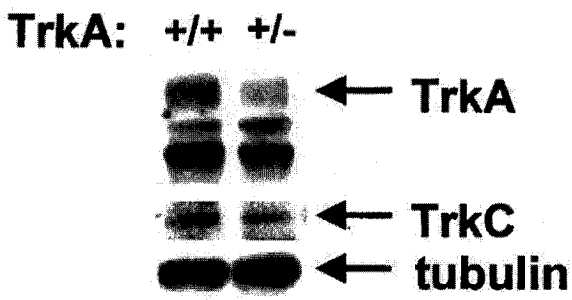
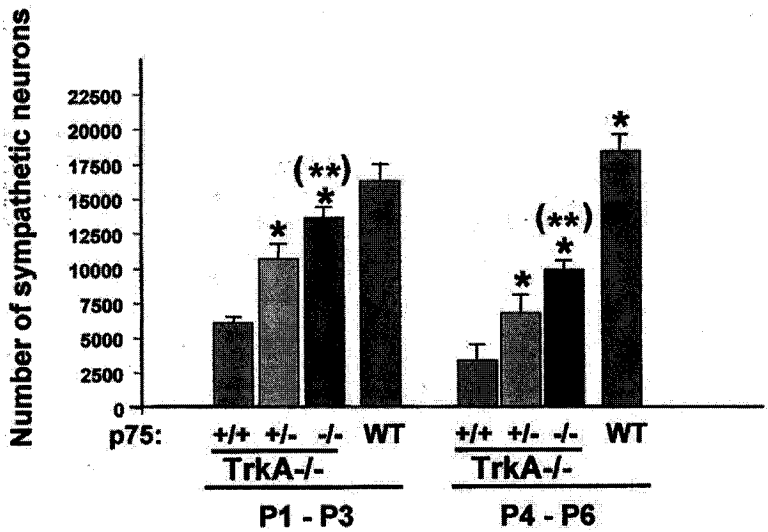
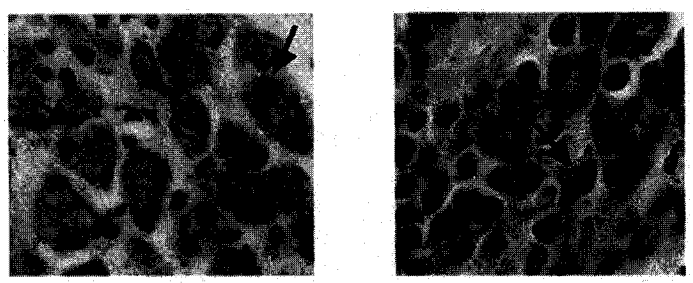

TrkA: +/+ +/-
TrkA
TrkC
tubulin

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

A



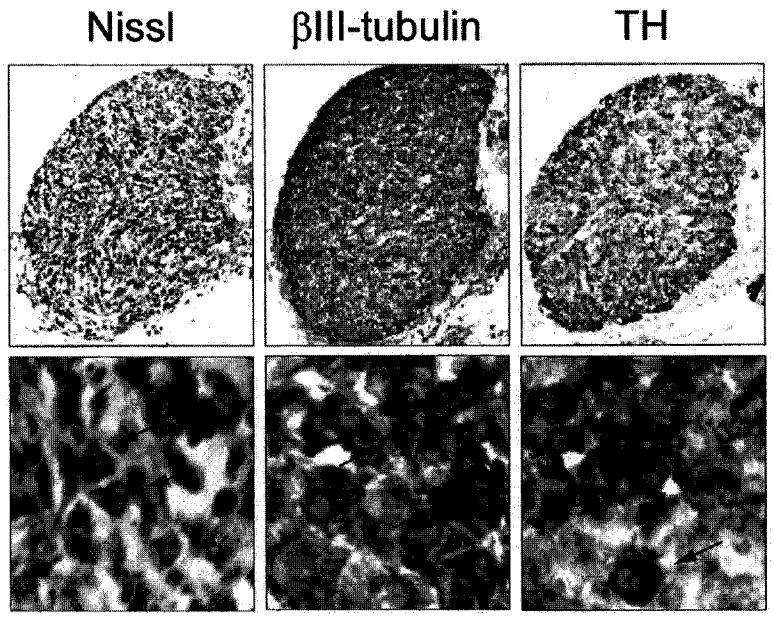
B



p75+/+ P4 SCG

p75-/- x TrkA-/- P4 SCG

Figure 7. ***p75NTR -/- , TrkA -/- sympathetic neurons express neuron-specific ßIII-tubulin and tyrosine hydroxylase.*** Photomicrographs of alternate sections taken from a p75NTR -/-, TrkA -/- ganglia and then either Nissl-stained or immunostained for the neuron-specific protein ßIII-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).


Nissl
βIII-tubulin
TH

CHAPTER 3

EVIDENCE THAT A JNK-p53 APOPTOTIC PATHWAY IS ESSENTIAL FOR NEURONAL DEATH AFTER TROPHIC FACTOR WITHDRAWAL AND FOLLOWING NERVE INJURY

Gregory S. Walsh[1,2], Freda D. Miller[1].

[1]Developmental Biology, Hospital for Sick Children, University of Toronto

[2]Center for Neuronal Survival and Brain Tumor Research Centre, Montreal Neurological Institute, McGill University

ABSTRACT

Sympathetic neurons require nerve growth factor for survival and die by apoptosis in its absence. Both c-Jun N-terminal kinase (JNK) and the tumor suppressor p53 have been implicated in the apoptotic signal transduction pathway that is activated in response to NGF withdrawal in developing neurons. JNK inhibition has been achieved pharmacologically by inhibiting upstream kinases, but there has been no direct demonstration that inhibition of JNK can prevent neuronal death. We have therefore examined whether the JNK-interacting protein 1 (JIP1, a scaffold protein and specific inhibitor of JNK) can inhibit c-Jun phosphorylation and support neuronal survival of sympathetic neurons deprived of NGF. We show that expression of JIP1 is sufficient to prevent the phosphorylation of c-Jun as well as inhibit neuronal cell death induced by NGF withdrawal. We further show that JNK activation and c-Jun phosphorylation are key events following neonatal axotomy of facial motoneurons. We further tested whether p53 is required downstream of JNK for neuronal death. Neonatal facial motoneurons from p53-deficient mice survived disconnection from their targets by axotomy. Taken together, these results establish a JNK-p53 signaling pathway as an essential component of cell death signaling in multiple neuronal cell types during development and following injury.

INTRODUCTION

In the mammalian nervous system, neuronal apoptosis (programmed cell death) occurs extensively during normal development, and is required for precisely regulating the number of neurons that make appropriate connections with their targets. During this period of naturally-occurring neuron death, cell survival is dependent on the action of neurotrophic factors that prevent both intrinsic and extrinsic cues from inducing cellular apoptosis. In the peripheral nervous system, target-derived neurotrophic factors, such as NGF, retrogradely determine neuronal survival in part by suppressing p75NTR-mediated apoptotic signals (reviewed in Kaplan and Miller, 2000).

In the central nervous system (CNS), c-Jun N-terminal kinase (JNK) and the tumor suppressor protein p53 are two key determinants of neuronal cell death following injury. The JNK family of stress-activated protein kinases belongs to a family including JNK1 and JNK2, which are expressed ubiquitously, and JNK3, which shows restricted expression to the brain, suggesting that JNK3 may mediate nervous system specific stress-induced apoptotic signaling. Consistent with this, the combined loss of *jnk-1* and *-2* genes in mice leads to inappropriate neural tube closure as a result of impaired developmental apoptosis (Kuan et al., 1999), whereas *jnk-3* deficient mice show a reduced sensitivity to excitotoxic death, and are protected from brain injury after cerebral ischemia-hypoxia (Yang et al., 1997; Kuan et al., 2003). A role for the tumor suppressor p53 in neuronal apoptosis is supported by the observation that p53 protein levels become elevated in CNS neurons following injury (Li et al., 1994; Sakhi et al., 1994; Sakhi et al., 1996a; Naprielski et al., 1999; Watanabe et al., 1999), and that neurons lacking p53 function exhibit reduced neuronal cell death in response to a variety of CNS insults, including excitotoxicity, stroke, and traumatic brain injury (Crumrine et al., 1994; Morrison et al., 1996; Xiang et al., 1996, Sakhi et al., 1996b).

Do JNK and p53 play a similar essential role in developing neurons in the peripheral nervous system? A number of studies have attempted to confirm the role of JNK in mediating apoptotic death of sympathetic neurons following NGF withdrawal. First, activation of JNKs by expression of upstream kinases leads to apoptotic cell death (Aloyz et al., 1998; Eilers et al., 1998). Second, CEP-1347, a pharmacological inhibitor of the mixed lineage kinases (MLKs, a family of kinases that lie upstream of JNK and

function as JNK kinase kinases) protects sympathetic neurons from trophic deprivation (Maroney et al., 1999). In addition, functional blockade of c-Jun *in vitro* by microinjection of antibodies or dominant negative c-Jun protects sympathetic neurons from NGF withdrawal mediated neuronal death (Estus et al., 1994; Ham et al., 1995). However, a direct demonstration of JNKs involvement has not been demonstrated.

A number of studies indicate that p53 plays an apoptotic role for both p75NTR-mediated and NGF withdrawal-induced death of sympathetic neurons. First, overexpression of p53 was sufficient to cause the death of sympathetic neurons (Slack et al, 1996). Second, p53-/- sympathetic neurons showed enhanced survival upon NGF withdrawal, although this deficit in apoptosis was much more pronounced in embryonic than in neonatal neurons (Vogel and Parada, 1998; Besirli et al, 2003). Third, sympathetic neuron apoptosis was rescued by concomitant expression of the adenoviral E1B55K protein, which binds to and inhibits p53 (Aloyz et al, 1998). Lastly, in p53-/- mice, the rate of developmental cell death was reduced (albeit not eliminated) in sympathetic neurons *in vivo* (Aloyz et al, 1998; Lee et al, 2004). It has been suggested that JNK acts upstream of p53 in the apoptotic pathway, since JNK can bind and phosphorylate p53 (Fuchs et al., 1998) and MEKK1-induced activation of JNK is sufficient to elevate p53 protein levels and induce cell death of cultured sympathetic neurons (Aloyz et al., 1998).

Here we extend these findings, showing that inhibition of the JNK signaling pathway in primary cultures of sympathetic neurons protects these cells from apoptosis following removal of NGF. In addition, we provide evidence for a JNK-p53 apoptotic pathway in nerve injured motoneurons. The phosphorylation of JNK and c-Jun was markedly elevated in the nuclei of injured facial motoneurons, and these neurons were rescued from injury-induced death in p53-/- mice.

MATERIAL AND METHODS

Sympathetic Neuron Cultures

Mass cultures of sympathetic neurons were derived from the superior cervical ganglia of postnatal day 1 (P1) Sprague-Dawley rats, as previously described (Ma et al. 1992), except that neurons were dissected into plating Ultraculture medium (serum free) containing 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Biowhittaker). Neurons were plated in the medium supplemented with 3% rat serum (Harlan Sprague Dawley Inc.), 50 ng/ml mouse 2.5 S NGF prepared from mouse salivary gland (Cedarlane Labs, Ltd.), and 0.5% cytosine arabinoside (Sigma Chemical Co.). For 3[4,5-dimethylthio-zol-2-yl]2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co.) assays, cell death ELISA, and biochemistry, neurons were plated on tissue culture dishes (Falcon Plastics) coated with rat-tail collagen. For microscopy, cells were plated on 8-well chamber slides (Nunc Inc.) coated twice with collagen. Neurons were plated at a density of 2,500–3,000/well of a 96-well plate for the MTT assays and cell death ELISA, and 40,000 cells/well of a 6-well plate for biochemistry. In all cases, neurons were cultured for 5 d in the above-mentioned medium. After 5 d of culture, cells were washed three times for 1 h in Ultraculture supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. After the washes, cells were induced in the same media used for the washes, supplemented with or without NGF.

Adenoviral Infection

The adenovirus coding for human JNK-interacting protein-1 (JIP1) was constructed as described (He et al., 1998). Briefly, the Jip1 cDNA was cloned into the pAdTrack shuttle vector, recombined with the pAdEasy adenoviral vector in bacteria, transfected, and amplified in human embryonic kidney (HEK)293 cells. The viruses were purified on CsCl gradients and titered in HEK293 cells, as we have described previously (Slack et al., 1996). The other adenoviruses have been described previously and encode green fluorescent protein (GFP) (Pozniak et al., 2000a; Toma et al., 2000) (Quantum Biotechnologies), and Escherichia coli β-galactosidase (Ad-lacZ)(Slack et al., 1996).

For adenoviral infection, cells were grown for 3 d in plating medium as described

earlier and then switched into similar media containing 50 mM KCl and the desired multiplicity of infection (MOI) of adenovirus and no cytosine arabinoside. Infection was allowed to proceed for 24 h, after which the cells were switched back to plating media containing cytosine arabinoside without virus for an additional 24 h before treatment.

Survival Assays and TUNEL

Survival assays were performed 48 h after washout and induction of neurons as previously described (Aloyz et al. 1998; Bamji et al. 1998). In brief, 20 μl of MTT reagent was added to the medium in each well of a 96-well plate containing the cultured neurons. After a 2.5 h incubation at 37°C, the medium/MTT mixture was removed and the cells were lysed with 100 μL of isopropanol containing 2 μL/mL of concentrated HCl. The absorbance of the lysate at 570 and 630 nm was determined using a Biotek model ELX-800 UV plate reader (Mandel Scientific Inc.). Apoptosis was also assessed by a quantitative sandwich-enzyme linked immunoassay (ELISA) that measures apoptotic cell death using monoclonal antibodies directed against DNA and histone, and measures the enrichment of nucleosomes, a hallmark of cells undergoing apoptosis (Roche Molecular Biochemicals). For the TUNEL experiments, cells were briefly rinsed in phosphate-buffered saline (PBS), pH 7.2, and fixed for 15 min in 4% paraformaldehyde (Sigma Chemical Co.), 0.25% glutaraldehyde (Fluka AG), and 0.2% Triton X-100 (Sigma Chemical Co.) in PBS, pH 7.2. Cells were then permeabilized with 0.5% Triton X-100 for 5 min and washed three times with PBS. TUNEL reaction was performed for 1 h at 37°C. Each 100 μl of TUNEL reaction mixture contained 20 μl of TdT buffer, 1.5 μl of TdT enzyme (both from Promega Corp.), and 1 μl of biotin-16-dUTP (Boehringer Mannheim Corp.). After the TUNEL reaction, cells were rinsed three times in PBS and incubated for 45 min at room temperature with Cy3-conjugated streptavidin (Jackson ImmunoResearch Laboratories) diluted 1:500 in PBS. Cells were then counterstained for 1 min with Hoechst 33258 (Sigma Chemical Co.) diluted 1:1,000 in PBS. Cells were washed three times with PBS after each of these incubations and then mounted. For each treatment, random images were captured and processed. Digital image acquisition and analysis was performed with the Northern Eclipse software (Empix Inc.) using a Sony XC-75CE CCD video camera.

Surgical procedures

One day after birth (P1) neonatal rats were anesthetized by hypothermia on ice. The branches of the left facial nerve were axotomized as they exited the stylomastoid foramen, the wound closed with sutures, the animals warmed on a heating pad and returned to their mother. The neonates were then killed at 3 days post-surgery for immunohistochemistry experiments and 7 days post-surgery for neuronal survival experiments.

Immunohistochemistry

Neurons were cultured in eight-well chamber slides as described above. After treatment, cells were briefly rinsed in PBS (pH 7.2) and fixed for 15 min in 4% paraformaldehyde in PBS (pH 7.2) Cells were then permeabilized with 0.5% Triton X-100 (Sigma) in PBS for 5 min and washed three times with PBS. Cells were incubated 1 hr with mouse monoclonal anti-FLAG M2 antibody (Sigma). This was followed by a 3×5 min PBS wash and incubation in goat anti-mouse CY3 (Jackson Laboratories) diluted 1:400 in PBS. Cells were then washed with PBS and mounted. Fluorescent labeling was visualized on an Axioscope (Zeiss, Inc) inverted microscope equipped with a Sony CCD camera. Digital image acquisition and analysis was performed with the Northern Eclipse software (Empix Inc.).

For facial nucleus histology, animals were terminally anesthetized and perfused transcardially with 4% paraformaldehyde in 0.1M phosphate buffer. Brainstems were removed and cryoprotected in graded sucrose solutions. 10 μm–thick sections were serially cut on a cryostat, and every section was collected on chromium/aluminum/gelatin–coated slides. 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 for phospho-JNK (1:1,000; Promega) or phospho-c-Jun (ser 73) (1:1,000; Cell Signaling). 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 facial motoneuron counting, coronal sections through the entire facial nucleus were stained with Cresyl Violet and mounted as described above. The number of facial motoneuron profiles displaying a nucleus was counted in every fifth section through the facial nucleus. Data is expressed as percent survival of ipsilateral facial motoneurons relative to contralateral uninjured facial motoneurons within the same animal.

Western Blot Analysis

For Western blot analysis, cultured sympathetic neurons were lysed and analyzed as previously described (Aloyz et al., 1998; Vaillant et al., 1999). Briefly, cultures were washed three times with cold PBS and collected by centrifugation, and proteins were extracted in RIPA lysis buffer, (containing 50 mM Tris-Hcl (pH 7.4), 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/mL/each of pprotinin, leupeptin, pepstatin, 1mM Na_3VO_4, and 1 mM NaF). Proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and probed with specific antibodies. The antibodies used for these analyses were anti-Erk 1 (1:5,000; Santa Cruz Biotechnology, Inc.), anti-JNK (1:2,000; Santa Cruz Biotechnology), anti-phospho-JNK (1:5000; Promega), anti-c-Jun (1:1,000; Transduction Laboratories), anti-phospho-c-Jun (ser 73) (1:1000; Cell Signaling Technology), anti-cleaved caspase-3 (1:2000; Cell Signaling Technology), anti-FLAG (M2) (1:5000; Sigma), anti-β-galactosidase (1:1000; Santa Cruz Biotechnology), and anti-GFP (1:1000; Clonetech). Blots were then processed using the appropriate HRP-linked secondary antibody (Boehringer) and visualized using enhanced chemiluminescence.

Protein Extraction from Whole Ganglia

SCG were rapidly dissected and placed into Eppendorf tubes containing the same lysis buffer used for cultured cells described above. The ganglia were sheared using a miniature Dounce homogenizer and mixed on a rotator for 20 to 30 min at 4°C. After clearing the lysates of insoluble material by centrifugation, the cleared lysates were subjected to Western analysis as described above. For each developmental time point, 4–8 SCG from rats were lysed in 400-600 μl of lysis buffer.

RESULTS

JIP overexpression inhibits the activity of JNK in sympathetic neurons

Recently, two cytoplasmic proteins that bind selectively to JNK but not to other MAP kinases (MAPK), including p38 and Erk, were identified as JNK-interacting protein 1 and 2 (JIP1 and JIP2) (Dickens et al., 1997; Yasuda et al., 1999). JIP proteins are scaffold proteins that bind several components of the JNK signaling cascade including the JNK group of MAPKs, the MAPKK isoform MKK7, and members of the MLK group of MAPKKKs (Whitmarsh et al., 1998). JIP1 binds JNK family members by virtue of an N-terminal JNK-binding domain (JBD), a domain that is similar to the JBD found in c-Jun, a well-known JNK substrate (Dickens et al., 1997). Overexpression of JIP1 causes the cytoplasmic retention of JNK disrupts JNK-substrate interactions and thereby inhibits signal transduction through the JNK pathway (Disckens et al., 1997). Since JIP1 binds all three JNK family members (JNK1, JNK2, JNK3), we reasoned that ectopic expression of JIP1 should be an effective inhibitor of JNK and thus can be used to examine whether JNK inhibition is sufficient to prevent neuronal death.

In order to study the role of JNK in sympathetic neuron apoptosis, we generated recombinant adenoviruses expressing JIP1. FLAG-tagged JIP1 and green fluorescent protein (GFP) were each cloned downstream of separate cytomegalovirus (CMV) promoters in the adenovirus (hereafter referred to as Ad-JIP) (see Fig. 1A). The expression of JIP is evident in the majority of Ad-JIP infected neurons as assessed by immunocytochemistry using the FLAG epitope-specific M2 monoclonal antibody (Fig. 1B). In addition, GFP fluorescence is co-localized in the vast majority of sympathetic neurons displaying FLAG immunofluorescence (Fig. 1B).

When sympathetic neurons are deprived of NGF, JNKs are activated, and c-Jun N-terminal phosphorylation increases (Virdee et al., 1997; Eilers et al., 1998). JNK regulates c-Jun via binding to the N-terminal transactivation domain of c-Jun and phosphorylate serines 63 and 73 (Ip and Davis, 1998). Phosphorylation of these sites increases the ability of c-jun to activate the transcription of target genes, including the *c-Jun* gene itself (Angel et al., 1988, Ip and Davis, 1998). To determine if JIP1 overexpression could inhibit JNK activity in sympathetic neurons as we had

hypothesized, sympathetic neurons were cultured for 4 days in 50 ng/mL NGF and then infected with Ad-JIP or, as controls, Ad-GFP or Ad-lacZ. Two days later, neurons were washed free of NGF, and then treated for 8 hours with or without 20 ng/mL NGF. Western blot analysis with anti-c-Jun revealed that NGF deprivation caused an increase in c-jun protein levels and an apparent shift in molecular weight of c-Jun, indicative of a change in phosphorylation (Fig. 2A). However, infection with Ad-JIP blocked the NGF withdrawal-induced molecular weight shift in c-Jun (Fig 2A). Western blot analysis with antibodies that recognize the phosphorylated, activated state of c-Jun revealed that NGF deprivation induced a substantial increase in c-Jun phosphorylation at the 8 hour timepoint in uninfected neurons (as previously described, Virdee et al., 1997), as well as in neurons infected with control Ad-lacZ. In contrast, infection with Ad-JIP completely abrogated c-Jun phosphorylation in response to NGF deprivation (Fig. 2B). Expression of Ad-JIP, Ad-GFP, and Ad-lacZ in NGF-deprived sympathetic neurons was confirmed using anti-FLAG, anti-GFP, and anti-βgalactosidase antibodies, respectively (Fig 2A,B).

It has been proposed that JIP1 inhibits JNK pathway signal transduction by causing the cytoplasmic retention of JNK and by disrupting JNK substrate interactions (Dickens et al., 1997). Alternatively, since JIP binds to the upstream kinases, MLKs and MKK7 (Whitmarsh et al., 1998), overexpression of JIP1 may sequester JNK signaling module components, thereby inhibiting the phopshorylation of JNK by upstream kinases. To determine whether JIP overexpression inhibits the phosphorylation and activation of JNK, sympathetic neurons were cultured for 4 days in 50 ng/mL NGF and then infected with Ad-JIP, or control Ad-lacZ. Two days later, NGF levels were reduced to insignificant levels using three successive changes of culture medium and neurons were maintained in this medium for 8 hours. Western blot analysis with antibodies directed against the phosphorylated, activated form of JNK revealed a basal phosphorylation state of JNK in sympathetic neurons maintained in neurotrophin. NGF deprivation induced an increase in JNK phosphorylation within 8 hours in uninfected sympathetic neurons as well as neurons that had been infected with control Ad-lacZ (Fig. 2C). However, infection with Ad-JIP inhibited the NGF withdrawal-induced phosphrylation of JNK (Fig. 2C). Thus, these data suggest that JIP overexpression can effectively inhibit JNK pathway

signal transduction, in part by blocking the phosphorylation and subsequent activation of JNK.

JIP overexpression protects sympathetic neurons from NGF deprivation

To investigate whether JIP overexpression can prevent NGF withdrawal-induced apoptosis, sympathetic neurons were established for 3 days in 50 ng/mL NGF and then infected with the appropriate amount of Ad-JIP, or control Ad-lacZ. Two days later, uninfected and infected neurons were washed free of NGF and treated with or without 50 ng/mL NGF. After 48 hours, survival was quantified using MTT, which measures mitochondrial function (Manthorpe et al., 1986). NGF deprivation reduced survival to approximately 25% relative to neurons maintained in 50 ng/mL NGF, regardless of whether they were uninfected or infected with control lacZ virus (Fig. 3A). In contrast, JIP overexpression increased the survival of NGF-deprived neurons to about 60% (100 MOI) relative to neurons maintained in 50 ng/mL NGF (Fig. 3A).

To quantify the extent of apoptosis, a cell death ELISA was used, which measures the amount of nucleosomes produced by apoptotic cells. Sympathetic neurons were grown in 50 ng/ml NGF for 3 days and then infected with Ad-JIP, or control Ad-lacZ virus. Two days later, neurons were washed free of NGF and treated with or without 50 ng/mL NGF for 48 hours. At 48 hours after NGF withdrawal, both uninfected neurons and neurons infected with control lacZ virus displayed an increase in apoptosis as compared to sister cultures treated with 50 ng/mL NGF (Fig. 3B). In contrast, sympathetic neurons infected with Ad-JIP exhibited no significant increase in apoptosis following NGF deprivation. As a third measure of cell survival, we examined the extent of apoptosis of cells by TUNEL staining (Fig. 3C). By 24 hours following NGF deprivation, the majority of Ad-GFP infected sympathetic neurons were also TUNEL-positive. In contrast, there was a marked reduction in TUNEL-labeling of GFP-positive, Ad-JIP-infected neurons following NGF withdrawal (Fig. 3C). Taken together, these findings indicate JNK activity is essential for NGF withdrawal-induced sympathetic neuron death.

Developmental Profile of JNK phosphorylation in whole SCG in vivo

To determine whether there is an *in vivo* requirement for the JNK pathway in neuronal apoptosis, we first examined the phosphorylation of JNK in the SCG during the period of sympathetic naturally occurring cell death. SCG ganglia were rapidly dissected from postnatal day 2 (P2), P8, P14, and adult (AD; 4 weeks), and were subjected to detergent extraction; the extracts were separated on SDS-PAGE gels and immunoblotted for phospho-JNK and cleaved caspase-3 (Fig. 4). JNK phosphorylation was maximal just after birth, but starting at P8, the level of JNK phosphorylation began decreasing and was at its lowest in adulthood (Fig. 4). Because the amounts of extracts were adjusted so that similar amounts of JNK was analyzed at all ages (Fig. 4), this phosphorylation represents an increase in the degree of JNK phosphorylation and not simply an increase in the total amount of JNK protein. The higher level of JNK phosphorylation in early postnatal SCG is correlated with a greater extent of caspase-3 cleavage (Fig. 4), coinciding with the peak of naturally occurring cell death for sympathetic neurons. Thus, in SCG, the amount of phosphorylated, and presumably activated, JNK was maximal during the period of developmental sympathetic neuron death *in vivo*, suggesting that JNK is involved in the development death of these neurons.

JNK pathway is activated in injured Facial Motoneurons after neonatal axotomy

The data presented here indicate that JNK is essential for NGF withdrawal-induced death of sympathetic neurons. We have previously reported several lines of evidence to support the notion that p53 lies downstream of JNK in sympathetic neuron apoptosis, i) activation of JNK leads to p53 stabilization in sympathetic neurons, ii) preventing the rise in p53 levels protects sympathetic neurons from NGF-deprivation induced death, and iii) the rate of developmental sympathetic neuron death is decreased in p53-/- animals (Aloyz et al., 1998; Lee et al., 2004). Taken together, these finding suggest an essential JNK-p53 apoptotic pathway functions in mediating trophic factor withdrawal-induced cell death of sympathetic neurons. We therefore sought to determine whether these findings would extend to other neuronal cell death paradigms. We have used the paradigm of facial nerve axotomy in which the motoneurons in the facial nucleus are disconnected from the trophic support provided by the facial muscles. Previous studies in rodents have shown

that injury to the facial nerve within the first week of life leads to rapid and extensive apoptotic death of facial motoneurons; most (>80%) of the neuronal loss has occurred by 7 days post-axotomy. In order to determine the extent of JNK activation in injured motoneurons, the facial nerve was unilaterally transected in one-day old (P1) rat pups, and 3 days later, the brainstem was processed for immunohistochemistry with antibodies specific for the phosphorylated, activated form of JNK (pJNK). Figure 5 shows a marked increase in pJNK immunoreactivity in injured facial motoneurons as compared to contralateral, uninjured facial motoneurons (Fig. 5B,D vs Fig. 5A,C). This increase in immunoreactivity was noticeable within the nuclei of injured motoneurons, consistent with previous reports showing a translocation of JNK upon activation (Dickens et al., 1997). Similarly, immunohistochemistry using an antibody that specifically recognizes c-Jun that is phosphorylated on serine 73 (ser 73) revealed an increase in c-Jun phosphorylation in injured facial motoneurons three days following neonatal axotomy (Fig. 6B,D). Although there was a basal level of phospho-c-Jun-like immunoreactivity in uninjured facial motoneurons (Fig. 6A,C), there was a clear increase in immunoreactivity specifically within the nuclei of injured facial motoneurons (Fig. 6B,D). Taken together, these findings demonstrate that the JNK signaling pathway is activated in axotomized facial neurons, and suggests that JNK may be required for motoneuron apoptosis following peripheral nerve injury *in vivo*.

p53 is required for Facial Motoneuron death following neonatal axotomy

We next examined whether the p53 tumor suppressor protein is necessary for neuronal cell death following peripheral nerve injury. To accomplish this, we unilaterally transected the facial nerve in neonatal p53+/+, p53+/-, and p53-/- mice. When performed on control p53+/+ P1 mice, this procedure results in the loss of ~75% of motoneurons by the seventh postoperative day. In striking contrast, in p53-/- mice, 80% of the facial motoneurons survived nerve transection (Fig. 7). Interestingly, p53+/- mice displayed an intermediate phenotype, with approximately 55% of neurons surviving neonatal nerve transection, at the timepoints assessed (Fig. 7). These results demonstrate that p53 is a critical component of the apoptotic cascade that is required for the death of facial motoneurons following neonatal peripheral nerve injury.

DISCUSSION

In the present study, we present data indicating that a JNK-p53 pathway constitutes a key component of apoptotic signaling in developing and injured neurons in the peripheral nervous system. More specifically, our data support three major conclusions. First, we provide evidence that direct inhibition of JNK activity via ectopic expression of JIP-1 rescues developing sympathetic neurons from NGF withdrawal-induced death. Second, we show that JNK may play a similar role in nerve-injury induced neuronal cell death since the JNK pathway is robustly activated facial motoneurons following neonatal axotomy. Third, we demonstrate that the tumor suppressor protein p53 is required *in vivo* for neuronal cell death of facial motoneurons after axonal injury in neonates. Thus, a JNK-p53 pathway is an essential component of apoptotic signaling in peripheral neurons both during development and following injury.

Developing sympathetic neurons are perhaps the best-studied primary neuron culture model of trophic factor withdrawal-induced apoptosis. It has been previously hypothesized that the activation of JNK is fundamental to the process of apoptotic cell death of sympathetic neurons induced by NGF withdrawal (Eilers et al., 1994; Ham et al., 1995). A number of studies have attempted to confirm the role of JNK in mediating apoptotic cell death. For instance, activation of JNKs by expression of upstream kinases leads to apoptotic cell death (Aloyz et al., 1998; Mota et al., 2001; Xu et al., 2001), and preventing the phosphorylation of JNK using a pharmacological inhibitor of MLKs protects cells NGF deprivation (Maroney et al., 1999). However, JNK activity has not been inhibited directly in an NGF deprivation model. Although each of the three *Jnk* genes have been knocked out by homolgous recombination, studies with *Jnk*-/- mice have thus far not provided evidence that JNKs play a role in neurotrophin-regulated developmental neuron death (Yang et al., 1997; Kuan et al., 1999). It is not surprising that no neuronal phenotype was reported in JNK single knockouts, since developing neurons express more than one JNK isoform and compensation likely occurs. In the case of *Jnk1*-/-*Jnk2*-/- double knockout mice, the animals die between embryonic days 11 and 12, well before the period of developmental cell death for sympathetic neurons (Kuan et al., 1999).

In the present study, we sought to directly inhibit JNK activity in sympathetic neurons *in vitro*, using ectopic expression of JIP1, a scaffold protein that has been found

to inhibit all of the JNK isoforms (JNK1, JNK2, and JNK3) (Dickens et al., 1997). We show here that ectopic expression of JIP1 is sufficient to prevent apoptotic death of NGF-deprived sympathetic neurons. The anti-apoptotic effect of JIP1 overexpression was correlated with its ability to not only prevent stress-induced phosphorylation of JNK, but also block the phosphorylation of downstream targets, such as c-Jun, demonstrating its effectiveness at directly inhibiting JNK signaling. At the time that we finished these experiments, two reports were published that are in agreement with our results. JIP1 interacts with JNK via the N-terminal JNK binding domain (JBD) that is homologous to the JBD found in the JNK substrate, c-Jun. Reasoning that overexpression of the JBD should inhibit JNK's ability to phosphorylate c-Jun, both Harding et al. (2001) and Eilers et al. (2001) showed that JBD expression alone was sufficient protect sympathetic neurons from NGF withdrawal-induced death. Since JNK may have other pro-apoptotic actions that do not require the ability to bind a JBD, we reasoned that overexpression of the whole JIP1 protein would have a maximal effect on inhibiting JNK activity. Our result that expression of whole JIP1 prevented both the phosphorylation of JNK and the subsequent phosphorylation of downstream substrates supports this notion. In the present study, we found that overexpression of JIP rescues ~60% of neurons as assessed by a mitochondrial viability assay, whereas the survival effect of JIP overexpression was more dramatic using survival assays that measure various nuclear events [ie., nucleosome cleavage (cell death ELISA) and DNA double strand breaks (TUNEL)]. One reason for this discrepancy may be that although JIP overexpression rescues the neuron from cell death, it may not rescue neurite degeneration that occurs following NGF withdrawal in sympathetic neurons. This is reminiscent of the observation that Bax-/- neurons survive NGF deprivation, but with reduced elaboration of neurites (Deckwerth et al., 1996).

We also show that JNK is phosphorylated, and presumably activated, in the SCG in vivo, at a timepoint coinciding with the peak of sympathetic naturally occurring neuron death. Although this finding is consistent with the hypothesis that JNK is required for developmental death of sympathetic neurons, it remains unclear which JNK family member (JNK1, JNK2, or JNK3) is responsible for developmental death of this neuronal population. Future experiments analyzing mice with compound mutations in the *Jnk* genes will likely resolve this question. Our data also show that cell death induced by

neonatal axotomy of motoneurons is also associated with phosphorylation of JNK and one of its downstream targets, c-Jun. Consistent with our results, a recent study has now shown that *Jnk2*, *Jnk3*, and *Jnk2/3* double deficient mice exhibit reduced cell death of facial neurons after neonatal axotomy (Keramaris et al., 2004).

How does JNK activation lead to cell death of trophic factor deprived neurons? Previous studies have indicated that the NGF withdrawal-induced cell death program likely involves the up-regulation of proapoptotic genes because this apoptotic program is transcription and translation dependent (Martin et al., 1988). The requirement for transcription has been attributed to the activation of c-Jun, a component of the AP-1 transcription factor and the stabilization of the tumor suppressor p53. For instance, transactivation of c-Jun requires the docking of JNK to an N-terminal sequence followed by the phosphorylation on serines 63 and 73 (Mechta-Grigoriou et al., 2001). Overexpression of c-Jun (Ham et al., 1995) can induce neuronal cell death, whereas ablating c-Jun function in sympathetic neurons protects these cells from trophic factor withdrawal induced death (Estus et al., 1994; Ham et al., 1995; Whitfield et al., 2001). Unfortunately, analysis of the role of c-Jun in sympathetic developmental death *in vivo* has not been possible due to the early embryonic lethality of *c-jun-/-* mice (Hilberg et al., 1993; Johnson et al., 1993). One relevant target gene for the JNK pathway and the AP-1 transcriptional response in trophic-deprived neurons may be BIM, a pro-apoptotic BH3-only member of the Bcl-2 family (Putcha et al., 2001; Whitfield et al., 2001; Putcha et al., 2003). Although we show in this study that phosphorylation of c-Jun occurs in injured motoneurons, it remains unclear whether JNK-mediated neuronal death necessarily occurs through c-Jun or whether other death signals may also be of central importance. The significance of this question is also supported by observations that c-Jun activation can occur without concomitant neuronal death. This is most clear in cases of neuronal axotomy in adult animals, where neuronal loss does not occur but prolonged c-Jun activation has been observed (Herdegen et al., 1998) and it has been suggested that c-Jun may in fact participate in regenerative processes (Herdegen et al., 2001; Raivich et al., 2004).

In addition to c-Jun, JNK are also known to regulate other transcription factors, including p53. JNK has also been shown to bind, phosphorylate, and stabilize the cellular

levels of the tumor suppressor protein p53 (Fuchs et al., 1998; Buschmann et al., 2001). The proapoptotic function of p53 appears to be dependent on its ability to modulate transcription, since p53 mutants lacking the transactivation domain are incapable of inducing apoptosis (Cregan et al., 2004). Similar to c-Jun, overexpression of p53 is sufficient to induce cell death of sympathetic neurons even in the presence of NGF (Slack et al., 1996). In contrast, expression of the viral p53 inhibitor (E1B55K) protects cultured sympathetic neurons from cell death induced either by NGF withdrawal or MEKK1-induced activation of JNK (Aloyz et al., 1998). We have previously shown that naturally occurring cell death of sympathetic neurons is inhibited in *p53*+/- and *p53*-/- mice (Aloyz et al., 1998). In the current study, cell death induced by neonatal axotomy of motoneurons was nearly completely prevented, at least for seven days, by the absence of p53. This latter finding importantly generalizes our result, as it constitutes a second neuronal cell death paradigm in which apoptosis is initiated after loss of target-derived trophic support. Moreover, *p53*+/- mice also demonstrated slightly enhanced numbers of motoneurons following neonatal axotomy when compared with wild-type mice (Fig. 7). This gene-dosage effect may indicate that the level of cellular p53 protein that accumulates in neurons in response to apoptotic challenges may ultimately determine survival and death decisions.

What are the molecular mechanisms responsible for the activation of the JNK-p53 apoptotic signal in axotomized motoneuron cell death? There is accumulating evidence that activation of the p75 neurotrophin receptor (p75NTR) induces apoptosis in a variety of neural cell types, both during development and after insult (Frade and Barde, 1998; Bamji et al., 1998; Roux et al., 1999; Syroid et al., 2000; Beattie et al., 2002). Moreover, ligand-mediated activation of p75NTR in numerous cell types leads to activation of both JNK (Cassacia-Bonnefil et al., 1996; Yoon et al., 1998; Harrington et al., 2002; Bamji et al., 1998) and p53 (Aloyz et al., 1998; Pozniak et al., 2000). That p75NTR plays a role in motoneuron cell death was supported by the finding that transgenic overexpression of the intracellular domain of p75NTR enhanced lesion-induced cell death of facial motoneurons in adult mice (Majdan et al. 1997). For facial motoneurons, p75NTR expression is upregulated following axotomy, and when the expression of this receptor was abolished in adult p75NTR–/– mice, survival and regeneration of axotomized

motoneurons was improved in comparison to control animals (Ferri et al. 1998). Moreover, application of NGF to axotomized facial motoneurons significantly increased the rate of cell death (Sendtner et al. 1992; Wiese et al. 1999). These results are compatible with the idea that p75NTR is a cell death receptor in developing motoneurons.

Similar to the findings in NGF-deprived sympathetic neurons, the apoptotic effector proteins downstream of JNK-p53 in axotomized facial motoneurons include BAX, a pro-apoptotic member of the Bcl-2 family, and caspases. Specifically, apoptosis of neonatal facial motoneurons after axotomy is prevented in Bax-/- mice (Deckwerth et al., 1996). Evidence for the involvement of caspases is supported by the finding that the rate of death of axtomoized facial motoneurons is significantly delayed in caspase-3 deficient mice (Vanderluit et al., 2000). Taken together, results from this and other studies support the notion that the apoptotic actions JNK-p53 are essential for neuronal cell death both during development and following injury.

Figure 1: ***Generation of Ad-JIP and its expression in sympathetic neurons***
(A) Structure of Ad-JIP adenoviral construct in which *Jip1* gene was cloned downstream of a cytomegalovirus (CMV) promoter and upstream of a second CMV promoter driving expression of green fluorescent protein (GFP). Also shown are control virus constructs that use CMV promoters to drive GFP or □-galactosidase (lacZ). (B) Photomicrographs of cultured sympathetic neurons that have been infected with Ad-JIP. These neurons show GFP fluorescence (top panel) and are positive for Flag-immunostaining (bottom panel). (C) Western blot analysis of sympathetic neurons infected with Ad-JIP, Ad-GFP, and Ad-lacZ. Note the expression of flag-JIP, GFP, and □-galactosidase in infected neurons.

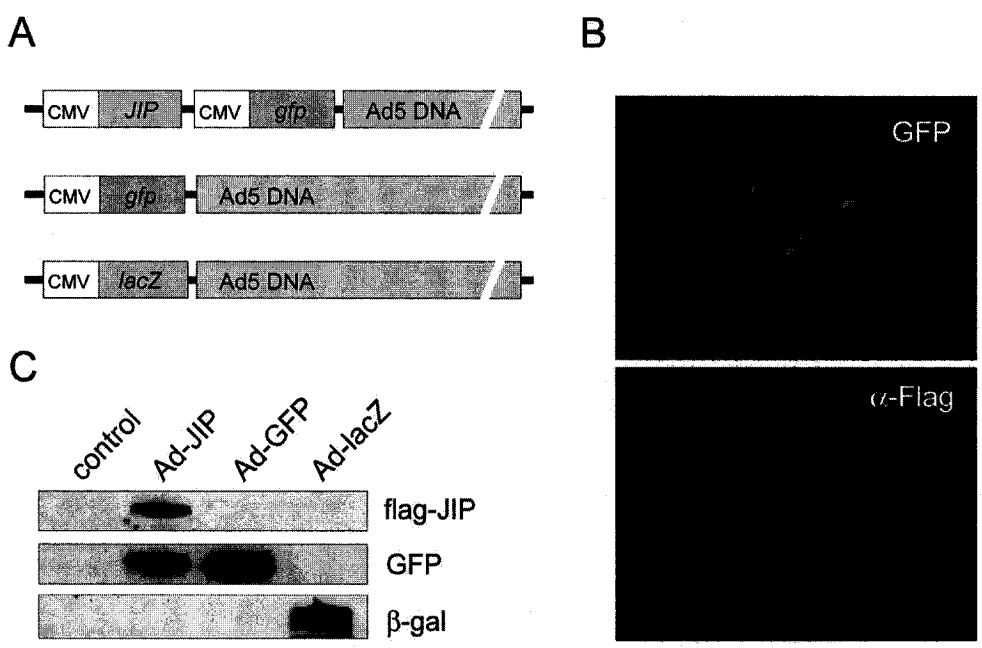

A
CMV
JIP
CMV
gfp
Ad5 DNA
CMV
gfp
Ad5 DNA
CMV
lacZ
Ad5 DNA
B
GFP
α-Flag
C
control
Ad-JIP
Ad-GFP
Ad-lacZ
flag-JIP
GFP
β-gal

Figure 2: ***Overexpression of JIP inhibits the activity of JNK in sympathetic neurons.***

(A-C) SCG neurons were infected with Ad-JIP, Ad-lacZ, or left uninfected. After 48 hours, neurons were maintained in medium containing NGF or were washed free of NGF for 8 hours. Proteins were extracted and analyzed by Western blotting using antibodies specific for c-Jun (A), for phospho-c-jun (p-c-Jun) (B) or for phospho-JNK (p-JNK) (C). Blots were reprobed for the ERKs to demonstrate equal loading, and adenoviral gene transfer was confirmed by reprobing with antibodies specific for Flag-JIP (M2 monoclonal), β-galactosidase, and for GFP.

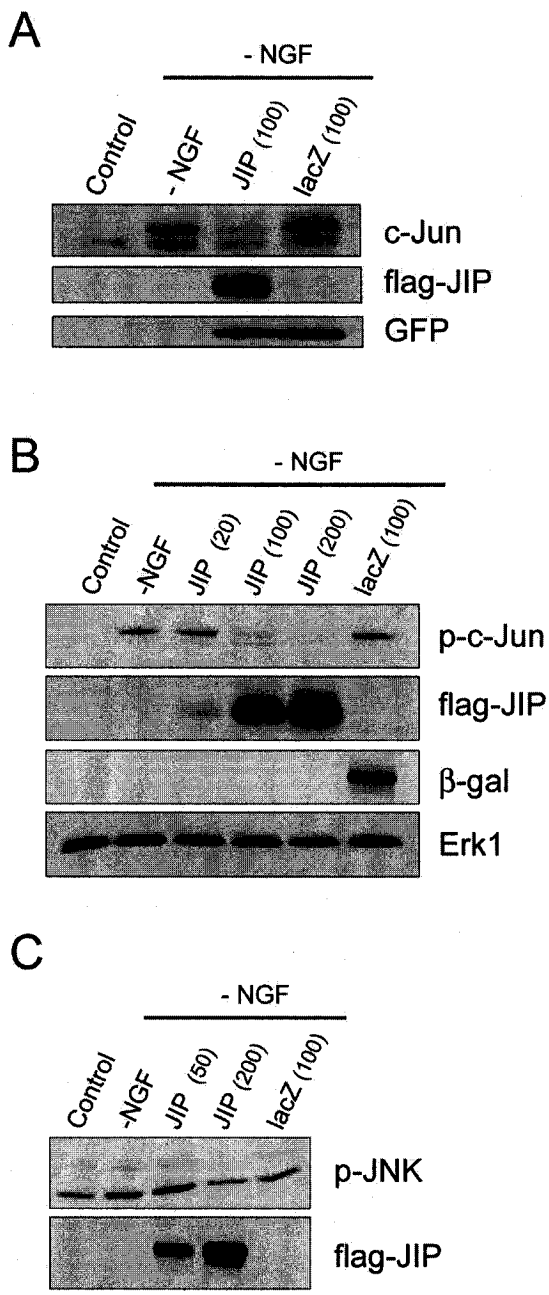

A
- NGF
Control
- NGF
JIP (100)
lacZ (100)
c-Jun
flag-JIP
GFP
B
- NGF
Control
-NGF
JIP (20)
JIP (100)
JIP (200)
lacZ (100)
p-c-Jun
flag-JIP
β-gal
Erk1
C
- NGF
Control
-NGF
JIP (50)
JIP (200)
lacZ (100)
p-JNK
flag-JIP

Figure 3: ***Overexpression of JIP protects sympathetic neurons from NGF deprivation.***

(A-B) Sympathetic neurons were infected with varying amounts of Ad-JIP or the control Ad-lacZ adenovirus, and left for 2 days, after which they were either maintained in the presence or deprived of NGF for 48 hours before being assessed for survival. (A) MTT assay; survival is expressed as a percentage relative to neurons treated with 50 ng/mL NGF. (B) Cell death ELISA; survival is expressed as a percentage of apoptotic neurons relative to neurons treated with 50 ng/mL NGF. (C) Sympathetic neurons were infected with control AD-GFP or Ad-JIP adenovirus, and left for 2 days, after which they were either maintained in the presence or deprived of NGF for 48 hours. Cells were fixed and stained for TUNEL. Note that AD-JIP-infected neurons are not TUNEL-positive following NGF withdrawal.

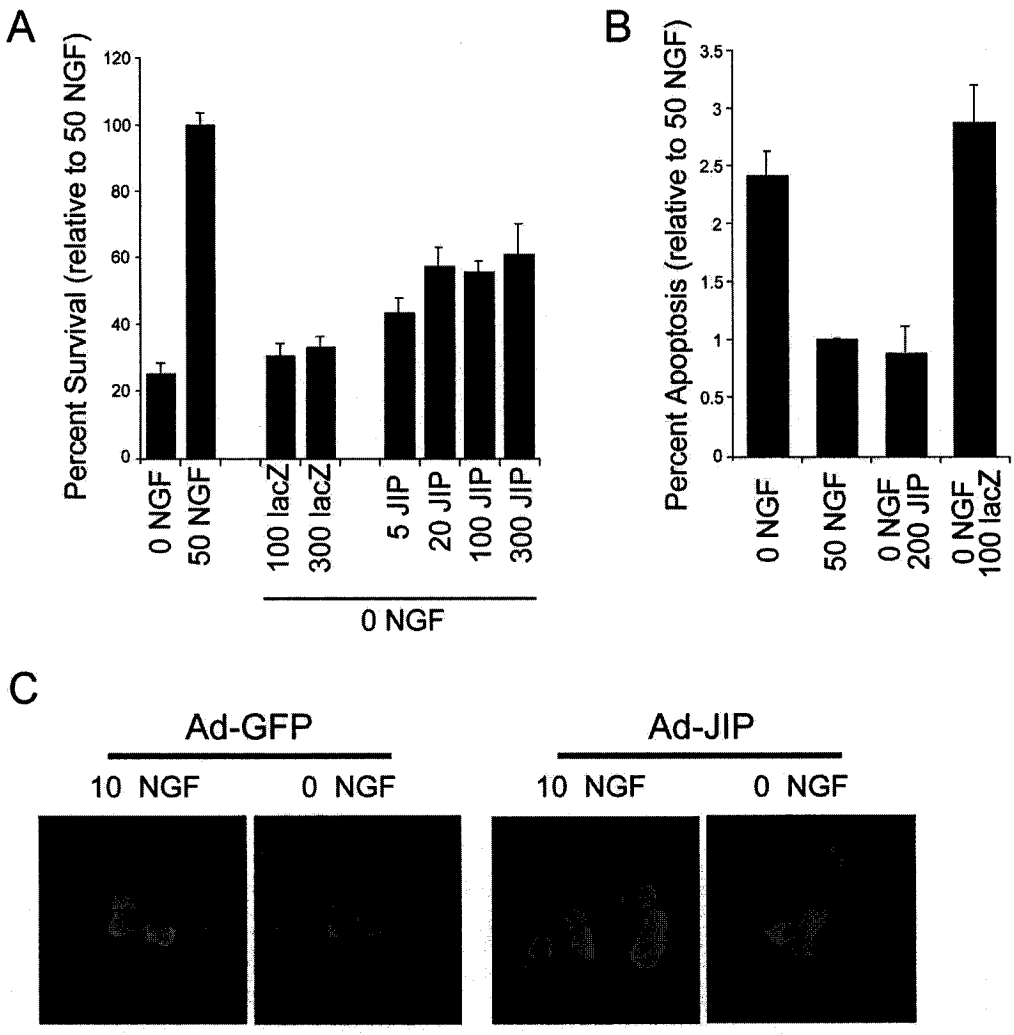

A
Percent Survival (relative to 50 NGF)
120
100
80
60
40
20
0
0 NGF
50 NGF
100 lacZ
300 lacZ
5 JIP
20 JIP
100 JIP
300 JIP
0 NGF
B
Percent Apoptosis (relative to 50 NGF)
3.5
3
2.5
2
1.5
1
0.5
0
0 NGF
50 NGF
0 NGF 200 JIP
0 NGF 100 lacZ
C
Ad-GFP
Ad-JIP
10 NGF
0 NGF
10 NGF
0 NGF

Figure 4: ***Developmental profile of JNK phosphorylation in SCG in vivo***
SCGs were rapidly dissected from P2, P8, P14, and adult rats and detergent extracts produced. Protein lysates were analyzed by phosphoJNK (p-JNK) immunoblotting (top panel). The blots were then stripped and reprobed with JNK antibodies (middle panel) and antibodies that recognize the cleaved form of caspase-3 (bottom panel). Similar results were obtained in 3 separate experiments. Note that the maximal phosphorylation of JNK and cleavage of caspase-3 occurs just after birth in SCG in vivo.

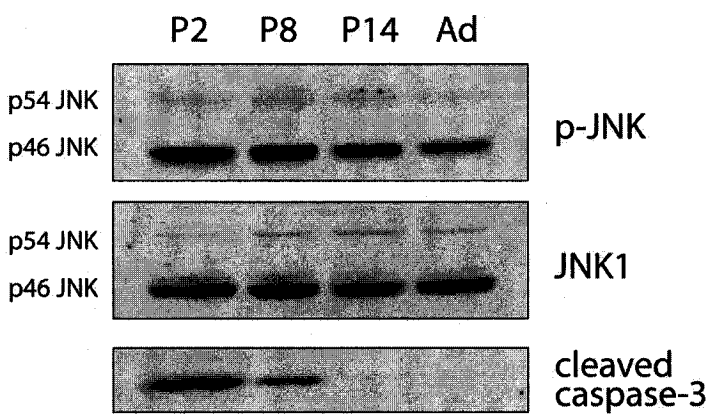

P2
P8
P14
Ad
p54 JNK
p46 JNK
p-JNK
p54 JNK
p46 JNK
JNK1
cleaved
caspase-3

Figure 5: ***JNK is phosphorylated in Facial motoneurons following neonatal axotomy***

Coronal sections through the hindbrain, three days following unilateral facial nerve axotomy performed on P2, stained immunohistochemically with antibodies specific for the phosphorylated form of JNK (pJNK). While there is little immunoreactivity for pJNK in the uninjured Facial nucleus (A), there is a dramatic increase in pJNK immunoreactivity specifically within the nuclei of injured Facial motoneurons (B). (C and D) High power magnification of images shown in A and B, respectively.

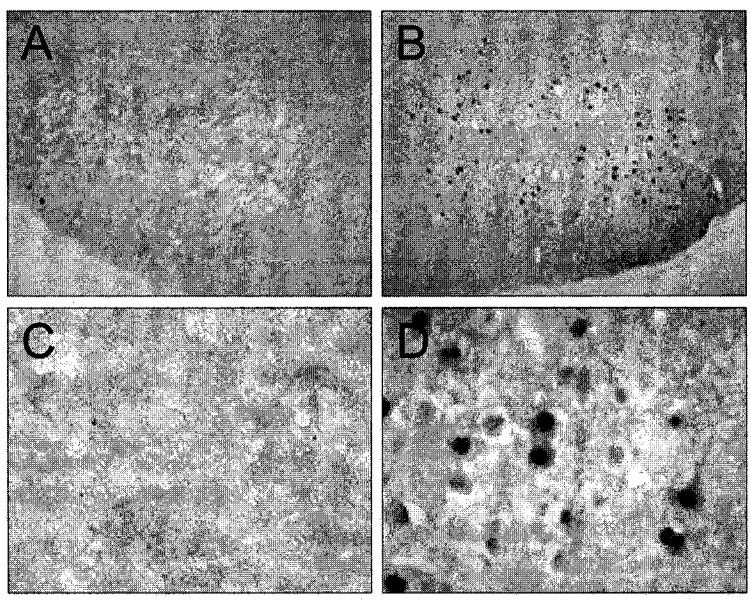

A
B
C
D

Figure 6: c-Jun *is phosphorylated in Facial motoneurons following neonatal axotomy*

Coronal sections through the hindbrain, three days following unilateral facial nerve axotomy performed on P2, stained immunohistochemically with antibodies specific for the phosphorylated form of c-Jun (ser73; p-c-Jun). Whereas there is low levels of immunoreactivity for p-c-Jun in the uninjured Facial nucleus (A,C), there is a dramatic increase in p-c-Jun immunoreactivity specifically within the nuclei of injured Facial motoneurons (B,D). (C and D) High power magnification of images shown in A and B, respectively.

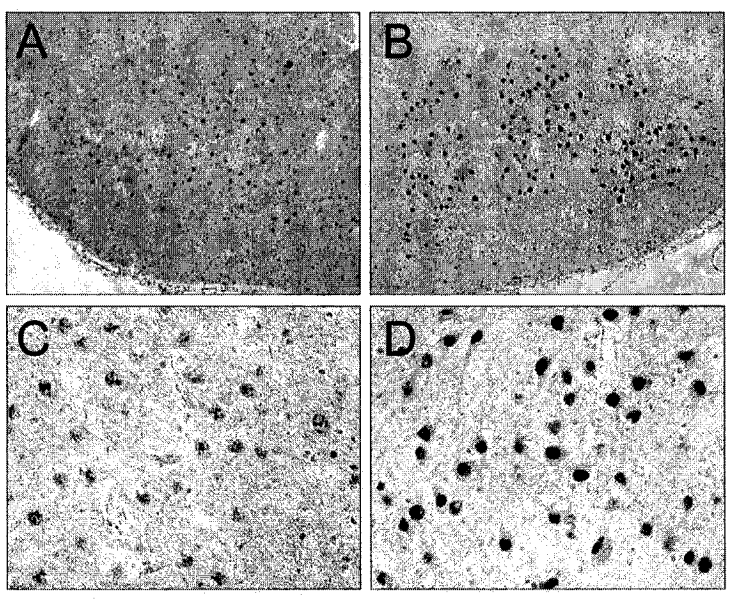
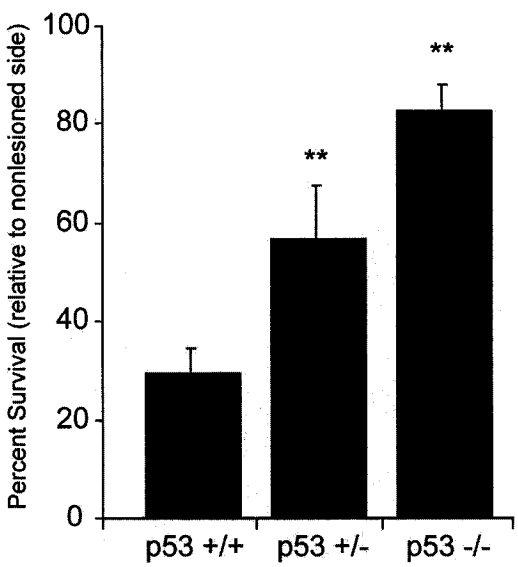

A
B
C
D

Figure 7: ***p53 is required for Facial motoneuron death following neonatal axotomy***

Quantitation of the number of facial motoneurons after a facial nerve transection in *p53*+/+, *p53*+/-, and *p53*-/- mice. Motor neuron survival is presented as mean ± SD of the percentage of viable neurons on the lesioned side relative to the nonlesioned side. The means for *p53*+/- and *p53*-/- animals were compared with the mean of *p53*+/+ mice using the Student's *t* test (** $p < 0.001$).


Percent Survival (relative to nonlesioned side)
100
80
60
40
20
0
**
**
p53 +/+
p53 +/-
p53 -/-

CHAPTER 4

THE INVULNERABILITY OF ADULT NEURONS: A CRITICAL ROLE FOR P73

Gregory S. Walsh,[1,2]* Nina Orike,[2]*‡ David R. Kaplan,[1,2,3] and Freda D. Miller[1,2,3]

[1]Developmental Biology and Cancer Research, Hospital for Sick Children, Departments of Medical Genetics and Physiology, University of Toronto

[2]Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University

[3]Corresponding authors.

ABSTRACT

Here we have investigated the intracellular mechanisms that underlie the relative invulnerability of adult versus developing DRG sensory neurons. In culture, adult neurons were resistant to stimuli that caused apoptosis of their neonatal counterparts. In both adult and neonatal neurons, death stimuli induced the apoptotic JNK pathway, but JNK activation only caused death of neonatal neurons, indicating that adult neurons have a downstream block to apoptosis. Expression of the dominant-inhibitory p53 family member, ΔNp73, rescued JNK-induced apoptosis of neonatal neurons, suggesting that it might participate in the downstream apoptotic block in adult neurons. To test this possibility, we examined adult DRG neurons cultured from p73+/- mice. Adult p73+/- DRG neurons were more vulnerable to apoptotic stimuli than their p73+/+ counterparts, and that invulnerability could be restored to the p73+/- neurons by increased expression of ΔNp73. Moreover, although DRG neuron development was normal in p73+/- animals in vivo, axotomy caused death of adult p73+/- but not p73+/+ DRG neurons. Thus, one way adult neurons become invulnerable is to enhance endogenous survival pathways, and one component of these survival pathways is p73.

INTRODUCTION

A fundamental question is how developing neurons, which are programmed to undergo apoptosis unless they establish the appropriate connectivity, become resistant to apoptotic stimuli as they mature, a transition that ensures their survival for the remainder of an animal's lifetime. This transition is perhaps best-characterized in the peripheral nervous system. During the developmental cell death period, peripheral neurons are absolutely dependent on trophic factors produced by their targets, and those developing neurons that fail to compete successfully for sufficient target support die by apoptosis. Moreover, any insult that interrupts the supply of target-derived growth factors causes apoptosis of newly-connected developing neurons. For example, axotomy or target removal leads to rapid and extensive cell death in both central and peripheral neurons (for review, see Snider et al., 1992), and exogenous replacement of target-derived growth factors rescues these axotomized neurons (Hendry and Campbell, 1976; Yip et al., 1984). This contrasts to the situation in adulthood, where peripheral neurons no longer depend upon target-derived growth factors for survival (Angeletti et al., 1971; Bjerre et al., 1975; Goedert et al., 1978; Otten et al., 1979) and axotomy results only in delayed and restricted neuronal loss (Tandrup et al., 2000; Ma et al., 2001). The mechanisms that underlie the relative invulnerability of adult neurons in vivo are not well-understood.

Culture studies have confirmed that the enhanced invulnerability of adult peripheral neurons is a cell-intrinsic phenomenon. For example, acutely isolated adult sympathetic or sensory neurons do not require exogenous neurotrophins for survival in culture (Lazarus et al., 1976; Chun and Patterson, 1977; Lindsay, 1988), a finding that might be partially explained by endogenous neurotrophin synthesis in older neurons (Acheson et al., 1995). However, these older neurons are also less sensitive to apoptosis induced by ionizing radiation (Tong et al., 1997), and removal of exogenous neurotrophins activates the same initial apoptotic signaling events in neonatal sympathetic neurons as in neurons that have been "aged" three weeks in culture (Easton et al., 1997; Vogelbaum et al., 1998), suggesting the existence of additional cell-intrinsic mechanisms that confer apoptotic resistance on the older neurons.

What might these cell-intrinsic mechanisms be? Potential differences might include an impairment in apoptotic signaling pathways and/or an enhancement in intrinsic

neuronal survival pathways that might "block" apoptosis. Potential insights into the specifics of such pathways derive from extensive work on naturally-occurring cell death in NGF-dependent sympathetic neurons. During development, the ultimate survival of any given sympathetic neuron is a function of survival signals deriving from the tyrosine receptor kinase A (TrkA)/NGF receptor and apoptotic signals deriving from the p75 panneurotrophin receptor (Bamji et al., 1998; Majdan et al., 2001; reviewed in Miller and Kaplan, 2001). TrkA mediates survival largely via a phosphatidylinositol 3 (PI3)-kinase-Akt-dependent pathway, while activation of a JNK-Bax apoptotic pathway occurs following either p75NTR activation or NGF withdrawal. Interestingly, work with sympathetic neurons that have been "aged" for three weeks in culture and that lose their NGF-dependence show that following NGF withdrawal, one of the major downstream apoptotic events, translocation of Bax to the mitochondria, no longer occurs (Easton et al., 1997; Putcha et al., 2000). These findings suggest that in the mature, resistant neurons, either an essential link between the cell surface and Bax is no longer present and/or that a potent survival signal inhibits the apoptotic pathway at this level.

In this regard, we have previously identified a key apoptotic checkpoint in developing sympathetic neurons that involves the p53 tumor suppressor family. Specifically, we have shown that p53 functions as a proapoptotic molecule during developmental sympathetic neuron death (Aloyz et al., 1998), and that a second family member, ΔNp73, is an essential prosurvival protein during the same developmental window (Pozniak et al., 2000). The p73 gene encodes two classes of protein products; full-length isoforms that are structurally homologous to p53 (TAp73α, TAp73β) and N-terminal truncated isoforms that lack the transactivation domain (ΔNp73α, ΔNp73β) (for review, see Irwin and Kaelin, 2001). In developing sympathetic neurons, NGF potently upregulates the prosurvival ΔNp73 isoform, and ΔNp73 maintains sympathetic neuron survival both in vivo and in culture by p53-dependent and independent mechanisms (Pozniak et al., 2000; Lee et al., 2004).

In the CNS, ΔNp73 variants are also the predominant isoforms, acting as potent prosurvival proteins in cultured cortical neurons exposed to a variety of apoptotic insults (Pozniak et al., 2002). In this regard, we have recently shown that p73-/- animals display a prolonged and steady loss of cortical neurons from the first to the eighth postnatal week

in vivo, suggesting that p73 is important for the maintenance of mature cortical neurons (Pozniak et al., 2002). On the basis of these findings, we hypothesized that ΔNp73 may well provide a key prosurvival mechanism for adult neurons. In this manuscript, we have tested this hypothesis, and show that ΔNp73 is an essential component of a cell-intrinsic pathway that is required for the survival of adult sensory neurons both in culture and in vivo following axonal injury.

MATERIAL AND METHODS

Animals

Mice deficient in p73 have been previously described (Yang et al., 1999; Pozniak et al., 2000; Pozniak et al, 2002). These mice were maintained through heteroygyote breeding pairs in a C3H background, and genotyping was performed as described (Pozniak et al., 2000).

Primary neuronal cultures

Newborn DRGs were removed rapidly, placed in F12 media (Gibco), and digested in 0.125% collagenase in F12 media for 20 minutes followed by 10 minutes in 0.25% trypsin, all at 37°C. For adult (6 week old) animals, DRGs were dissected and digested in 0.125% collagenase in F12 media for 1.5 hours followed by 30 minutes in 0.25% trypsin. Ganglia were washed in F12 media and then gently triturated through a glass-polished pipette and the cell suspension was centrifuged through a 15% BSA solution (Sigma). The cell pellet was resuspended in Ultraculture media containing 2 mM glutamine, 100 U/mL penicillin, 100 ug/mL streptomycin, 50 ng/mL NGF and 5 μM cytosine arabinoside and 20 μM fluorodeoxyuridine. Neurons were maintained for 24 hours and then treated as follows. For NGF deprivation, cultures were rinsed with Ultraculture, followed by the addition of Ultraculture containing goat anti-NGF antibodies (Sigma). For cell survival experiments, cultures were rinsed with Ultraculture, followed by the addition of Ultraculture containing 50 ng/mL NGF alone, or in combination with either 50 μM 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one LY294002, or 10 μM camptothecin. For cultures treated with anti-NGF, LY294002, or camptothecin, the number of phase bright neurons within a defined area was counted on the day of treatment and at 24 hour intervals thereafter. Cells with round, phase-bright bodies and intact neurites were counted as surviving neurons. Survival is expressed as a percentage of the original number of neurons. Approximately 200 neurons were counted for each experiment. Statistical differences between conditions was examined with a Student's t test.

Cultures of sympathetic neurons were prepared from newborn p73+/+ and p73+/- mice. SCG ganglia from individual mice were dissected and triturated as previously

described (Majdan et al., 2001). 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 g/ml streptomycin, 3% FBS (Life Technologies), and 50 ng/ml mouse 2.5 S NGF prepared from mouse salivary gland (Cedarlane Labs, Ltd.). One day after plating, neurons were fed with the same media containing 0.5% cytosine arabinoside (Sigma-Aldrich). For cell survival experiments, cultures were rinsed with Ultraculture, followed by the addition of Ultraculture containing 50 ng/mL NGF alone, or in combination with either 50 μM LY294002, or 10 μM campthothecin. Two days later, cultures were rinsed and fixed in 4% paraformaldehyde and stained with Hoechst. Apoptosis was assessed by counting the number of neurons displaying evidence of condensed apoptotic nuclei. Statistical differences between values were examined with a Student's t test.

Adenovirus infections

For JNK activation experiments, semi-confluent human embryonic kidney (HEK) 293A were rinsed with DMEM and infected with 20 multiplicity of infection (MOI) of adenovirus expressing wildtype mixed-lineage kinase 3 (MLK3)/GFP (described in Roux et al., 2002) or GFP alone. Alternatively, DRG cultures were rinsed with Ultraculture 24 hours after plating, followed by the addition of Ultraculture containing 50 ng/mL NGF and 100 MOI of adenovirus expressing wildtype MLK3/GFP or GFP alone. 24 hours later, cells lysates were prepared and the level of JNK phophorylation was assessed by immunoblotting (see below). For survival experiments with DRG neurons, cultures were rinsed with Ultraculture 24 hours after plating, followed by the addition of Ultraculture containing 50 ng/mL NGF and 100 MOI of adenovirus expressing wildtype MLK3/GFP or GFP alone. Two days later, cultures were switched to media containing 50 ng/mL NGF alone for a further three days. Cultures were then rinsed and fixed in 4% paraformaldehyde and stained with Hoechst. Apoptosis was assessed by counting 200 infected neurons and determining the number of infected neurons displaying evidence of condensed apoptotic nuclei. Statistical differences between values were examined with a Student's t test. For the ΔNp73 rescue experiments, cultures of adult p73+/- neurons were rinsed with Ultraculture 24 hours after plating, followed by the addition of

Ultraculture containing 50 ng/ml NGF and 100 MOI of adenovirus expressing ΔNp73β/GFP (described in Pozniak et al., 2000) or GFP alone. Two days later, cultures were switched to media either lacking NGF or containing NGF with LY294002 or camptothecin. Apoptosis was assessed as for the MLK3 experiments. For double-infection experiments with sympathetic neurons, cultures were rinsed with Ultraculture 4 days after plating, followed by the addition of Ultraculture containing 10 ng/ml NGF, 50 mM KCl and 100 MOI of adenovirus expressing wildtype MLK3/GFP either alone or in combination with 100 MOI of ΔNp73β/GFP or 100 MOI of GFP. One day later, cultures were washed free of virus and replaced with Ultraculture media containing 10 ng/mL NGF. Two days later, cultures were then rinsed and fixed in 4% paraformaldehyde and stained with Hoechst, and apoptosis was assessed as described above.

RT-PCR

Whole DRGs were dissected from neonatal and adult (6 week old) wild-type mice and quickly frozen. Total RNA was isolated from tissues using the Trizol reagent and was reverse transcribed (RT) using M-MuLV reverse transcriptase (Fermentas). PCR was performed using site-specific primers. Primer sequences were as follows: p73 core: 5'-TGCCCCGGCTAGCCACCTCATCC-3' and 5'-CCTCGTCCCCGTGGCGTCTCTTCT-3'; TAp73: 5'-GAGCACCTGTGGAGTTCTCTAGAG-3' and 5'-GGTATTGGAAGGGATGACAGGCG-3'; ΔNp73: 5'-GTCGGTGACCCCATGAGACAC-3' and 5'-GGGGCAGCGCTTAACAATGT-3'; p53: 5'-AACCGCCGACCTATCCTTACCATC-3' and 5'-AGGCCCCACTTTCTTGACCATTGT-3'; GAPDH: 5'-ACGGCAAGTTCAATGGCACAGTCA-3' and 5'-GCTTTCCAGAGGGGCCATCCACAG-3' . Expected PCR product sizes were as follows: p73 core - 434 bp; TAp73 - 297 bp; ΔNp73 - 429 bp; p53 - 432 bp; GAPDH – 425 bp.

Surgical procedures

Sciatic nerve resections were performed on adult (6 week old) p73 heterozygous mice and their wild type littermates. Adult animals were anesthetized with an intraperitoneal

injection of a ketamine/xylazine mixture (100mg/kg/10mg/kg). The right sciatic nerve was exposed at mid thigh level and a 5mm segment of the nerve was resected. The wound was sutured, and the animals were allowed to recover for one month.

Immunohistochemistry

Mice were terminally anesthetized with sodium pentobarbital and then perfused transcardially with a solution of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The L4 DRGs were removed, post-fixed for 2 hr in the same fixative, and then immersed for three days in 30% phosphate-buffered sucrose. Ganglia were embedded in OCT compound, frozen in isopentane, and cryosectioned at 10 μm onto chrom-alum gelatin–coated slides. Sections were stained with cresyl violet or incubated for 1 hour in 0.1M PBS containing 10% normal goat serum, and 0.25% Triton-X, followed by incubation with primary antibody overnight at 4°C. Primary antibodies were mouse anti-neurofilament 200 (Sigma) and rabbit anti-CGRP (Chemicon). After rinsing in PBS, sections were incubated with either alexa594-conjugated anti-mouse or alexa350-conjugated anti-rabbit secondary antibodies (Molecular Probes) for 2 hours at room temperature. Sections were washed in PBS, mounted, and viewed under a Zeiss fluorescence microscope at 20x magnification. For isolectin B4 (IB4) staining, after the blocking step, sections were incubated for 2 hours with FITC-labeled IB4 lectin (Sigma) in 0.1 M PBS containing 0.25% Triton-X. Sections were then rinsed and coverslipped with geltol (Immunon), and analyzed with a Zeiss Axioplan microscope using 20X objective, and the Northern Eclipse computer-based image analysis software (Empix Inc.).

Electron microscopy

The L4 dorsal (sensory) spinal roots from adult p73 heterozygous mice and their wild-type littermates were examined for the number of nerve fibers in plastic-embedded material. Doral root specimens were fixed in 4% paraformaldehyde and 1% gluteraldehyde in 0.1M phosphate buffer. They were then post-fixed in aqueous 1% OsO4 and dehydrated through a graded series of acetone followed by infiltration and embedding in the Epon-Araldite epoxy resin (Canemco Inc. Quebec, Canada). Semi-thin sections

were cut and stained with Toluidene Blue for light microscopy examination. Specimen blocks with appropriate cross sections were selected for ultrathin sectioning. Sections of about 80 nm thick were cut with a diamond knife and stained with uranyl acetate and lead citrate for transmission electron microscopy (TEM) imaging.

The sections were examined in a JEM-1230 TEM (JEOL USA, Inc.) operating at 80kV. Digital electron micrographs were recorded directly using CCD camera with a computer system (AMT Advantage CCD ORCA camera, AMT Corp.). Images with offset overlapping edges covering the entire area of the dorsal root were systematically recorded. The total number of nerve fibers (both myelinated and unmyelinated) in the L4 dorsal roots of heterozygous and wild-type mice was determined from electron micrographs at 5000×. Statistical differences between wild-type and heterozygous values were examined with a Student's t test.

Western Blot Analysis

HEK293A cells or DRG neurons were rinsed briefly in cold TBS and then lysed in TBS lysis buffer (137 mM NaCl, 20 mM Tris, pH 8.0, 1% vol/vol NP-40, and 10% vol/vol glycerol; Knusel et al., 1994) supplemented with Mini Complete protease inhibitor cocktail (Boehringer Mannheim Corp.) and 1.5 mM sodium vanadate. Lysates were scraped into Eppendorf tubes and rocked for 10 min at 4°C. Samples were then cleared by centrifugation. Protein concentration was determined by the BCA assay (Pierce Chemical Co.) using BSA as a standard. Samples were diluted in sample buffer (Laemmli, 1974) and placed in a 95°C heat block for 5 min. Equal amounts of protein were separated on 10% SDS-PAGE gels and transferred onto nitrocellulose membranes. For all antibodies, blots were blocked in 3% skim milk (Carnation) in TBS overnight at 4°C. Primary antibodies used included anti-JNK1 (Santa Cruz Biotechnology Inc.), anti-phospho-JNK (Promega), anti-MLK3 (Santa Cruz Biotechnology Inc.), anti-GFP (Clonetech). For Western blots, secondary antibodies used were HRP-conjugated anti-mouse (1:10,000) and anti-rabbit (1:10,000) pAbs (Boehringer Mannhiem Corp.). All incubations were performed in 3% skim milk in TBS + 0.1% Tween-20 (Sigma Chemical Co.). For detection, blots were washed with TBS and antibody localization visualized using the ECL chemiluminescence kit (Nycomed Amersham Inc.).

RESULTS

Adult sensory neurons are resistant to stimuli that induce apoptosis of neonatal sensory neurons

To study the intracellular mechanisms that promote the survival of adult neurons, *in vitro* studies have relied largely on embryonic neurons that have been aged in culture (Easton et al., 1997; Vogelbaum et al., 1998; Putcha et al., 2000), despite the possibility that neurons may develop differently in culture than in vivo. To overcome this limitation, we have instead examined the survival requirements of sensory neurons acutely isolated from adult (6 week old) rat DRGs and directly compared them to cultured neurons isolated from neonatal (postnatal day 0) DRGs. In all of these experiments, neurons were isolated from lumbar DRGs and low density cultures were established and maintained in serum-free culture conditions containing few non-neuronal cells. Under these conditions, the adult cultures were comprised of phase bright neurons of varying sizes, consistent with neuronal heterogeneity in the DRG.

Initially, we confirmed the previously-reported finding (Lindsay, 1988; Vogelbaum et al., 1998) that adult DRG neurons no longer require neurotrophins for their survival. Adult or neonatal DRG neurons were cultured in the presence of NGF for one day, and then were switched into media lacking NGF, but with the addition of function-blocking anti-NGF antibodies. Quantitation of surviving, phase-bright neurons two days later revealed that while only 40 ± 9% of neonatal neurons were still alive, the large majority of adult neurons (77 ± 11%) survived the NGF withdrawal (Fig. 1A). We then asked whether this enhanced survival of adult DRG neurons in the absence of NGF reflected a more general resistance of adult neurons to apoptotic stimuli. We first asked whether adult DRG neurons were more resistant to apoptosis caused by inhibition of the PI3-kinase survival pathway; established cultures of neonatal or adult neurons were switched into media containing NGF plus 50 μM of the pharmacological PI3-kinase inhibitor LY294002. Western blot analysis demonstrated the specificity of this concentration of LY294002 to inhibit the phosphorylation of the PI3-kinase target Akt in these neurons (data not shown), as we have previously published for sympathetic neurons (Vaillant et al., 1999). We then quantitated survival; after two days of PI3-kinase

inhibition, only 25 ± 14% of neonatal DRG neurons remained alive, while the majority (62 ± 6%) of adult neurons survived (Fig. 1B). As a second, more robust apoptotic insult, we treated neurons with the DNA damaging agent camptothecin, which causes p53-dependent neuronal apoptosis (Xiang et al., 1998). Established cultures of adult or neonatal DRG neurons were treated with 10 μM camptothecin, and survival was quantitated two days later. This analysis demonstrated that, surprisingly, adult DRG neurons were relatively resistant to even this apoptotic stimulus; after two days, 72 ± 8% of adult neurons survived versus 26 ± 7% of neonatal neurons (Fig. 1C).

Activation of the JNK pathway is sufficient to cause apoptosis of neonatal, but not adult, neurons, and JNK-mediated neonatal neuron apoptosis can be rescued by ΔNp73

A generalized apoptotic resistance in adult neurons could result from an impairment in apoptotic signaling pathways and/or enhanced "adult" survival pathways. To distinguish these two possibilities, we examined one common component of the apoptotic pathways induced by a variety of stimuli, activation of the N-terminal jun-kinase. Specifically, adult or neonatal DRG neurons were established and then were either withdrawn from NGF, or were treated with 50 μM LY294002 or 10 μM camptothecin for 24 hours. Western blot analysis revealed that JNK phosphorylation was increased in neonatal DRG neurons in all of these conditions (Fig. 2A). An increase in JNK phosphorylation was also observed in adult DRG neurons treated in the same way (Fig. 2B), although the magnitude of the increase was smaller. Thus, apoptotic pathways upstream of JNK were intact in both populations, but adult neurons apparently had a downstream block to apoptosis.

To directly test this hypothesis, we exogenously activated JNK and asked whether this was, on its own, sufficient to cause apoptosis of neonatal versus adult neurons. To perform these experiments, we utilized a bicistronic recombinant adenovirus that coexpressed GFP and wild-type MLK3, an upstream kinase that efficiently activates JNK when overexpressed in peripheral neurons (Maroney et al., 1999). Confirmation of the efficacy of this adenovirus was obtained by transducing HEK293A cells or neonatal DRG cultures and assaying for expression of MLK3 and activation of JNK by Western blot

analysis (Fig. 2C). We then infected established neonatal or adult neurons with adenoviruses expressing MLK3/GFP or GFP alone, and scored GFP-positive neurons for apoptotic nuclei as indicated by Hoechst staining. This analysis revealed that GFP alone had no effect on neuronal survival. Overexpression of MLK3 was, however, sufficient to cause the apoptosis of the majority of neonatal, but not adult, neurons (Fig. 2D), suggesting that adult neurons have a relative impairment in the apoptotic pathways downstream of JNK.

One protein that is downstream of JNK in neonatal sympathetic neurons, and that plays an essential prosurvival role is the dominant-inhibitory p53 family member, ΔNp73 (Pozniak et al., 2000; Lee et al., 2004). To ask whether enhanced ΔNp73 levels or function could explain the downstream block to JNK-mediated apoptosis in adult neurons, we overexpressed ΔNp73 in neonatal neurons, and asked whether this could rescue MLK3-mediated apoptosis. To perform this experiment, we turned to neonatal sympathetic neurons, which apoptose in response to JNK pathway activation (Aloyz et al., 1998; Eilers et al., 1998), and which can be reliably double-infected by recombinant adenovirus. Neonatal neurons were infected with recombinant adenoviruses expressing GFP, MLK3 and/or ΔNp73, and neuronal survival was quantitated by assessing nuclear morphology with Hoechst three days following infection. These experiments demonstrated that, as seen with neonatal sensory neurons (Fig. 2D), overexpression of MLK3 in neonatal sympathetic neurons was sufficient to induce apoptosis in the presence of NGF (Fig. 2E). The magnitude of this apoptotic effect was unaffected by coinfection with a control, GFP-expressing adenovirus. In contrast, when neurons were coinfected with adenoviruses expressing MLK3 and ΔNp73, infected sympathetic neurons were largely rescued from apoptosis (Fig. 2E). Thus, enhanced levels of ΔNp73 were sufficient to block neonatal neuron apoptosis in response to JNK pathway activation, suggesting that upregulation of ΔNp73 function would be sufficient to uncouple extracellular apoptotic stimuli from an apoptotic response in adult neurons.

p73 is required for the relative invulnerability of cultured adult DRG neurons

These studies indicated that enhanced ΔNp73 levels and/or function would be sufficient to confer relative invulnerability to apoptotic stimuli on adult neurons. We therefore asked whether ΔNp73 was necessary for the invulnerability of adult sensory neurons. To do this, we initially characterized the expression of full-length (TAp73) versus N-terminal truncated (ΔNp73) isoforms in dorsal root ganglia isolated from adult mice. Reverse transcription-polymerase chain reaction (RT-PCR) analysis with specific primers demonstrated that mRNAs encoding both TAp73 and ΔNp73 isoforms were expressed in adult DRGs as they are in the develping brain (Fig. 3A,B) (Pozniak et al., 2002). A similar analysis demonstrated that these isoforms are also both expressed in the neonatal DRG (Fig. 3A, data not shown). The specificity of this analysis was demonstrated by showing that these RT-PCR products were not present in the p73-/- brain (Fig. 3B).

We next asked whether p73 was required for the relative resistance of adult sensory neurons to death-inducing stimuli. To perform these experiments, we chose to directly compare adult DRG neurons from p73+/+ and p73+/- mice. To ensure that p73 heterozygosity did not developmentally influence sensory neuron repertoire, we characterized 6 week old p73+/+ versus p73+/- L4 DRGs (most p73-/- animals die at P10). Sectioning and Nissl-staining of ganglia from 6 week old p73+/+ and p73+/- mice revealed that they were similar in size and morphology (Fig. 4A). Immunostaining of these sections for NF200, which marks predominantly large diameter myelinated neurons, CGRP, which marks predominantly small diameter peptidergic nociceptors, and the isolectin B4 (IB4), which marks small diameter non-peptidergic nociceptors, demonstrated that the sensory neuron repertoire was also similar (Fig. 4B). Confirmation that the actual number of neurons was similar in p73+/+ versus p73+/- L4 DRGs was obtained by quantitating axons in dorsal root transverse sections, using electron microscopy, a method that provides a reliable index of neuronal number in the L4 DRG (Coggeshall et al., 1997). This analysis revealed that p73+/+ and p73+/- dorsal roots contain 3411 ± 170 (n=3) and 3301 ± 179 (n=3) unmyelinated and 2376 ± 104 (n=3) and 2335 ± 131 (n=3) myelinated axons, respectively, numbers that were statistically similar between the genotypes (Fig. 4C,D).

Having demonstrated that p73 heterozygosity had no impact on the repertoire or number of DRG neurons in vivo, we then asked whether the decreased levels of p73 in p73+/- neurons made them more vulnerable to apoptotic insults in culture. Adult DRG neurons were isolated from p73+/+ and p73+/- mice, established in the presence of NGF for 24 hours, treated with the DNA damaging agent, camptothecin (10μM), and the number of phase-bright neurons counted over the ensuing two days. As seen with adult rat DRG neurons (Fig. 1B), the survival of adult p73+/+ mouse DRG neurons was only slightly diminished (80 ± 7% surviving neurons) by camptothecin at 48 hours (Fig. 5A). In contrast, p73+/- neurons showed a significantly enhanced sensitivity to camptothecin treatment relative to the p73+/+ neurons (Fig. 5A), with only 54 ± 6% surviving at 48 hours. A similar difference was seen when we inhibited the PI3-kinase survival pathway in these two populations of neurons using 50 μM LY294002. At 48 hours, survival was 72 ± 7% versus 40 ± 6% for p73+/+ versus p73+/- neurons, respectively (Fig. 5B). Thus, adult DRG neurons from p73+/- heterozygous mice are more vulnerable to apoptotic stimuli than their wild-type counterparts.

While these data argued that p73 was essential for the relative invulnerability of adult neurons, and that even a small decrease in levels was sufficient to make them more sensitive to apoptotic stimuli, it was still formally possible that the p73+/- neurons differed from their wildtype counterparts in some other parameter. To address this possibility, we performed a rescue experiment; if the increased sensitivity of p73+/- adult neurons to apoptotic stimuli was due to a decrease in levels of ΔNp73, then we should be able to rescue this phenotype by exogenously increasing expression of ΔNp73. To perform this rescue experiment, established cultures of adult p73 +/- DRGs were infected with recombinant adenoviruses expressing ΔNp73/GFP or GFP alone, and one day later, neurons were either withdrawn from NGF, or treated with 10 μM camptothecin or 50 μM LY294002. Quantitation of the number of GFP-positive, phase-bright neurons 48 hours later revealed that the overexpression of ΔNp73, but not GFP, rescued the enhanced susceptibility of p73+/- neurons (Fig. 5C), arguing that ΔNp73 was essential for making adult DRG neurons resistant to apoptotic stimuli, at least in culture.

One other population of neurons that requires ΔNp73 for their survival is neonatal sympathetic neurons (Pozniak et al., 2000; Lee et al., 2004). Moreover, exogenously

increased expression of ΔNp73 is sufficient to rescue sympathetic neurons from apoptosis induced by NGF withdrawal (Pozniak et al., 2000; Lee et al., 2004). We therefore asked whether p73+/- neonatal sympathetic neurons were also more sensitive to apoptotic insults than their wildtype counterparts. Neurons were cultured from neonatal p73+/- versus p73+/+ littermates, these neurons were then exposed to either 10 μM camptothecin or 50 μM LY294002, and apoptosis was quantified 48 hours later by assessing nuclear morphology. As previously shown (Crowder and Freeman, 1998; Park et al., 1998; Vaillant et al., 1999), both of these apoptotic stimuli were sufficient to cause sympathetic neuron apoptosis in the presence of NGF (Fig. 5D). However, unlike adult DRG neurons, apoptosis of p73+/- neurons was not enhanced relative to p73+/+ neurons (Fig. 5D).

p73 is necessary for the survival of adult DRG neurons following peripheral nerve injury in vivo

The anatomical analysis of the L4 DRG presented above demonstrated that the development of sensory neurons is normal in the p73+/- animals, but our culture data implied that adult neurons might be impaired in their survival following injury. To directly test this idea, we resected the sciatic nerve of 6 week old p73+/+ versus p73+/- animals and, one month later, asked whether sensory neuron survival was affected by counting myelinated and unmyelinated axons in electron micrographs of the L4 DRG dorsal roots. This analysis revealed that, as previously reported in rats (Coggeshall et al., 1997; Tandrup et al., 2000), the amount of axonal loss in wild-type mice (p73+/+ littermates) was minimal one month following this type of axonal injury (Fig. 6A,B). The number of small, unmyelinated axons was reduced from 3411 ± 170 to 3061 ± 309 (n=3) (a decrease of ~10%), while the number of large, myelinated axons was unaffected (2376 ± 105, n=3 vs. 2314 ±132, n=3). In contrast, while the number of myelinated axons was unaffected following axotomy of p73+/- mice (2335 ± 130, n=3 vs. 2292 ± 154, n=3), there was a substantive and significant 30% loss of unmyelinated axons in p73+/- animals (2144 ± 209, n=3 versus 3061 ± 309; n=4; p73+/- versus p73+/+, respectively). Thus, a decrease in the levels of p73 is sufficient to cause enhanced neuronal vulnerability both in vivo and in vitro, arguing that this molecule plays a key role in rendering adult neurons relatively invulnerable to injury.

DISCUSSION

In this paper we present data indicating that the relative invulnerability of adult neurons is due to a cell-intrinsic enhancement of neuronal survival pathways, and that a key component of these survival pathways is ΔNp73. More specifically, our data support three major conclusions. First, we provide evidence that adult sensory neurons are much more resistant to a variety of apoptotic stimuli than are their neonatal counterparts, and that this resistance is mediated downstream of JNK. Second, we show that increased expression of ΔNp73 is sufficient to rescue JNK-mediated apoptosis of neonatal neurons, and that the loss of one allele of p73 renders adult sensory neurons more vulnerable to apoptotic stimuli in culture. This enhanced vulnerability is due to decreased ΔNp73 because the invulnerability of adult sensory neurons can be restored by exogenous expression of ΔNp73 in p73+/- neurons. Third, we demonstrate that these findings are relevant in vivo; the loss of even one p73 allele, while having no overt effects on developing sensory neurons, enhanced the death of small unmyelinated DRG neurons following axonal injury in adults. Thus, p73 represents one key mechanism used by neurons to ensure that they survive the lifetime of the animal.

We have previously demonstrated that the ΔNp73 isoforms are highly potent survival proteins, inhibiting the death of cultured sympathetic and cortical neurons as mediated by a variety of stimuli, including NGF withdrawal and DNA damage (Pozniak et al., 2000; Pozniak et al., 2002). Moreover, we previously demonstrated enhanced death of p73-/- postnatal sympathetic and cortical neurons in vivo indicating that this gene was essential for the survival of at least some populations of developing neurons (Pozniak et al., 2000; Pozniak et al., 2002; Lee et al., 2004). However, the first indication that p73 might be important for survival of mature neurons was our finding of ongoing loss of cortical neurons between 3 and 8 weeks postnatally in p73-/- animals. While there are many potential explanations for such a finding, this observation suggested that p73 might play a previously-unsuspected role in determining the maintenance of adult neurons. In that regard, the data documented here demonstrates such a cell-intrinsic role for p73 in peripheral neurons, and our previous in vivo work on the p73-/- cortex suggests that this role may well generalize to CNS neurons.

How does p73 determine the long-term survival of adult neurons? We propose that it is the ΔNp73 isoforms that are important for this function since (i) ΔNp73 isoforms are the only isoforms detectable in the brain at the protein level, (ii) as discussed above, ΔNp73 is a very potent survival protein when overexpressed, whereas overexpression of TAp73 causes neuronal apoptosis (Lo et al., 2003), and (iii) as shown here, ΔNp73 rescues the enhanced vulnerability of p73+/- adult sensory neurons. We also propose that one of the ways that ΔNp73 determines survival of mature neurons is by functionally antagonizing p53. p53 has been shown to play a key role in mediating the apoptosis of mature neurons in vivo in response to a wide variety of stimuli, including excitotoxicity, DNA damage (reviewed in Miller et al., 2000; Morrison and Konoshita, 2000) and, with regard to the current study, axonal injury (Martin and Liu, 2002). Support for this model derives from the fact that ΔNp73 rescues sympathetic neurons from apoptosis induced by p53 overexpression (Pozniak et al., 2000), and from our recent finding that deletion of even one p53 allele is sufficient to partially rescue the enhanced sympathetic neuron apoptosis seen in developing p73-/- animals (Lee et al., 2004). Together, we believe these findings make a compelling argument that p53 is one of the targets of ΔNp73. However, it is also clear that ΔNp73 has additional targets (Lee et al., 2004), and we propose that these additional targets include other full-length, proapoptotic p53 family members such as p63, the third p53 family member (Yang et al., 1998; 1999), which is predominantly expressed in the nervous system as a full-length TAp63 isoform (Jacobs et al., submitted). In that regard, we propose that it is ultimately the balance of full-length, proapoptotic p53/TAp63 family members relative to the prosurvival N-terminal truncated ΔNp73/ΔNp63 isoforms that determines the life versus death of any given neuron.

How does such a p53 family-dependent checkpoint integrate with our current knowledge of adult neuron survival and apoptosis? Although most of what we know about neuronal survival and death pathways derives from studies of developing neurons, studies on peripheral neurons "aged" in culture have provided some insight. In particular, "aged", NGF-independent sympathetic neurons still activate the JNK pathway upon NGF withdrawal, but they do not translocate Bax in the mitochondrion, and Bcl2+/- neurons are just as resistant to NGF withdrawal as Bcl2+/+ neurons, implying that a major block to apoptosis occurs between JNK and Bax (Greenlund et al., 1995; Putcha et al., 2000).

Is there any evidence that the p53 family might act at such an upstream point? While p53 is thought to mediate at least some of its proapoptotic actions at the transcriptional level, for example by enhanced transcription of Bax (Miyashita and Reed, 1995), there is also evidence that it can promote apoptosis directly at the level of the mitochondrion in a cell-free system (Schuler et al., 2000; Mihara et al., 2003). Moreover, we demonstrate here that ΔNp73 can rescue apoptosis induced by JNK pathway activation, and our recent studies in sympathetic neurons demonstrate that ΔNp73 can block the mitochondrial apoptotic transition following NGF withdrawal, potentially by inhibiting full-length p53 family members, and/or by binding directly to JNK itself (Lee et al., 2004). All of these data then, are consistent with the hypothesis that adult neurons have a major block to apoptosis downstream of JNK but upstream of the mitochondrion, and that this major checkpoint involves ΔNp73. It is likely that additional downstream checkpoints also exist, potentially involving the Bcl2 and/or IAP families (Wiese et al., 1999; Yuan and Yanker, 2000). In that regard, if one way that adult neurons become resistant to apoptosis is by enhancing survival pathways, than it makes sense to have multiple checkpoints at different levels to ensure that mature neurons are only eliminated when they have received multiple and continuous apoptotic insults.

Our ongoing studies on the role of p73 in the nervous system indicate that in addition to the essential role in maintenance of mature neurons that is demonstrated here, ΔNp73 is also important for the survival of a number of populations of developing neurons, including neonatal sympathetic and cortical neurons (Pozniak et al., 2000; Pozniak et al., 2002; Lee et al., 2004). Moreover, p73-/- mice display reduced numbers of more caudal CNS neurons at birth, including facial motor neurons and neurons of the deep cerebellar nuclei (Pozniak et al., 2002), implying that these neuronal populations might also have enhanced apoptosis during their embryonic developmental death period in the absence of p73. However, while our findings argue that p73 is important for neuronal survival both in development and in adulthood, data presented here suggest that the requirement for p73 somehow differs in developing versus adult neurons; neonatal sympathetic neurons are not sensitive to the loss of a single p73 allele, while adult sensory neurons are. One potential explanation for this difference is that developing versus adult neurons differ in how they regulate ΔNp73 levels. For example, when

neonatal sympathetic neurons are withdrawn from NGF, ΔNp73 levels fall dramatically (Pozniak et al., 2000), a response that is necessary to ensure the death of neurons that do not compete successfully for target-derived NGF in vivo. Disruption of the essential PI3-kinase survival pathway and/or DNA damage may lead to a similar loss of ΔNp73 expression in neonatal sympathetic neurons, thereby making p73+/- and p73-/- neurons equally vulnerable to apoptotic stimuli. In contrast, if adult neurons maintain higher basal levels of ΔNp73 through a cell-intrinsic mechanism that is essentially impervious to extrinsic cues, then these neurons would be relatively invulnerable to apoptotic stimuli. However, a decrease in basal ΔNp73 levels as a function of decreased p73 gene dosage would then be predicted to enhance neuronal vulnerability to these same apoptotic insults.

One of the major implications of this study derives from our finding that the lack of one p73 allele is sufficient to have a major impact on neuronal survival after injury. While the finding that relatively small potential alterations in protein levels would influence neuronal survival is a prediction of a "rheostat" model such as we have proposed, it is nonetheless surprising and has broad implications with regard to a genetic propensity for injury-induced damage to the adult nervous system, and even with regard to neurodegeneration. In particular, if the levels of ΔNp73 serve as a "buffer", then a small decrease in levels might well be sufficient to enhance the probability that an individual neuron would apoptose in response to an extrinsic cue, something that would ultimately result in an increased rate of neuronal loss over time (Clarke et al., 2000). This is precisely what is seen in the culture studies reported here, where the rate of cell death in response to apoptotic stimuli is increased in p73+/- neurons. Moreover, a similar increase in rate might be responsible for the axotomy results reported here, since the loss of small diameter unmyelinated sensory neurons seen in the p73+/- animals at one month is roughly similar in magnitude to the loss that is seen in rats after 8 months (Tandrup et al., 2000). Such a genetically determined increase in the rate of death would be particularly problematic in situations of acute injury, such as during ischemic stroke, and potentially even in the aging brain, where the DNA damage response, which is regulated by the p53 family, is now known to be elevated (Lu et al., 2004).

Figure 1. ***Adult sensory neurons are more resistant to apoptotic insults than are their neonatal counterparts.*** Adult (open squares) or neonatal (black squares) sensory neurons of the L4 DRG were cultured for 24 hours in NGF, and then were cultured for 2 additional days either (A) following NGF withdrawal and in the presence of anti-NGF antibodies, (B) in the presence of NGF, but with the addition of 50 μM LY294002, an inhibitor of PI3-kinase, or (C) in the presence of NGF, but with the addition of 10 μM camptothecin, a DNA-damaging agent. As controls, sister cultures were maintained in NGF over the same timeperiod (open circles). Phase-bright neurons with intact neurites in multiple identified fields were counted at 0, 1 and 2 days, for a total of at least 200 neurons per experiment, and the amount of survival was plotted relative to the initial number of neurons in the same fields at the time the treatment was initiated (Percent Survival). Results are the cumulative results of at least three independent experiments, and the error bars represent the standard error of the mean. (*t*-test; $P<0.005$, comparison at 2 day timepoint for all experiments).

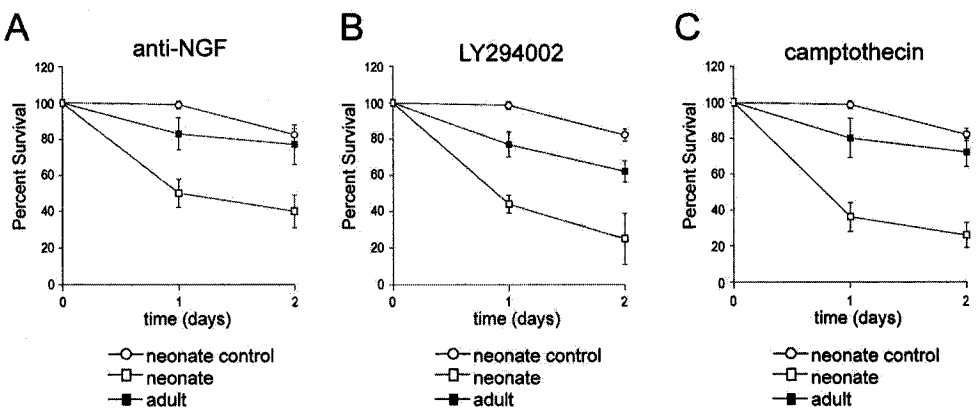

A
anti-NGF
B
LY294002
C
camptothecin
Percent Survival
120
100
80
60
40
20
0
0
1
2
time (days)
neonate control
neonate
adult

Figure 2. ***JNK activation occurs in both adult and neonatal sensory neurons, but exogenous JNK activation preferentially causes apoptosis of neonatal neurons.*** (A,B) Western blot analysis with an antibody specific for the phosphorylated, activated form of JNK (p-JNK) in lysates of neonatal (A) or adult (B) neurons that were established in NGF for one day, and then either withdrawn from NGF (anti-NGF) or treated with 50 μM LY294002 or 10 μM camptothecin for 24 hours. Equal amounts of protein were present in all lanes, as determined by reprobing the same blots for total JNK protein. The numbers to the left of the blots refer to the molecular weights of the two bands, which correspond to p46 and p54 isoforms of JNK. (C) Western blot analysis for the activated, phosphorylated form of JNK (pJNK) in equal amounts of protein from lysates of HEK293 cells (left panel) or neonatal DRG cultures (right panel) that were infected with replication-deficient recombinant adenoviruses expressing either GFP alone (Ad-GFP) or both GFP and the upstream kinase MLK3 (Ad-MLK3). Uninfected cells were also examined as an additional control. Blots were reprobed with anti-JNK to demonstrate that equal amounts of protein were present as well as for MLK3 and GFP. Note that overexpression of MLK3 causes activation of both p46 and p54 isoforms of JNK (D) Quantitation of the percentage of GFP-positive, infected neurons with apoptotic nuclei, as determined by Hoechst staining 3 days following infection. Neonatal or adult sensory neurons were established, infected with adenoviruses expressing either GFP alone or GFP plus MLK3, and then apoptosis was quantitated three days later. Note that the majority of neonatal neurons become apoptotic in response to MLK3 overexpression. (*t*-test; **P<0.05). Error bars represent standard deviation. (E) MLK3-induced neonatal neuron death is rescued by ectopic expression of ΔNp73. Quantitation of the percentage of GFP-positive, infected neonatal sympathetic neurons with apoptotic nuclei, as determined by Hoechst staining 3 days following infection. Neonatal sympathetic neurons were established, infected with adenoviruses expressing GFP, MLK3/GFP, and/or ΔNp73/GFP, and then apoptosis was quantitated 3 days later. Note that ΔNp73 rescued MLK3-mediated death. (ANOVA; *P<0.001). Error bars represent standard deviation.

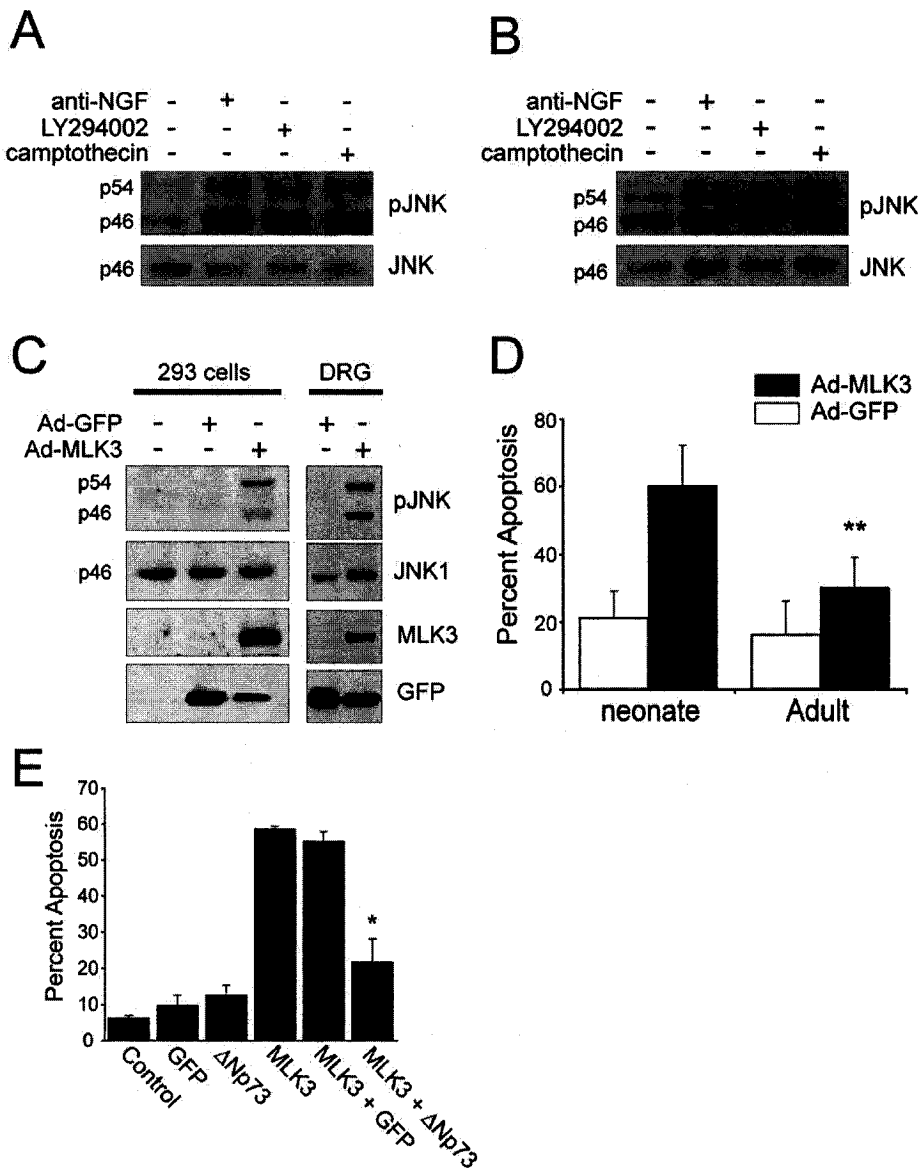

A
anti-NGF - + - -
LY294002 - - + -
camptothecin - - - +
p54
p46
pJNK
p46
JNK
B
anti-NGF - + - -
LY294002 - - + -
camptothecin - - - +
p54
p46
pJNK
p46
JNK
C
293 cells
DRG
Ad-GFP - + - + -
Ad-MLK3 - - + - +
p54
p46
pJNK
p46
JNK1
MLK3
GFP
D
Ad-MLK3
Ad-GFP
Percent Apoptosis
80
60
40
20
0
**
neonate
Adult
E
Percent Apoptosis
70
60
50
40
30
20
10
0
*
Control
GFP
ΔNp73
MLK3
MLK3 + GFP
MLK3 + ΔNp73

Figure 3. ***Expression of p73 in L4 DRG.*** RT-PCR analysis for p73 mRNA in total RNA isolated from the neonatal (P1 DRG) and adult (6wk DRG) mouse dorsal root ganglia. Total RNA from the E16 cortex, or from the total brain (P1) of p73+/+ versus p73-/- animals were all used as controls. Primers were specific to the core region that is shared amongst all p73 isoforms (p73 core) (A), to the full-length TA-containing isoforms (TAp73) (A), to the N-terminal truncated isoforms (ΔNp73) (B), or as a positive control, to p53. RT-PCR for GAPDH was used to demonstrate that RNA was present in all samples. Note that all p73 isoforms are expressed in the developing brain, as we have previously reported (Pozniak et al., 2000; 2002), and are not present in the p73-/- brain RNA samples.

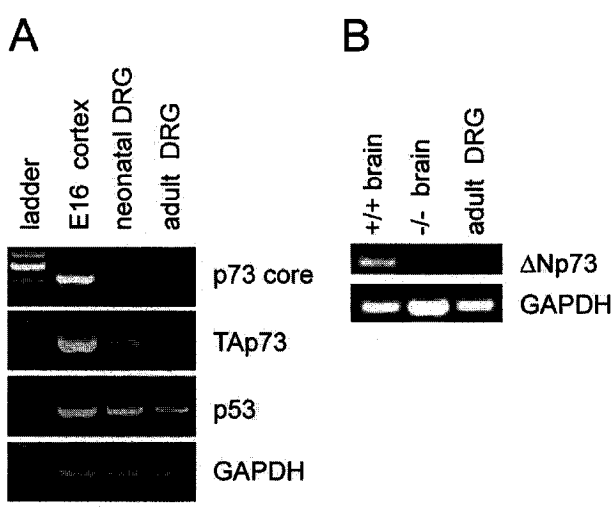

A
ladder
E16 cortex
neonatal DRG
adult DRG
p73 core
TAp73
p53
GAPDH
B
+/+ brain
-/- brain
adult DRG
ΔNp73
GAPDH

Figure 4. ***Deletion of one p73 allele does not affect the development of sensory neurons of the L4 DRG.*** (A) Cresyl violet-stained sections of the L4 DRG from p73+/+ versus p73+/- adult littermates. Scale bar, 100 μm. (B) Immunocytochemical analysis of sections through the p73+/+ and p73+/- L4 DRG with antibodies specific to NF200 (upper panels), which marks predominantly large diameter myelinated neurons, CGRP (middle panels), which marks predominantly small diameter peptidergic nociceptors, and IB4 (lower panels), which marks small diameter non-peptidergic nociceptors. Scale bars, 50 μm (C) Electron micrographs of transverse sections through the L4 dorsal roots of adult p73+/+ versus p73+/- animals. Scale bars, 2 μm (D) Quantitation of sections similar to those shown in panel C for the number of myelinated and unmyelinated axons in the L4 dorsal roots of p73+/+ and p73+/- animals.

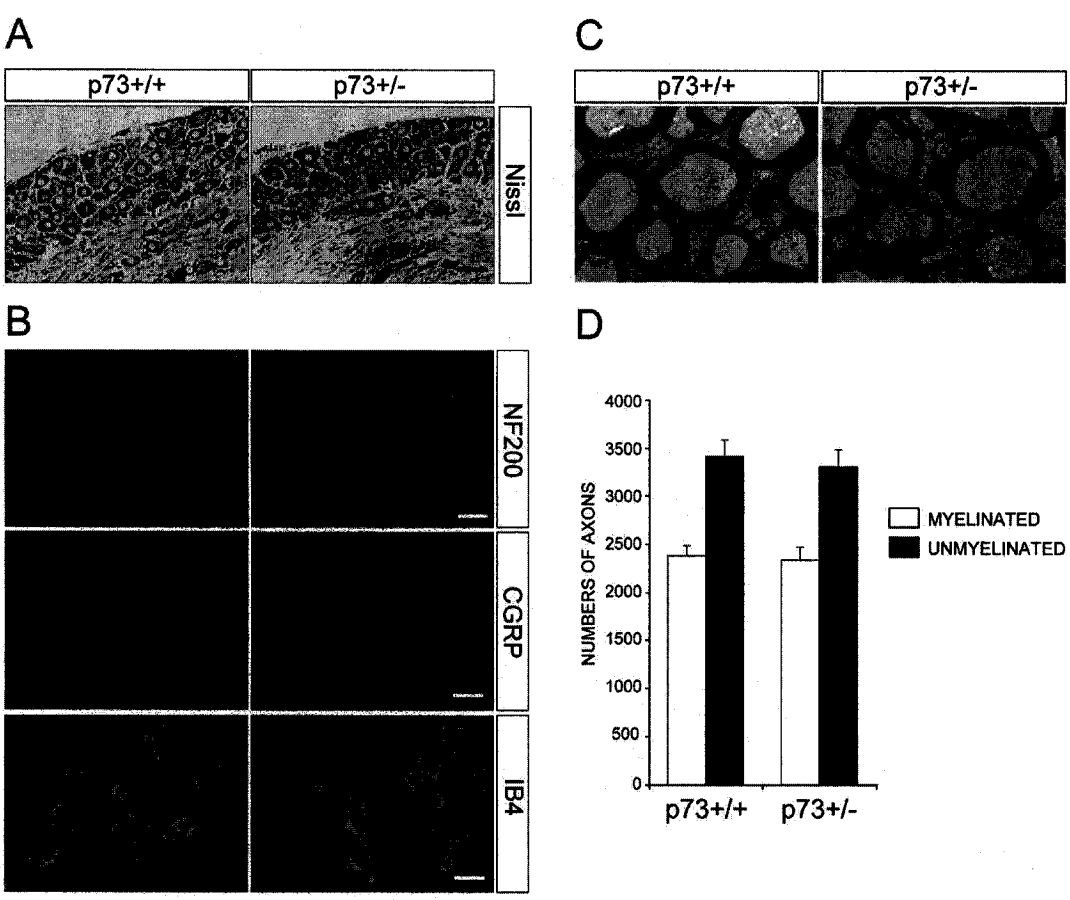

A
p73+/+
p73+/-
Nissl
B
NF200
CGRP
IB4
C
p73+/+
p73+/-
D
NUMBERS OF AXONS
4000
3500
3000
2500
2000
1500
1000
500
0
p73+/+
p73+/-
MYELINATED
UNMYELINATED

Figure 5. (A,B) ***p73+/- adult sensory neurons are more vulnerable to apoptotic stimuli.*** Adult sensory neurons cultured from p73+/+ versus p73+/- animals, were established in NGF for one day, and then were maintained in NGF, but with the addition of 10 μM camptothecin, a DNA-damaging agent (A) or 50 μM LY294002, an inhibitor of PI3-kinase (B). Phase-bright neurons with intact neurites in multiple identified fields were counted at 0, 1 and 2 days, and the amount of survival plotted relative to the initial number of neurons in the same fields at the time the treatment was initiated (Percentage Survival). Results are the cumulative results of at least three independent experiments, and the error bars represent the standard error of the mean. (*t*-test; $P<0.005$, comparison between groups at 2 day timepoint). (C) The enhanced vulnerability of p73+/- adult sensory neurons can be rescued by expression of ΔNp73. Adult p73+/- neurons were established in NGF, infected with recombinant adenoviruses expressing either GFP or ΔNp73β and GFP, and then either withdrawn from NGF (anti-NGF) or maintained in the presence of NGF with the addition of 50 μM LY294002 or 10 μM camptothecin. Two days later, the survival of GFP-positive neurons was quantitated, and expressed as a percentage of the total number of GFP-positive neurons. Error bars represent standard error of the mean. (D) p73+/- and p73+/+ neonatal sympathetic neurons are equally sensitive to apoptotic stimuli. Neonatal sympathetic neurons cultured from p73+/+ versus p73+/- animals, were established in NGF for 5 days, and then maintained in either 10 ng/mL NGF, or 10 ng/mL NGF with the addition of either 10 μM camptothecin or 50 μM LY294002. The number of apoptotic nuclei was quantified as determined by Hoechst staining 48 hours after treatment. Error bars represent standard error of the mean.

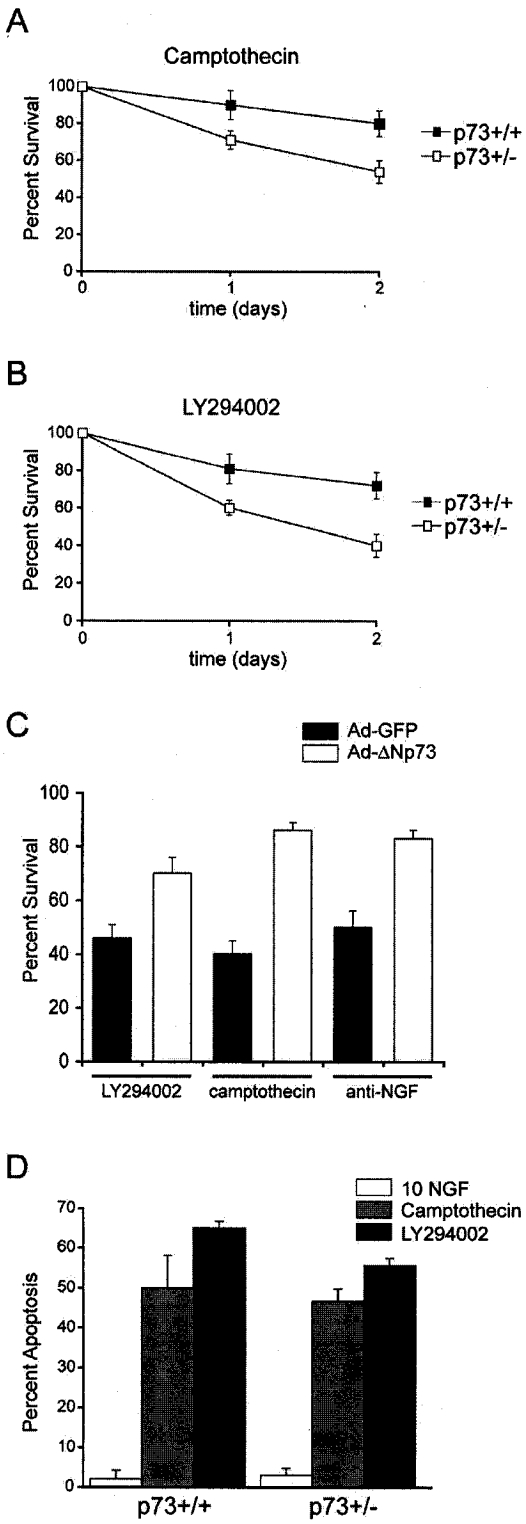
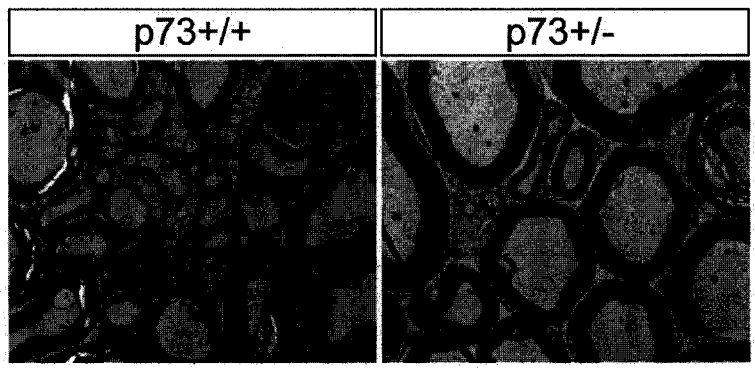
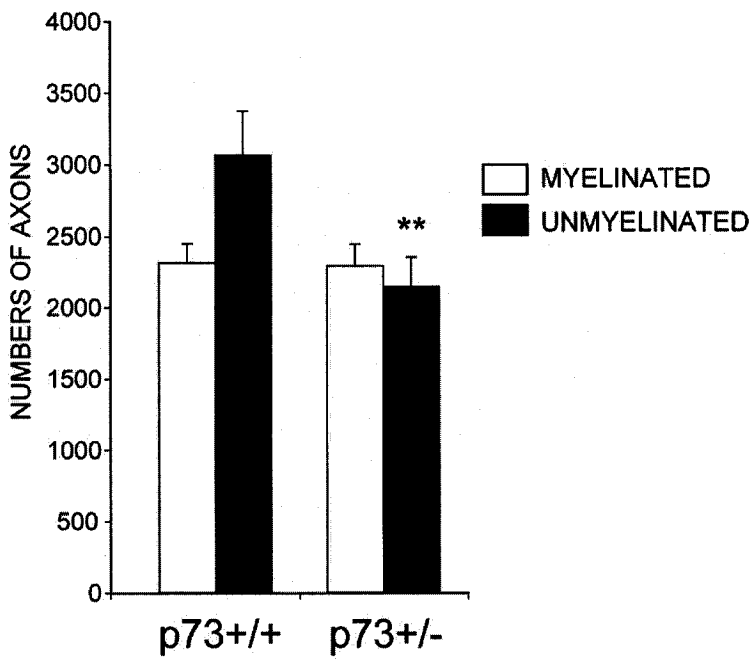

A
Camptothecin
Percent Survival
100
80
60
40
20
0
0
1
2
time (days)
p73+/+
p73+/-
B
LY294002
Percent Survival
100
80
60
40
20
0
0
1
2
time (days)
p73+/+
p73+/-
C
Ad-GFP
Ad-ΔNp73
Percent Survival
100
80
60
40
20
0
LY294002
camptothecin
anti-NGF
D
10 NGF
Camptothecin
LY294002
Percent Apoptosis
70
60
50
40
30
20
10
0
p73+/+
p73+/-

Figure 6. ***The absence of one p73 allele leads to enhanced death of small diameter, unmyelinated adult sensory neurons following axonal injury.*** (A) Electron micrographs of transverse sections through the L4 dorsal roots of p73+/- versus p73+/+ animals one month following sciatic nerve resection. Scale bar, 2 μm (B) Quantitation of the number of myelinated and unmyelinated axons present in cross-sections similar to those shown in (A). (*t*-test;**P<0.005). Error bars represent standard deviation.

A


p73+/+
p73+/-

B


4000
3500
3000
2500
2000
1500
1000
500
0
NUMBERS OF AXONS
**
MYELINATED
UNMYELINATED
p73+/+
p73+/-

CHAPTER 5

GENERAL DISCUSSION

Programmed cell death plays a fundamental role in the morphogenesis of multicellular organisms (Ellis et al., 1991). The nervous system is no exception, and a substantial proportion of the neurons initially formed die in later phases of neuronal development (Barde, 1989; Oppenheim, 1991). In the peripheral nervous system, programmed cell death comes into play when outgrowing axons reach their target tissues and compete there for neurotrophic molecules, which are produced in limited quantities (Levi-Montalcini, 1987; Barde, 1989; Oppenheim, 1991). At this time, sympathetic neurons extend their axons to their target tissues, where they compete for limiting amounts of target-derived NGF; those neurons that compete successfully survive, and go on to innervate their target tissues in an NGF-dependent fashion, while those neurons that fail are rapidly eliminated by apoptosis (Majdan and Miller, 1999). NGF promotes survival by binding to TrkA receptors on axonal terminals and mediating a retrograde survival signal (Miller and Kaplan, 2001), predominantly through activation of a phospho-inositide-3 kinase (PI3K) - Akt signaling pathway (Kaplan and Miller, 2000). Somewhat paradoxically, this pathway mediates survival in part by blocking apoptotic signals that initiate from a second neurotrophin receptor, the p75 neurotrophin receptor (p75NTR) (Kaplan and Miller, 2000). The functional antagonism between these two neurotrophin receptors is perhaps best exemplified by considering mice with targeted mutations in TrkA and/or p75NTR. In TrkA-/- mice, developing sympathetic neurons die due to the lack of prosurvival signals (Smeyne et al., 1994), while in the p75NTR-/- mice, there is a deficit in sympathetic neuron apoptosis during this same developmental window (Bamji et al., 1998). Importantly, studies presented in this thesis support the notion that the increased sympathetic neuron number reported in p75NTR-/- mice is due to a dramatic deficit in apoptosis, and not due to an increase in neuroblast proliferation. Nor is there an increase in Trk receptor levels or an increase in Trk receptor activation in response to NGF in p75NTR-/- sympathetic neurons. Thus, our findings indicate that p75NTR does

not mediate its proapoptotic effects by modulating TrkA and /or TrkA signaling cascades. Instead, our data strongly argue that p75NTR plays a major role in directly signaling apoptosis in sympathetic neurons in a TrkA-independent fashion. Cultured p75NTR-/- sympathetic neurons are delayed in their apoptosis in the absence of all Trk receptor signaling, and the absence of p75NTR in mice substantially rescues neonatal TrkA-/- sympathetic neurons in vivo (Majdan et al., 2001). Taken together, our findings support a model of naturally occurring neuronal death where sympathetic neurons are 'destined to die' as a consequence of an ongoing, p75NTR-mediated apoptotic signal, and survive only if they sequester sufficient NGF to robustly activate TrkA. 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.

However, the finding that the absence of p75NTR only partially inhibits the death seen in TrkA-/- mice also argues that other, p75NTR-independent pathways are also important for apoptosis in the absence of TrkA signaling. What might these p75NTR-independent pathways be? Previous work indicates that a second pathway shown to be important for NGF withdrawal involves the activation of the cell cycle regulatory molecules CDK4/6, which activate the retinoblastoma protein (pRb) by phosphorylation and subsequently contribute to sympathetic neuron apoptosis (Park et al., 1996; Park et al., 1997). This cell cycle pathway may well represent a p75NTR-independent pathway that is responsible for the delayed death seen in p75NTR-/- sympathetic neurons.

It should be noted that the $p75NTR^{ExonIII}$ -/- mice are not fully deficient for p75NTR expression (Dechant et al., 1997; von Schack et al., 2001). These studies show that the p75NTR locus produces an alternatively spliced isoform of p75NTR (s-p75NTR) that lacks Exon III. Alternative splicing of Exon III occurs in both wildtype and $p75NTR^{ExonIII}$ -/- mice, and produced a protein product that lacks the portion of the extracellular domain responsible for neurotrophin binding. This finding has prompted some researchers to suggest that the $p75NTR^{ExonIII}$ -/-mice is not a true knockout, but rather a p75NTR-hypomorphic mouse, and that caution is warranted in interpreting previous papers using these mice. The continued presence of a splice form of p75NTR that has intrinsic apoptotic activity could potentially explain the delayed death of sympathetic neurons in $p75NTR^{ExonIII}$ -/- mice. There is, however, little evidence showing

that the splice form of p75 (s-p75NTR) is capable of modulating neurotrophin signaling in either wildtype or p75NTR^{ExonIII} -/- cells, either by negatively regulating Trk activity or by initiating an apoptotic response. The presence of s-p75NTR has been detected in some, but not all p75NTR expressing cells, and it is unclear if it is expressed at sufficiently high levels to elicit a biological effect (von Schack et al., 2001). Indeed in Chapter 2, we examined the remaining expression of p75NTR in whole SCG lysates from p75NTR^{ExonIII} -/- mice, and found no detectable protein corresponding to this smaller variant. Taken together, the p75NTR-independent cell death is not likely to be due to the presence of a splice variant of s-p75NTR in p75NTR^{ExonIII} -/- mice.

Recent studies have described the discovery of two novel genes that encode proteins with substantial homology to p75NTR in cytoplasmic and transmembrane domains, defining a gene subfamily. These have been referred to as neurotrophin receptor homology 1 (NRH1) and NRH2 (Hutson and Bothwell, 2001). NRH1 is a *Xenopus* gene, also known as "fullback" and NRH2 was cloned from rat and has also been called PLAIDD, for "p75-like apoptosis-inducing death domain protein" (Frankowski et al., 2002). NRH2 contains a similar transmembrane and intracellular domain as p75NTR but lacks the characteristic cysteine-rich repeats in the extracellular domain (Frankowski et al., 2001; Hutson and Bothwell, 2001). NRH2 is expressed in several neuronal populations that also express p75NTR and Trk receptors, including sympathetic SCG neurons (Murray et al., 2004). Although unable to bind to neurotrophins, NRH2 maintains p75-like qualities. Namely, NRH2 can physically interact with Trk receptors and co-expression of NRH2 and Trk resulted in the formation of NGF high-affinity binding sites *in vitro* (Murray et al., 2004). Recent evidence suggests a role for a membrane-tethered form of p75NTR in forming an association with TrkA (Jung et al., 2003). Trk receptors, therefore, may have the ability of forming multimeric complexes with NRH2 and full-length or cleaved forms of p75NTR. NRH2 may also be capable of independent signaling through proteins known to associate with p75NTR, because their cytoplasmic sequences display homology. Certainly, the expression of p75NTR intracellular domain (ICD) in developing neurons of transgenic mice induced extensive neuronal apoptosis (Majdan et al., 1997), and proapoptotic signaling capacity of the p75NTR ICD has been demonstrated in cultured cells (Coulson et al., 2000; Roux et al., 2001). That NRH2

contributes to apoptotic signaling in neurons is still unclear. Bredesen's group reported that ectopic expression of NRH2/PLAIDD in SCG neurons was cytotoxic (Frankowski et al., 2001), whereas Schecterson's group reported that overexpression of soluble forms NRH2 in HEK293 cells actively stimulates NF-κB signaling (Kanning et al., 2003). Future loss-of-function studies using either RNAi or knockout mice will be required to determine the specific role NRH2 plays in neurotrophin-mediated neuronal survival during development, and whether these homologs contribute to p75NTR-independent pathways for sympathetic neuron apoptosis.

Nevertheless, our findings support the conclusion that p75NTR signaling constitutes a major component of the apoptotic signaling pathways activated after NGF withdrawal in sympathetic neurons. What is this apoptotic signaling pathway? Evidence from this thesis and other studies indicates that NGF withdrawal and p75NTR activation involves the recruitment of a JNK-p53-BAX apoptotic pathway in sympathetic neurons. These studies have shown that i) JNK activity is essential for sympathetic neuron apoptosis after NGF withdrawal (this thesis), ii) JNK function upstream of p53 in p75NTR mediated apoptosis (Bamji et al., 1998), while cdc42/Rac1 (Bazanet et al., 1998), Ask1 (Kanamoto et al., 2000), Mkk, JNK, c-jun (Ham et al., 1995), and p53 (Aloyz et al., 1998) have been shown to act in a signaling pathway regulating NGF withdrawal induced cell death. Consistent with this, we show in this thesis that JNK phosphorylation is elevated in the SCG in vivo during the period of naturally occurring cell death. We also show that levels of p53 and the amount of cleaved caspase-3 are decreased in the developing p75NTR-/- SCG, supporting the idea p75NTR contributes to the activation of this apoptotic pathway in vivo. In sympathetic neurons, the JNK-p53 arm of this apoptotic signaling pathway can be silenced by optimal TrkA survival signaling via Ras activation (Mazzoni et al., 1999).

As mentioned earlier, our findings 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. Although our studies demonstrate a major role for p75NTR, we did not focus our investigations on the nature of the ligand that mediates p75NTR activation in vivo. With the discovery that pro-neurotrophins are bonafide agonists for p75NTR-mediated cell death, we need to

redefine what the relevant ligand for p75NTR might be for developmental death of sympathetic neurons. It now appears that the *NGF* gene can give rise to both a pro-survival ligand (mature NGF) that binds and activates TrkA and a pro-apoptotic ligand (proNGF) that binds Sortilin/p75NTR and induces apoptosis. Several reports have now confirmed that proNGF can mediate p75NTR-induced cell death of non-TrkA expressing cells, such as smooth muscle cells and oligodendrocytes (Lee et al., 2001; Beattie et al., 2002). Interestingly, cultured sympathetic neurons undergo a more rapid rate of cell death if treated with pro-NGF (Lee et al., 2001). This was a surprising result showing that a proNGF could induce apoptosis of a cell that expresses both TrkA and p75NTR. In this study, however, proNGF was added to cultured neurons in the absence of other exogenous growth factors. Thus, while this study indicates that proNGF acting through p75NTR is sufficient to mediate cell death in TrkA-expressing sympathetic neurons, it is as yet unclear whether proNGF-mediated p75NTR activation is capable of inducing apoptosis in the presence of optimal TrkA receptor activation. It is likely that proBDNF and proNT-4 would also be in vivo ligands for p75NTR-mediated cell death. Our group has previously reported that BDNF-/- mice exhibit an increase in sympathetic number (albeit not as large as in p75NTR-/- mice). Since sympathetic neurons express BDNF, we had previously proposed a BDNF-mediated autocrine cell death loop (Bamji et al., 1998). Instead, the phenotype of BDNF-/- mice may well represent the presence of a proBDNF-p75NTR mediated autocrine death loop in sympathetic neurons. How sympathetic neurons regulate the release of mature- versus pro-forms of BDNF will be the subject of future investigations.

Role of p53 family members in developmental death of sympathetic neurons

As mentioned earlier, a number of studies indicate that p53 plays an apoptotic role for both p75NTR and NGF withdrawal induced death of sympathetic neurons. In particular, p53-/- sympathetic neurons showed enhanced survival upon NGF withdrawal, although this deficit in apoptosis was much more pronounced in embryonic than in neonatal neurons (Vogel and Parada, 1998). Second, sympathetic neurons show reduced apoptosis in p53-/- SCG in vivo (Aloyz et al., 1998), although this deficit is not of the same magnitude as that seen in p75NTR-/- mice (Bamji et al., 1998; Majdan et al., 2001). This

intermediate cell phenotype seen in p53-/- mice suggests that developmental compensation occurs in a p53-/- background, potentially via other members of the p53 family, p73 or p63.

With that in mind, our group has recently asked whether the third family member, p63 might collaborate with p53 in sympathetic neurons. In this regard, our data indicate that sympathetic neurons express TAp63, and that overexpression of TAp63 is sufficient to cause neuronal apoptosis (Jacobs et al., 2004). Importantly, we have also found that cultured p63-/- sympathetic neurons are significantly rescued from apoptosis following NGF withdrawal (Jacobs et al., 2004). We therefore propose that TAp63 is an essential pro-apoptotic mediator of sympathetic neuron death and that expression of TAp63 may compensate for p53 deficiency in the nervous system of p53-/- animals. We further propose that the ultimate survival of sympathetic neurons during developmental cell death is dependent on the balance of the proapoptotic family members p53/TAp63 versus the anti-apoptotic ΔNp73 and ΔNp63 isoforms

How do TAp73/TAp63 and ΔNp73/ΔNp63 mediate their proapoptotic and prosurvival effects, respectively? With regard to apoptosis, TAp73, TAp63 and p53 bind to similar sequences in DNA, and emerging evidence indicates that they have shared and distinct target genes. Recent evidence support the idea that these proapoptotic family members collaborate with each other to induce neuronal apoptosis, potentially via convergence on to the same apoptotic mechanisms. With regard to the ΔN prosurvival isoforms, it is clear that one way they function is by antagonizing the proapoptotic actions of p53 and/or full-length p73/p63 (Irwin and Kaelin, 2001), and our recent genetic studies of p73-/-, p53-/- sympathetic neurons argue that this is also true in neurons (Lee et al., 2004). However, this same study provided evidence that ΔNp73 mediates at least some of its prosurvival effects upstream of the mitochondrion in a p53-independent fashion (Lee et al., 2004), arguing that ΔNp73 acts to inhibit neuronal apoptosis at multiple points via multiple mechanisms.

p73 is an essential pro-survival protein in the nervous system

As neurons mature, they become less dependent on target-derived trophic factors for their survival, although they remain responsive to trophic factors with regard to axon sprouting and phenotypic gene expression. Once neurons have made the transition to maturity, mechanisms must be in place to allow postmitotic neurons to survive for the duration of an organism's lifespan. The presence of the ΔNp73 protein in the mammalian nervous system during the period of NOCD and its maintenance beyond this period (Pozniak et al., 2000; Pozniak et al., 2002; study presented here) is consistent with an essential role played by ΔNp73 in the control of neuronal survival during multiple phases throughout a neuron's lifespan. Our data presented here and combined with previous reports indicate that ΔNp73 satisfies many criteria that would be predicted for an essential pro-survival protein in the nervous system.

First, ΔNp73 appears to mediate, at least in part, the survival effects of neurotrophins during the period of naturally occurring cell death. Accordingly, ΔNp73 protein levels are regulated by NGF, and decrease rapidly in the absence of NGF (Pozniak et al., 2000). Moreover, the degeneration of sympathetic neurons in response to NGF deprivation can be delayed if ΔNp73 levels are kept high by adenoviral-mediated expression (Pozniak et al., 2000). Significantly, the elimination of p73 in mice augments the degeneration of sympathetic neurons during the period of naturally occurring cell death suggesting a crucial role for ΔNp73 in mediating the survival of peripheral neurons during this early developmental period.

Second, ΔNp73 appears to be important for the maintenance of neuronal survival in the early postnatal period, subsequent to the period of naturally occurring cell death. Our group previously demonstrated that elimination of p73 in mice causes a gradual loss of cortical neurons in the weeks and months after birth (Pozniak et al., 2002). This decrease in CNS neuron number in p73-/- mice was not confined to cortex, but also included facial motorneurons and the olfactory bulb (Pozniak et al., 2002), indicating that ΔNp73 is important for the long-term maintenance of at least some adult neurons in the CNS. Although p73-/- mice display hippocampal dysgenesis before birth, no significant neuronal cell loss was observed between E15 and birth, indicating that ΔNp73 is likely not required for the formation of neural progenitor cells or the regulation of CNS

neuronal survival during naturally occurring cell death. Either ΔNp73 is one of several players with broadly overlapping function, or its absence is compensated for by appropriately modified expression of related apoptotic/anti-apoptotic molecules, since no significant loss of cortical or facial motorneurons became apparent before birth.

Third, data presented in this thesis support the notion that ΔNp73 may be an essential component of the pathway used by adult neurons to resist axotomy. Therefore, one might predict that the absence of p73 protein would render adult neurons more vulnerable to injury. Given the fact that p73-/- animals do not live into adulthood, we used p73 heterozygous mice to examine what role p73 plays in the survival of adult sensory neurons. Interestingly, p73 heterozygous animals display no significant degeneration in sensory neuron number, suggesting that haploinsufficiency of p73 has no developmental phenotypic consequences (Walsh et al., 2004). However, we demonstrate that elimination of one allele of p73 renders adult sensory neurons more vulnerable to apoptotic stressors, including genotoxic injury and peripheral nerve axotomy. These findings imply that ΔNp73 is an essential component of the survival mechanisms that protect adult neurons from stress-induced apoptosis, presumably an important function for the maintenance and survival of such long-lived cells (Walsh et al., 2004).

Could a loss of ΔNp73 function explain the degeneration of neurons in neurodegenerative diseases? This question remains unanswered, but it is an intriguing hypothesis that will surely be tested in the near future. Given that ΔNp73 protects neurons in part by blocking p53-mediated cell death, (Lee et al., 2004), and the evidence that p53 is increased in appropriate regions in the brains of individuals suffering from a number of neurodegenerative disorders (De La Monte et al., 1997; Kitamura et al., 1997), suggest the possibility that one reason for increased p53 activity in degenerating neurons could be from a decrease in the function of ΔNp73.

The p53 Family: A Key Survival Checkpoint for Developing and Mature Mammalian Neurons

Together, the findings summarized here strongly support the hypothesis that the p53 family provides a fundamental survival checkpoint in neurons, and that this checkpoint lies upstream of the mitochondrion, in a key location to act as a "sensor" responsible for

integrating multiple proapoptotic and prosurvival cues. These studies support a model wherein the ultimate life or death of any given neuron is determined by the relative levels of the proapoptotic p53/TAp73/TAp63 family members versus the prosurvival ΔNp73/ΔNp63 members. We propose that proapoptotic signals deriving from cues as diverse as the p75 neurotrophin receptor to DNA damage will upregulate the levels/activity of the proapoptotic family members while, conversely, survival signals would converge on to the ΔN isoforms. We further propose that both the proapoptotic and prosurvival members mediate their effects on the intrinsic mitochondrial death pathway and its upstream activators at multiple points in the cascade, thereby ensuring the fidelity of a survival/apoptosis decision. One of the major implications of such a "rheostat" model is that subtle alterations in the levels of any of the family members could have a major impact on neuronal survival. Such an implication has broad implications, particularly with regard to a genetic propensity for injury-induced damage to the adult nervous system, and even with regard to neurodegeneration. As one example, if the levels of a protein like ΔNp73 serve as a "buffer" for the proapoptotic family members, as our ongoing studies suggest, then a small decrease in levels might well be sufficient to enhance the probability that an individual neuron would apoptose in response to a detrimental environmental stimulus, something that would ultimately result in an increased rate of neuronal loss over time (Clarke et al., 2000). This is precisely what is seen in a dramatic form in the p73-/- CNS (Pozniak et al., 2002), and what we have recently seen in p73+/- adult sensory neurons, both in culture and following axonal injury (Walsh et al., 2004). These studies indicate that loss of even a single allele of the p73 gene genetically determines the propensity for injury-induced neuronal death, a propensity that would be particularly problematic in situations of acute injury, such as during ischemic stroke, and potentially even with normal aging.

REFERENCE LIST

Acheson A, Conover JC, Fandl JP, DeChiara TM, Russell M, Thadani A, Squinto SP, Yancopoulos GD, Lindsay RM (1995) A BDNF autocrine loop in adult sensory neurons prevents cell death. Nature 374:450-453.

Agerman K, Baudet C, Fundin B, Willson C, Ernfors P (2000) Attenuation of a caspase-3 dependent cell death in NT4- and p75-deficient embryonic sensory neurons. Mol Cell Neurosci 16:258-268.

Albers KM, Wright DE, Davis BM (1994) Overexpression of nerve growth factor in epidermis of transgenic mice causes hypertrophy of the peripheral nervous system. J Neurosci 14:1422-1432.

Aloyz RS, Bamji SX, Pozniak CD, Toma JG, Atwal J, Kaplan DR, Miller FD (1998) p53 is essential for developmental neuron death as regulated by the TrkA and p75 neurotrophin receptors. J Cell Biol 143:1691-1703.

Anderson CN, Tolkovsky AM (1999) A role for MAPK/ERK in sympathetic neuron survival: protection against a p53-dependent, JNK-independent induction of apoptosis by cytosine arabinoside. J Neurosci 19:664-673.

Angel P, Allegretto EA, Okino ST, Hattori K, Boyle WJ, Hunter T, Karin M (1988) Oncogene jun encodes a sequence-specific trans-activator similar to AP-1. Nature 332:166-171.

Angel P, Hattori K, Smeal T, Karin M (1988) The jun proto-oncogene is positively autoregulated by its product, Jun/AP-1. Cell 55:875-885.

Angeletti PU, Levi-Montalcini R, Caramia F (1971a) Analysis of the effects of the antiserum to the nerve growth factor in adult mice. Brain Res 27:343-355.

Angeletti PU, Levi-Montalcini R, Caramia F (1971b) Ultrastructural changes in sympathetic neurons of newborn and adult mice treated with nerve growth factor. J Ultrastruct Res 36:24-36.

Armstrong JF, Kaufman MH, Harrison DJ, Clarke AR (1995) High-frequency developmental abnormalities in p53-deficient mice. Curr Biol 5:931-936.

Atwal JK, Massie B, Miller FD, Kaplan DR (2000) The TrkB-Shc site signals neuronal survival and local axon growth via MEK and P13-kinase. Neuron 27:265-277.

Baker SJ, Reddy EP (1998) Modulation of life and death by the TNF receptor superfamily. Oncogene 17:3261-3270.

Bamji SX, Majdan M, Pozniak CD, Belliveau DJ, Aloyz R, Kohn J, Causing CG, Miller FD (1998) The p75 neurotrophin receptor mediates neuronal apoptosis and is essential for naturally occurring sympathetic neuron death. J Cell Biol 140:911-923.

Banasiak KJ, Haddad GG (1998) Hypoxia-induced apoptosis: effect of hypoxic severity and role of p53 in neuronal cell death. Brain Res 797:295-304.

Banfield MJ, Naylor RL, Robertson AG, Allen SJ, Dawbarn D, Brady RL (2001) Specificity in Trk receptor:neurotrophin interactions: the crystal structure of TrkB-d5 in complex with neurotrophin-4/5. Structure (Camb) 9:1191-1199.

Barde YA (1989) Trophic factors and neuronal survival. Neuron 2:1525-1534.

Barker PA, Shooter EM (1994) Disruption of NGF binding to the low affinity neurotrophin receptor p75LNTR reduces NGF binding to TrkA on PC12 cells. Neuron 13:203-215.

Barrett GL, Bartlett PF (1994) The p75 nerve growth factor receptor mediates survival or death depending on the stage of sensory neuron development. Proc Natl Acad Sci U S A 91:6501-6505.

Bazenet CE, Mota MA, Rubin LL (1998) The small GTP-binding protein Cdc42 is required for nerve growth factor withdrawal-induced neuronal death. Proc Natl Acad Sci U S A 95:3984-3989.

Beal MF (1996) Mitochondria, free radicals, and neurodegeneration. Curr Opin Neurobiol 6:661-666.

Beattie MS, Farooqui AA, Bresnahan JC (2000) Review of current evidence for apoptosis after spinal cord injury. J Neurotrauma 17:915-925.

Beattie MS, Harrington AW, Lee R, Kim JY, Boyce SL, Longo FM, Bresnahan JC, Hempstead BL, Yoon SO (2002) ProNGF induces p75-mediated death of oligodendrocytes following spinal cord injury. Neuron 36:375-386.

Behrens A, Sibilia M, Wagner EF (1999) Amino-terminal phosphorylation of c-Jun regulates stress-induced apoptosis and cellular proliferation. Nat Genet 21:326-329.

Belliveau DJ, Krivko I, Kohn J, Lachance C, Pozniak C, Rusakov D, Kaplan D, Miller FD (1997) NGF and neurotrophin-3 both activate TrkA on sympathetic neurons but differentially regulate survival and neuritogenesis. J Cell Biol 136:375-388.

Benedetti M, Levi A, Chao MV (1993) Differential expression of nerve growth factor receptors leads to altered binding affinity and neurotrophin responsiveness. Proc Natl Acad Sci U S A 90:7859-7863.

Bengzon J, Mohapel P, Ekdahl CT, Lindvall O (2002) Neuronal apoptosis after brief and prolonged seizures. Prog Brain Res 135:111-119.

Bentley CA, Lee KF (2000) p75 is important for axon growth and schwann cell migration during development. J Neurosci 20:7706-7715.

Bergmann I, Priestley JV, McMahon SB, Brocker EB, Toyka KV, Koltzenburg M (1997) Analysis of cutaneous sensory neurons in transgenic mice lacking the low affinity neurotrophin receptor p75. Eur J Neurosci 9:18-28.

Besirli CG, Deckwerth TL, Crowder RJ, Freeman RS, Johnson EM, Jr. (2003) Cytosine arabinoside rapidly activates Bax-dependent apoptosis and a delayed Bax-independent death pathway in sympathetic neurons. Cell Death Differ 10:1045-1058.

Bibel M, Hoppe E, Barde YA (1999) Biochemical and functional interactions between the neurotrophin receptors trk and p75NTR. Embo J 18:616-622.

Bjerre B, Bjorklund A, Mobley W, Rosengren E (1975) Short- and long-term effects of nerve growth factor on the sympathetic nervous system in the adult mouse. Brain Res 94:263-277.

Blaschke AJ, Staley K, Chun J (1996) Widespread programmed cell death in proliferative and postmitotic regions of the fetal cerebral cortex. Development 122:1165-1174.

Bonni A, Brunet A, West AE, Datta SR, Takasu MA, Greenberg ME (1999) Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. Science 286:1358-1362.

Bonny C, Nicod P, Waeber G (1998) IB1, a JIP-1-related nuclear protein present in insulin-secreting cells. J Biol Chem 273:1843-1846.

Brandoli C, Shi B, Pflug B, Andrews P, Wrathall JR, Mocchetti I (2001) Dexamethasone reduces the expression of p75 neurotrophin receptor and apoptosis in contused spinal cord. Brain Res Mol Brain Res 87:61-70.

Bredesen DE, Ye X, Tasinato A, Sperandio S, Wang JJ, Assa-Munt N, Rabizadeh S (1998) p75NTR and the concept of cellular dependence: seeing how the other half die. Cell Death Differ 5:365-371.

Bredesen DE, Ye X, Tasinato A, Sperandio S, Wang JJ, Assa-Munt N, Rabizadeh S (1998) p75NTR and the concept of cellular dependence: seeing how the other half die. Cell Death Differ 5:365-371.

Brennan C, Rivas-Plata K, Landis SC (1999) The p75 neurotrophin receptor influences NT-3 responsiveness of sympathetic neurons in vivo. Nat Neurosci 2:699-705.

Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME (1999) Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell 96:857-868.

Bui NT, Konig HG, Culmsee C, Bauerbach E, Poppe M, Krieglstein J, Prehn JH (2002) p75 neurotrophin receptor is required for constitutive and NGF-induced survival signalling in PC12 cells and rat hippocampal neurones. J Neurochem 81:594-605.

Bunone G, Mariotti A, Compagni A, Morandi E, Della Valle G (1997) Induction of apoptosis by p75 neurotrophin receptor in human neuroblastoma cells. Oncogene 14:1463-1470.

Burstein DE, Greene LA (1982) Nerve growth factor has both mitogenic and antimitogenic activity. Dev Biol 94:477-482.

Buschmann T, Potapova O, Bar-Shira A, Ivanov VN, Fuchs SY, Henderson S, Fried VA, Minamoto T, Alarcon-Vargas D, Pincus MR, Gaarde WA, Holbrook NJ, Shiloh Y, Ronai Z (2001) Jun NH2-terminal kinase phosphorylation of p53 on Thr-81 is important for p53 stabilization and transcriptional activities in response to stress. Mol Cell Biol 21:2743-2754.

Carter BD, Kaltschmidt C, Kaltschmidt B, Offenhauser N, Bohm-Matthaei R, Baeuerle PA, Barde YA (1996) Selective activation of NF-kappa B by nerve growth factor through the neurotrophin receptor p75. Science 272:542-545.

Casaccia-Bonnefil P, Carter BD, Dobrowsky RT, Chao MV (1996) Death of oligodendrocytes mediated by the interaction of nerve growth factor with its receptor p75. Nature 383:716-719.

Casademunt E, Carter BD, Benzel I, Frade JM, Dechant G, Barde YA (1999) The zinc finger protein NRIF interacts with the neurotrophin receptor p75(NTR) and participates in programmed cell death. Embo J 18:6050-6061.

Casciano I, Ponzoni M, Lo Cunsolo C, Tonini GP, Romani M (1999) Different p73 splicing variants are expressed in distinct tumour areas of a multifocal neuroblastoma. Cell Death Differ 6:391-393.

Casha S, Yu WR, Fehlings MG (2001) Oligodendroglial apoptosis occurs along degenerating axons and is associated with FAS and p75 expression following spinal cord injury in the rat. Neuroscience 103:203-218.

Causing CG, Gloster A, Aloyz R, Bamji SX, Chang E, Fawcett J, Kuchel G, Miller FD (1997) Synaptic innervation density is regulated by neuron-derived BDNF. Neuron 18:257-267.

Cecconi F, Alvarez-Bolado G, Meyer BI, Roth KA, Gruss P (1998) Apaf1 (CED-4 homolog) regulates programmed cell death in mammalian development. Cell 94:727-737.

Cerezo A, Martinez AC, Lanzarot D, Fischer S, Franke TF, Rebollo A (1998) Role of Akt and c-Jun N-terminal kinase 2 in apoptosis induced by interleukin-4 deprivation. Mol Biol Cell 9:3107-3118.

Chao MV, Hempstead BL (1995) p75 and Trk: a two-receptor system. Trends Neurosci 18:321-326.

Chi SW, Ayed A, Arrowsmith CH (1999) Solution structure of a conserved C-terminal domain of p73 with structural homology to the SAM domain. Embo J 18:4438-4445.

Chong MJ, Murray MR, Gosink EC, Russell HR, Srinivasan A, Kapsetaki M, Korsmeyer SJ, McKinnon PJ (2000) Atm and Bax cooperate in ionizing radiation-induced apoptosis in the central nervous system. Proc Natl Acad Sci U S A 97:889-894.

Chopp M, Li Y, Zhang ZG, Freytag SO (1992) p53 expression in brain after middle cerebral artery occlusion in the rat. Biochem Biophys Res Commun 182:1201-1207.

Chun LL, Patterson PH (1977) Role of nerve growth factor in the development of rat sympathetic neurons in vitro. I. Survival, growth, and differentiation of catecholamine production. J Cell Biol 75:694-704.

Clarke G, Collins RA, Leavitt BR, Andrews DF, Hayden MR, Lumsden CJ, McInnes RR (2000) A one-hit model of cell death in inherited neuronal degenerations. Nature 406:195-199.

Coggeshall RE, Chung K, Greenwood D, Hulsebosch CE (1984) An empirical method for converting nucleolar counts to neuronal numbers. J Neurosci Methods 12:125-132.

Coggeshall RE, Lekan HA, Doubell TP, Allchorne A, Woolf CJ (1997) Central changes in primary afferent fibers following peripheral nerve lesions. Neuroscience 77:1115-1122.

Cohen S (1960) Purification and metabolic effects of a nerve growth-promoting protein from the mouse salivary gland and its neurotroxic antiserum. Proc Natl Acad Sci U S A 46:302-311.

Cordon-Cardo C, Tapley P, Jing SQ, Nanduri V, O'Rourke E, Lamballe F, Kovary K, Klein R, Jones KR, Reichardt LF, et al. (1991) The trk tyrosine protein kinase mediates the mitogenic properties of nerve growth factor and neurotrophin-3. Cell 66:173-183.

Coulson EJ, Reid K, Barrett GL, Bartlett PF (1999) p75 neurotrophin receptor-mediated neuronal death is promoted by Bcl-2 and prevented by Bcl-xL. J Biol Chem 274:16387-16391.

Coulson EJ, Reid K, Baca M, Shipham KA, Hulett SM, Kilpatrick TJ, Bartlett PF (2000) Chopper, a new death domain of the p75 neurotrophin receptor that mediates rapid neuronal cell death. J Biol Chem 275:30537-30545.

Cregan SP, MacLaurin JG, Craig CG, Robertson GS, Nicholson DW, Park DS, Slack RS (1999) Bax-dependent caspase-3 activation is a key determinant in p53-induced apoptosis in neurons. J Neurosci 19:7860-7869.

Cregan SP, Arbour NA, Maclaurin JG, Callaghan SM, Fortin A, Cheung EC, Guberman DS, Park DS, Slack RS (2004) p53 activation domain 1 is essential for PUMA upregulation and p53-mediated neuronal cell death. J Neurosci 24:10003-10012.

Crowder RJ, Freeman RS (1998) Phosphatidylinositol 3-kinase and Akt protein kinase are necessary and sufficient for the survival of nerve growth factor-dependent sympathetic neurons. J Neurosci 18:2933-2943.

Crowley C, Spencer SD, Nishimura MC, Chen KS, Pitts-Meek S, Armanini MP, Ling LH, MacMahon SB, Shelton DL, Levinson AD, et al. (1994) Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. Cell 76:1001-1011.

Crumrine RC, Thomas AL, Morgan PF (1994) Attenuation of p53 expression protects against focal ischemic damage in transgenic mice. J Cereb Blood Flow Metab 14:887-891.

D'Mello SR, Borodezt K, Soltoff SP (1997) Insulin-like growth factor and potassium depolarization maintain neuronal survival by distinct pathways: possible involvement of PI 3-kinase in IGF-1 signaling. J Neurosci 17:1548-1560.

Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 91:231-241.

Datta SR, Brunet A, Greenberg ME (1999) Cellular survival: a play in three Akts. Genes Dev 13:2905-2927.

Davey F, Davies AM (1998) TrkB signalling inhibits p75-mediated apoptosis induced by nerve growth factor in embryonic proprioceptive neurons. Curr Biol 8:915-918.

Davies AM, Lee KF, Jaenisch R (1993) p75-deficient trigeminal sensory neurons have an altered response to NGF but not to other neurotrophins. Neuron 11:565-574.

de la Monte SM, Sohn YK, Wands JR (1997) Correlates of p53- and Fas (CD95)-mediated apoptosis in Alzheimer's disease. J Neurol Sci 152:73-83.

De Laurenzi V, Costanzo A, Barcaroli D, Terrinoni A, Falco M, Annicchiarico-Petruzzelli M, Levrero M, Melino G (1998) Two new p73 splice variants, gamma and delta, with different transcriptional activity. J Exp Med 188:1763-1768.

De Laurenzi VD, Catani MV, Terrinoni A, Corazzari M, Melino G, Costanzo A, Levrero M, Knight RA (1999) Additional complexity in p73: induction by mitogens in lymphoid cells and identification of two new splicing variants epsilon and zeta. Cell Death Differ 6:389-390.

Dechant G, Barde YA (1997) Signalling through the neurotrophin receptor p75NTR. Curr Opin Neurobiol 7:413-418.

Deckwerth TL, Elliott JL, Knudson CM, Johnson EM, Jr., Snider WD, Korsmeyer SJ (1996) BAX is required for neuronal death after trophic factor deprivation and during development. Neuron 17:401-411.

DeFreitas MF, McQuillen PS, Shatz CJ (2001) A novel p75NTR signaling pathway promotes survival, not death, of immunopurified neocortical subplate neurons. J Neurosci 21:5121-5129.

Delcroix JD, Valletta JS, Wu C, Hunt SJ, Kowal AS, Mobley WC (2003) NGF signaling in sensory neurons: evidence that early endosomes carry NGF retrograde signals. Neuron 39:69-84.

Deng Y, Wu X (2000) Peg3/Pw1 promotes p53-mediated apoptosis by inducing Bax translocation from cytosol to mitochondria. Proc Natl Acad Sci U S A 97:12050-12055.

Deveraux QL, Roy N, Stennicke HR, Van Arsdale T, Zhou Q, Srinivasula SM, Alnemri ES, Salvesen GS, Reed JC (1998) IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. Embo J 17:2215-2223.

Dickens M, Rogers JS, Cavanagh J, Raitano A, Xia Z, Halpern JR, Greenberg ME, Sawyers CL, Davis RJ (1997) A cytoplasmic inhibitor of the JNK signal transduction pathway. Science 277:693-696.

Didier M, Bursztajn S, Adamec E, Passani L, Nixon RA, Coyle JT, Wei JY, Berman SA (1996) DNA strand breaks induced by sustained glutamate excitotoxicity in primary neuronal cultures. J Neurosci 16:2238-2250.

Dobrowsky RT, Werner MH, Castellino AM, Chao MV, Hannun YA (1994) Activation of the sphingomyelin cycle through the low-affinity neurotrophin receptor. Science 265:1596-1599.

Dobrowsky RT, Jenkins GM, Hannun YA (1995) Neurotrophins induce sphingomyelin hydrolysis. Modulation by co-expression of p75NTR with Trk receptors. J Biol Chem 270:22135-22142.

Dolcet X, Egea J, Soler RM, Martin-Zanca D, Comella JX (1999) Activation of phosphatidylinositol 3-kinase, but not extracellular-regulated kinases, is necessary to mediate brain-derived neurotrophic factor-induced motoneuron survival. J Neurochem 73:521-531.

Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA, Jr., Butel JS, Bradley A (1992) Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature 356:215-221.

Dowling P, Ming X, Raval S, Husar W, Casaccia-Bonnefil P, Chao M, Cook S, Blumberg B (1999) Up-regulated p75NTR neurotrophin receptor on glial cells in MS plaques. Neurology 53:1676-1682.

Dudek H, Datta SR, Franke TF, Birnbaum MJ, Yao R, Cooper GM, Segal RA, Kaplan DR, Greenberg ME (1997) Regulation of neuronal survival by the serine-threonine protein kinase Akt. Science 275:661-665.

Easton RM, Deckwerth TL, Parsadanian AS, Johnson EM, Jr. (1997) Analysis of the mechanism of loss of trophic factor dependence associated with neuronal maturation: a phenotype indistinguishable from Bax deletion. J Neurosci 17:9656-9666.

Eide FF, Vining ER, Eide BL, Zang K, Wang XY, Reichardt LF (1996) Naturally occurring truncated trkB receptors have dominant inhibitory effects on brain-derived neurotrophic factor signaling. J Neurosci 16:3123-3129.

Eilers A, Whitfield J, Babij C, Rubin LL, Ham J (1998) Role of the Jun kinase pathway in the regulation of c-Jun expression and apoptosis in sympathetic neurons. J Neurosci 18:1713-1724.

Eilers A, Whitfield J, Shah B, Spadoni C, Desmond H, Ham J (2001) Direct inhibition of c-Jun N-terminal kinase in sympathetic neurones prevents c-jun promoter activation and NGF withdrawal-induced death. J Neurochem 76:1439-1454.

Ellis RE, Yuan JY, Horvitz HR (1991) Mechanisms and functions of cell death. Annu Rev Cell Biol 7:663-698.

ElShamy WM, Linnarsson S, Lee KF, Jaenisch R, Ernfors P (1996) Prenatal and postnatal requirements of NT-3 for sympathetic neuroblast survival and innervation of specific targets. Development 122:491-500.

Ernfors P, Lee KF, Jaenisch R (1994a) Mice lacking brain-derived neurotrophic factor develop with sensory deficits. Nature 368:147-150.

Ernfors P, Lee KF, Kucera J, Jaenisch R (1994b) Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of limb proprioceptive afferents. Cell 77:503-512.

Esposito D, Patel P, Stephens RM, Perez P, Chao MV, Kaplan DR, Hempstead BL (2001) The cytoplasmic and transmembrane domains of the p75 and Trk A receptors regulate high affinity binding to nerve growth factor. J Biol Chem 276:32687-32695.

Estus S, Zaks WJ, Freeman RS, Gruda M, Bravo R, Johnson EM, Jr. (1994) Altered gene expression in neurons during programmed cell death: identification of c-jun as necessary for neuronal apoptosis. J Cell Biol 127:1717-1727.

Fagan AM, Zhang H, Landis S, Smeyne RJ, Silos-Santiago I, Barbacid M (1996) TrkA, but not TrkC, receptors are essential for survival of sympathetic neurons in vivo. J Neurosci 16:6208-6218.

Farinas I, Jones KR, Backus C, Wang XY, Reichardt LF (1994) Severe sensory and sympathetic deficits in mice lacking neurotrophin-3. Nature 369:658-661.

Ferri CC, Moore FA, Bisby MA (1998) Effects of facial nerve injury on mouse motoneurons lacking the p75 low-affinity neurotrophin receptor. J Neurobiol 34:1-9.

Ferri CC, Bisby MA (1999) Improved survival of injured sciatic nerve Schwann cells in mice lacking the p75 receptor. Neurosci Lett 272:191-194.

Fillippovich I, Sorokina N, Gatei M, Haupt Y, Hobson K, Moallem E, Spring K, Mould M, McGuckin MA, Lavin MF, Khanna KK (2001) Transactivation-deficient p73alpha (p73Deltaexon2) inhibits apoptosis and competes with p53. Oncogene 20:514-522.

Foltz IN, Gerl RE, Wieler JS, Luckach M, Salmon RA, Schrader JW (1998) Human mitogen-activated protein kinase kinase 7 (MKK7) is a highly conserved c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) activated by environmental stresses and physiological stimuli. J Biol Chem 273:9344-9351.

Fortin A, Cregan SP, MacLaurin JG, Kushwaha N, Hickman ES, Thompson CS, Hakim A, Albert PR, Cecconi F, Helin K, Park DS, Slack RS (2001) APAF1 is a key transcriptional target for p53 in the regulation of neuronal cell death. J Cell Biol 155:207-216.

Fortin A, MacLaurin JG, Arbour N, Cregan SP, Kushwaha N, Callaghan SM, Park DS, Albert PR, Slack RS (2004) The proapoptotic gene SIVA is a direct transcriptional target for the tumor suppressors p53 and E2F1. J Biol Chem 279:28706-28714.

Frade JM, Rodriguez-Tebar A, Barde YA (1996) Induction of cell death by endogenous nerve growth factor through its p75 receptor. Nature 383:166-168.

Frade JM, Barde YA (1998) Microglia-derived nerve growth factor causes cell death in the developing retina. Neuron 20:35-41.

Frade JM, Barde YA (1999) Genetic evidence for cell death mediated by nerve growth factor and the neurotrophin receptor p75 in the developing mouse retina and spinal cord. Development 126:683-690.

Francis N, Farinas I, Brennan C, Rivas-Plata K, Backus C, Reichardt L, Landis S (1999) NT-3, like NGF, is required for survival of sympathetic neurons, but not their precursors. Dev Biol 210:411-427.

Frankowski H, Castro-Obregon S, del Rio G, Rao RV, Bredesen DE (2002) PLAIDD, a type II death domain protein that interacts with p75 neurotrophin receptor. Neuromolecular Med 1:153-170.

Friedman WJ (2000) Neurotrophins induce death of hippocampal neurons via the p75 receptor. J Neurosci 20:6340-6346.

Fuchs SY, Adler V, Pincus MR, Ronai Z (1998) MEKK1/JNK signaling stabilizes and activates p53. Proc Natl Acad Sci U S A 95:10541-10546.

Garcia I, Martinou I, Tsujimoto Y, Martinou JC (1992) Prevention of programmed cell death of sympathetic neurons by the bcl-2 proto-oncogene. Science 258:302-304.

Gentry JJ, Casaccia-Bonnefil P, Carter BD (2000) Nerve growth factor activation of nuclear factor kappaB through its p75 receptor is an anti-apoptotic signal in RN22 schwannoma cells. J Biol Chem 275:7558-7565.

Ghosh A, Carnahan J, Greenberg ME (1994) Requirement for BDNF in activity-dependent survival of cortical neurons. Science 263:1618-1623.

Ghosh A, Greenberg ME (1995) Distinct roles for bFGF and NT-3 in the regulation of cortical neurogenesis. Neuron 15:89-103.

Gibson S, Widmann C, Johnson GL (1999) Differential involvement of MEK kinase 1 (MEKK1) in the induction of apoptosis in response to microtubule-targeted drugs versus DNA damaging agents. J Biol Chem 274:10916-10922.

Gilmore EC, Nowakowski RS, Caviness VS, Jr., Herrup K (2000) Cell birth, cell death, cell diversity and DNA breaks: how do they all fit together? Trends Neurosci 23:100-105.

Glebova NO, Ginty DD (2004) Heterogeneous requirement of NGF for sympathetic target innervation in vivo. J Neurosci 24:743-751.

Goedert M, Otten U, Thoenen H (1978) Biochemical effects of antibodies against nerve growth factor on developing and differentiated sympathetic ganglia. Brain Res 148:264-268.

Gorin PD, Johnson EM, Jr. (1980) Effects of exposure to nerve growth factor antibodies on the developing nervous system of the rat: an experimental autoimmune approach. Dev Biol 80:313-323.

Gotthardt M, Trommsdorff M, Nevitt MF, Shelton J, Richardson JA, Stockinger W, Nimpf J, Herz J (2000) Interactions of the low density lipoprotein receptor gene family with cytosolic adaptor and scaffold proteins suggest diverse biological functions in cellular communication and signal transduction. J Biol Chem 275:25616-25624.

Graham SH, Chen J (2001) Programmed cell death in cerebral ischemia. J Cereb Blood Flow Metab 21:99-109.

Green DR (1998) Apoptotic pathways: the roads to ruin. Cell 94:695-698.

Greenlund LJ, Korsmeyer SJ, Johnson EM, Jr. (1995) Role of BCL-2 in the survival and function of developing and mature sympathetic neurons. Neuron 15:649-661.

Grewal SS, York RD, Stork PJ (1999) Extracellular-signal-regulated kinase signalling in neurons. Curr Opin Neurobiol 9:544-553.

Gu C, Casaccia-Bonnefil P, Srinivasan A, Chao MV (1999) Oligodendrocyte apoptosis mediated by caspase activation. J Neurosci 19:3043-3049.

Hallbook F, Ibanez CF, Persson H (1991) Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in Xenopus ovary. Neuron 6:845-858.

Ham J, Babij C, Whitfield J, Pfarr CM, Lallemand D, Yaniv M, Rubin LL (1995) A c-Jun dominant negative mutant protects sympathetic neurons against programmed cell death. Neuron 14:927-939.

Ham J, Eilers A, Whitfield J, Neame SJ, Shah B (2000) c-Jun and the transcriptional control of neuronal apoptosis. Biochem Pharmacol 60:1015-1021.

Hamanoue M, Middleton G, Wyatt S, Jaffray E, Hay RT, Davies AM (1999) p75-mediated NF-kappaB activation enhances the survival response of developing sensory neurons to nerve growth factor. Mol Cell Neurosci 14:28-40.

Hantzopoulos PA, Suri C, Glass DJ, Goldfarb MP, Yancopoulos GD (1994) The low affinity NGF receptor, p75, can collaborate with each of the Trks to potentiate functional responses to the neurotrophins. Neuron 13:187-201.

Harding TC, Xue L, Bienemann A, Haywood D, Dickens M, Tolkovsky AM, Uney JB (2001) Inhibition of JNK by overexpression of the JNL binding domain of JIP-1 prevents apoptosis in sympathetic neurons. J Biol Chem 276:4531-4534.

Harrington AW, Kim JY, Yoon SO (2002) Activation of Rac GTPase by p75 is necessary for c-jun N-terminal kinase-mediated apoptosis. J Neurosci 22:156-166.

Harrington AW, Leiner B, Blechschmitt C, Arevalo JC, Lee R, Morl K, Meyer M, Hempstead BL, Yoon SO, Giehl KM (2004) Secreted proNGF is a pathophysiological death-inducing ligand after adult CNS injury. Proc Natl Acad Sci U S A 101:6226-6230.

Harris CA, Johnson EM, Jr. (2001) BH3-only Bcl-2 family members are coordinately regulated by the JNK pathway and require Bax to induce apoptosis in neurons. J Biol Chem 276:37754-37760.

Hayes RC, Wiley RG, Armstrong DM (1992) Induction of nerve growth factor receptor (p75NGFr) mRNA within hypoglossal motoneurons following axonal injury. Brain Res Mol Brain Res 15:291-297.

He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B (1998) A simplified system for generating recombinant adenoviruses. Proc Natl Acad Sci U S A 95:2509-2514.

Hempstead BL, Martin-Zanca D, Kaplan DR, Parada LF, Chao MV (1991) High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor. Nature 350:678-683.

Hendry IA, Campbell J (1976) Morphometric analysis of rat superior cervical ganglion after axotomy and nerve growth factor treatment. J Neurocytol 5:351-360.

Herdegen T, Claret FX, Kallunki T, Martin-Villalba A, Winter C, Hunter T, Karin M (1998) Lasting N-terminal phosphorylation of c-Jun and activation of c-Jun N-terminal kinases after neuronal injury. J Neurosci 18:5124-5135.

Herdegen T, Waetzig V (2001) AP-1 proteins in the adult brain: facts and fiction about effectors of neuroprotection and neurodegeneration. Oncogene 20:2424-2437.

Herzog KH, Chong MJ, Kapsetaki M, Morgan JI, McKinnon PJ (1998) Requirement for Atm in ionizing radiation-induced cell death in the developing central nervous system. Science 280:1089-1091.

Hetman M, Kanning K, Cavanaugh JE, Xia Z (1999) Neuroprotection by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol 3-kinase. J Biol Chem 274:22569-22580.

Hilberg F, Aguzzi A, Howells N, Wagner EF (1993) c-jun is essential for normal mouse development and hepatogenesis. Nature 365:179-181.

Himes BT, Tessler A (1989) Death of some dorsal root ganglion neurons and plasticity of others following sciatic nerve section in adult and neonatal rats. J Comp Neurol 284:215-230.

Holgado-Madruga M, Moscatello DK, Emlet DR, Dieterich R, Wong AJ (1997) Grb2-associated binder-1 mediates phosphatidylinositol 3-kinase activation and the promotion of cell survival by nerve growth factor. Proc Natl Acad Sci U S A 94:12419-12424.

Holland PM, Suzanne M, Campbell JS, Noselli S, Cooper JA (1997) MKK7 is a stress-activated mitogen-activated protein kinase kinase functionally related to hemipterous. J Biol Chem 272:24994-24998.

Hollyday M, Hamburger V (1976) Reduction of the naturally occurring motor neuron loss by enlargement of the periphery. J Comp Neurol 170:311-320.

Hollyday M, Hamburger V, Farris JM (1977) Localization of motor neuron pools supplying identified muscles in normal and supernumerary legs of chick embryo. Proc Natl Acad Sci U S A 74:3582-3586.

Huang EJ, Reichardt LF (2001) Neurotrophins: roles in neuronal development and function. Annu Rev Neurosci 24:677-736.

Huber LJ, Chao MV (1995) A potential interaction of p75 and trkA NGF receptors revealed by affinity crosslinking and immunoprecipitation. J Neurosci Res 40:557-563.

Hutson LD, Bothwell M (2001) Expression and function of Xenopus laevis p75(NTR) suggest evolution of developmental regulatory mechanisms. J Neurobiol 49:79-98.

Ip NY, Stitt TN, Tapley P, Klein R, Glass DJ, Fandl J, Greene LA, Barbacid M, Yancopoulos GD (1993) Similarities and differences in the way neurotrophins interact with the Trk receptors in neuronal and nonneuronal cells. Neuron 10:137-149.

Ip YT, Davis RJ (1998) Signal transduction by the c-Jun N-terminal kinase (JNK)--from inflammation to development. Curr Opin Cell Biol 10:205-219.

Irwin MS, Kaelin WG (2001) p53 family update: p73 and p63 develop their own identities. Cell Growth Differ 12:337-349.

Ito M, Yoshioka K, Akechi M, Yamashita S, Takamatsu N, Sugiyama K, Hibi M, Nakabeppu Y, Shiba T, Yamamoto KI (1999) JSAP1, a novel jun N-terminal protein kinase (JNK)-binding protein that functions as a Scaffold factor in the JNK signaling pathway. Mol Cell Biol 19:7539-7548.

Jacobs WB, Govoni G, Ho D, Mills AA, Miller FD, Kaplan DR (2004) p63 is an essential proapoptotic molecule during developmental neuronal death. Society for Neuroscience 837.9.

Jing S, Tapley P, Barbacid M (1992) Nerve growth factor mediates signal transduction through trk homodimer receptors. Neuron 9:1067-1079.

Johnson D, Lanahan A, Buck CR, Sehgal A, Morgan C, Mercer E, Bothwell M, Chao M (1986) Expression and structure of the human NGF receptor. Cell 47:545-554.

Johnson RS, van Lingen B, Papaioannou VE, Spiegelman BM (1993) A null mutation at the c-jun locus causes embryonic lethality and retarded cell growth in culture. Genes Dev 7:1309-1317.

Johnson MD, Xiang H, London S, Kinoshita Y, Knudson M, Mayberg M, Korsmeyer SJ, Morrison RS (1998) Evidence for involvement of Bax and p53, but not caspases, in radiation-induced cell death of cultured postnatal hippocampal neurons. J Neurosci Res 54:721-733.

Johnson MD, Wu X, Aithmitti N, Morrison RS (2002) Peg3/Pw1 is a mediator between p53 and Bax in DNA damage-induced neuronal death. J Biol Chem 277:23000-23007.

Johnstone RW (2002) Deamidation of Bcl-X(L): a new twist in a genotoxic murder mystery. Mol Cell 10:695-697.

Jones KR, Farinas I, Backus C, Reichardt LF (1994) Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development. Cell 76:989-999.

Jordan J, Galindo MF, Prehn JH, Weichselbaum RR, Beckett M, Ghadge GD, Roos RP, Leiden JM, Miller RJ (1997) p53 expression induces apoptosis in hippocampal pyramidal neuron cultures. J Neurosci 17:1397-1405.

Jost CA, Marin MC, Kaelin WG, Jr. (1997) p73 is a simian [correction of human] p53-related protein that can induce apoptosis. Nature 389:191-194.

Jung KM, Tan S, Landman N, Petrova K, Murray S, Lewis R, Kim PK, Kim DS, Ryu SH, Chao MV, Kim TW (2003) Regulated intramembrane proteolysis of the p75 neurotrophin receptor modulates its association with the TrkA receptor. J Biol Chem 278:42161-42169.

Jurgensmeier JM, Xie Z, Deveraux Q, Ellerby L, Bredesen D, Reed JC (1998) Bax directly induces release of cytochrome c from isolated mitochondria. Proc Natl Acad Sci U S A 95:4997-5002.

Kaghad M, Bonnet H, Yang A, Creancier L, Biscan JC, Valent A, Minty A, Chalon P, Lelias JM, Dumont X, Ferrara P, McKeon F, Caput D (1997) Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. Cell 90:809-819.

Kanamoto T, Mota M, Takeda K, Rubin LL, Miyazono K, Ichijo H, Bazenet CE (2000) Role of apoptosis signal-regulating kinase in regulation of the c-Jun N-terminal kinase pathway and apoptosis in sympathetic neurons. Mol Cell Biol 20:196-204.

Kaplan DR, Hempstead BL, Martin-Zanca D, Chao MV, Parada LF (1991a) The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. Science 252:554-558.

Kaplan DR, Martin-Zanca D, Parada LF (1991b) Tyrosine phosphorylation and tyrosine kinase activity of the trk proto-oncogene product induced by NGF. Nature 350:158-160.

Kaplan DR, Miller FD (2000) Neurotrophin signal transduction in the nervous system. Curr Opin Neurobiol 10:381-391.

Kelkar N, Gupta S, Dickens M, Davis RJ (2000) Interaction of a mitogen-activated protein kinase signaling module with the neuronal protein JIP3. Mol Cell Biol 20:1030-1043.

Keramaris E, Vanderluit JL, Bahadori M, Mousavi K, Davis RJ, Flavell R, Slack RS, Park DS (2004) c-Jun N-terminal kinase 3 deficiency protects neurons from axotomy induced death in vivo, through mechanisms independent of c-Jun phosphorylation. J Biol Chem.

Khursigara G, Orlinick JR, Chao MV (1999) Association of the p75 neurotrophin receptor with TRAF6. J Biol Chem 274:2597-2600.

Kim AH, Khursigara G, Sun X, Franke TF, Chao MV (2001) Akt phosphorylates and negatively regulates apoptosis signal-regulating kinase 1. Mol Cell Biol 21:893-901.

Kim AH, Yano H, Cho H, Meyer D, Monks B, Margolis B, Birnbaum MJ, Chao MV (2002) Akt1 regulates a JNK scaffold during excitotoxic apoptosis. Neuron 35:697-709.

Kimpinski K, Jelinski S, Mearow K (1999) The anti-p75 antibody, MC192, and brain-derived neurotrophic factor inhibit nerve growth factor-dependent neurite growth from adult sensory neurons. Neuroscience 93:253-263.

Kitamura Y, Shimohama S, Kamoshima W, Matsuoka Y, Nomura Y, Taniguchi T (1997) Changes of p53 in the brains of patients with Alzheimer's disease. Biochem Biophys Res Commun 232:418-421.

Klein R, Conway D, Parada LF, Barbacid M (1990) The trkB tyrosine protein kinase gene codes for a second neurogenic receptor that lacks the catalytic kinase domain. Cell 61:647-656.

Klein R, Jing SQ, Nanduri V, O'Rourke E, Barbacid M (1991) The trk proto-oncogene encodes a receptor for nerve growth factor. Cell 65:189-197.

Klein R, Smeyne RJ, Wurst W, Long LK, Auerbach BA, Joyner AL, Barbacid M (1993) Targeted disruption of the trkB neurotrophin receptor gene results in nervous system lesions and neonatal death. Cell 75:113-122.

Klein R, Silos-Santiago I, Smeyne RJ, Lira SA, Brambilla R, Bryant S, Zhang L, Snider WD, Barbacid M (1994) Disruption of the neurotrophin-3 receptor gene trkC eliminates la muscle afferents and results in abnormal movements. Nature 368:249-251.

Klesse LJ, Parada LF (1998) p21 ras and phosphatidylinositol-3 kinase are required for survival of wild-type and NF1 mutant sensory neurons. J Neurosci 18:10420-10428.

Ko LJ, Prives C (1996) p53: puzzle and paradigm. Genes Dev 10:1054-1072.

Kohn J, Aloyz RS, Toma JG, Haak-Frendscho M, Miller FD (1999) Functionally antagonistic interactions between the TrkA and p75 neurotrophin receptors regulate sympathetic neuron growth and target innervation. J Neurosci 19:5393-5408.

Koike T, Martin DP, Johnson EM, Jr. (1989) Role of Ca2+ channels in the ability of membrane depolarization to prevent neuronal death induced by trophic-factor deprivation: evidence that levels of internal Ca2+ determine nerve growth factor dependence of sympathetic ganglion cells. Proc Natl Acad Sci U S A 86:6421-6425.

Kokaia Z, Andsberg G, Martinez-Serrano A, Lindvall O (1998) Focal cerebral ischemia in rats induces expression of P75 neurotrophin receptor in resistant striatal cholinergic neurons. Neuroscience 84:1113-1125.

Kong XT, Valentine VA, Rowe ST, Valentine MB, Ragsdale ST, Jones BG, Wilkinson DA, Brodeur GM, Cohn SL, Look AT (1999) Lack of homozygously inactivated p73 in single-copy MYCN primary neuroblastomas and neuroblastoma cell lines. Neoplasia 1:80-89.

Korhonen JM, Said FA, Wong AJ, Kaplan DR (1999) Gab1 mediates neurite outgrowth, DNA synthesis, and survival in PC12 cells. J Biol Chem 274:37307-37314.

Kuan CY, Yang DD, Samanta Roy DR, Davis RJ, Rakic P, Flavell RA (1999) The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development. Neuron 22:667-676.

Kuan CY, Roth KA, Flavell RA, Rakic P (2000) Mechanisms of programmed cell death in the developing brain. Trends Neurosci 23:291-297.

Kuan CY, Whitmarsh AJ, Yang DD, Liao G, Schloemer AJ, Dong C, Bao J, Banasiak KJ, Haddad GG, Flavell RA, Davis RJ, Rakic P (2003) A critical role of neural-specific JNK3 for ischemic apoptosis. Proc Natl Acad Sci U S A 100:15184-15189.

Kuida K, Zheng TS, Na S, Kuan C, Yang D, Karasuyama H, Rakic P, Flavell RA (1996) Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. Nature 384:368-372.

Kuida K, Haydar TF, Kuan CY, Gu Y, Taya C, Karasuyama H, Su MS, Rakic P, Flavell RA (1998) Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. Cell 94:325-337.

Kuruvilla R, Zweifel LS, Glebova NO, Lonze BE, Valdez G, Ye H, Ginty DD (2004) A neurotrophin signaling cascade coordinates sympathetic neuron development through differential control of TrkA trafficking and retrograde signaling. Cell 118:243-255.

Ladiwala U, Lachance C, Simoneau SJ, Bhakar A, Barker PA, Antel JP (1998) p75 neurotrophin receptor expression on adult human oligodendrocytes: signaling without cell death in response to NGF. J Neurosci 18:1297-1304.

Lamballe F, Klein R, Barbacid M (1991) trkC, a new member of the trk family of tyrosine protein kinases, is a receptor for neurotrophin-3. Cell 66:967-979.

Lazarus KJ, Bradshaw RA, West NR, Bunge P (1976) Adaptive survival or rat sympathetic neurons cultured without supporting cells or exogenous nerve growth factor. Brain Res 113:159-164.

Lee KF, Li E, Huber LJ, Landis SC, Sharpe AH, Chao MV, Jaenisch R (1992) Targeted mutation of the gene encoding the low affinity NGF receptor p75 leads to deficits in the peripheral sensory nervous system. Cell 69:737-749.

Lee KF, Davies AM, Jaenisch R (1994a) p75-deficient embryonic dorsal root sensory and neonatal sympathetic neurons display a decreased sensitivity to NGF. Development 120:1027-1033.

Lee KF, Bachman K, Landis S, Jaenisch R (1994b) Dependence on p75 for innervation of some sympathetic targets. Science 263:1447-1449.

Lee TH, Abe K, Kogure K, Itoyama Y (1995) Expressions of nerve growth factor and p75 low affinity receptor after transient forebrain ischemia in gerbil hippocampal CA1 neurons. J Neurosci Res 41:684-695.

Lee CW, La Thangue NB (1999) Promoter specificity and stability control of the p53-related protein p73. Oncogene 18:4171-4181.

Lee R, Kermani P, Teng KK, Hempstead BL (2001) Regulation of cell survival by secreted proneurotrophins. Science 294:1945-1948.

Lee H, Kimelman D (2002) A dominant-negative form of p63 is required for epidermal proliferation in zebrafish. Dev Cell 2:607-616.

Lee AF, Ho DK, Zanassi P, Walsh GS, Kaplan DR, Miller FD (2004) Evidence that DeltaNp73 promotes neuronal survival by p53-dependent and p53-independent mechanisms. J Neurosci 24:9174-9184.

Leibrock J, Lottspeich F, Hohn A, Hofer M, Hengerer B, Masiakowski P, Thoenen H, Barde YA (1989) Molecular cloning and expression of brain-derived neurotrophic factor. Nature 341:149-152.

Levi-Montalcini R, Booker B (1960) Destruction of the sympathetic ganglia in mammals by an antiserum to a nerve growth protein. Proc Natl Acad Sci U S A 46:385-391.

Levi-Montalcini R, Angeletti PU (1963) Essential role of the nerve growth factor in the survival and maintenance of dissociated sensory and sympathetic embryonic nerve cells in vitro. Dev Biol 7:653-659.

Levi-Montalcini R, Angeletti PU (1966) Second symposium on catecholamines. Modification of sympathetic function. Immunosympathectomy. Pharmacol Rev 18:619-628.

Levi-Montalcini R (1987) The nerve growth factor 35 years later. Science 237:1154-1162.

Li Y, Chopp M, Zhang ZG, Zaloga C, Niewenhuis L, Gautam S (1994) p53-immunoreactive protein and p53 mRNA expression after transient middle cerebral artery occlusion in rats. Stroke 25:849-855; discussion 855-846.

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91:479-489.

Lievremont JP, Sciorati C, Morandi E, Paolucci C, Bunone G, Della Valle G, Meldolesi J, Clementi E (1999) The p75(NTR)-induced apoptotic program develops through a ceramide-caspase pathway negatively regulated by nitric oxide. J Biol Chem 274:15466-15472.

Lievremont JP, Sciorati C, Morandi E, Paolucci C, Bunone G, Della Valle G, Meldolesi J, Clementi E (1999) The p75(NTR)-induced apoptotic program develops through a ceramide-caspase pathway negatively regulated by nitric oxide. J Biol Chem 274:15466-15472.

Lindsay RM (1988) Nerve growth factors (NGF, BDNF) enhance axonal regeneration but are not required for survival of adult sensory neurons. J Neurosci 8:2394-2405.

Liu Y, Guyton KZ, Gorospe M, Xu Q, Lee JC, Holbrook NJ (1996) Differential activation of ERK, JNK/SAPK and P38/CSBP/RK map kinase family members during the cellular response to arsenite. Free Radic Biol Med 21:771-781.

Liu YZ, Boxer LM, Latchman DS (1999) Activation of the Bcl-2 promoter by nerve growth factor is mediated by the p42/p44 MAPK cascade. Nucleic Acids Res 27:2086-2090.

Lo WD, Akhmametyeva EM, Zhu L, Friesen PD, Chang LS (2003) Induction of apoptosis by the p53-related p73 and partial inhibition by the baculovirus-encoded p35 in neuronal cells. Brain Res Mol Brain Res 113:1-12.

Longo FM, Manthorpe M, Xie YM, Varon S (1997) Synthetic NGF peptide derivatives prevent neuronal death via a p75 receptor-dependent mechanism. J Neurosci Res 48:1-17.

Lonze BE, Riccio A, Cohen S, Ginty DD (2002) Apoptosis, axonal growth defects, and degeneration of peripheral neurons in mice lacking CREB. Neuron 34:371-385.

Lu T, Pan Y, Kao SY, Li C, Kohane I, Chan J, Yankner BA (2004) Gene regulation and DNA damage in the ageing human brain. Nature 429:883-891.

Ma Y, Campenot RB, Miller FD (1992) Concentration-dependent regulation of neuronal gene expression by nerve growth factor. J Cell Biol 117:135-141.

Ma J, Novikov LN, Wiberg M, Kellerth JO (2001) Delayed loss of spinal motoneurons after peripheral nerve injury in adult rats: a quantitative morphological study. Exp Brain Res 139:216-223.

MacInnis BL, Campenot RB (2002) Retrograde support of neuronal survival without retrograde transport of nerve growth factor. Science 295:1536-1539.

MacPhee IJ, Barker PA (1997) Brain-derived neurotrophic factor binding to the p75 neurotrophin receptor reduces TrkA signaling while increasing serine phosphorylation in the TrkA intracellular domain. J Biol Chem 272:23547-23551.
Mahadeo D, Kaplan L, Chao MV, Hempstead BL (1994) High affinity nerve growth factor binding displays a faster rate of association than p140trk binding. Implications for multi-subunit polypeptide receptors. J Biol Chem 269:6884-6891.

Maher PA (1988) Nerve growth factor induces protein-tyrosine phosphorylation. Proc Natl Acad Sci U S A 85:6788-6791.

Maisonpierre PC, Belluscio L, Squinto S, Ip NY, Furth ME, Lindsay RM, Yancopoulos GD (1990) Neurotrophin-3: a neurotrophic factor related to NGF and BDNF. Science 247:1446-1451.

Majdan M, Lachance C, Gloster A, Aloyz R, Zeindler C, Bamji S, Bhakar A, Belliveau D, Fawcett J, Miller FD, Barker PA (1997) Transgenic mice expressing the intracellular domain of the p75 neurotrophin receptor undergo neuronal apoptosis. J Neurosci 17:6988-6998.

Majdan M, Miller FD (1999) Neuronal life and death decisions functional antagonism between the Trk and p75 neurotrophin receptors. Int J Dev Neurosci 17:153-161.

Majdan M, Walsh GS, Aloyz R, Miller FD (2001) TrkA mediates developmental sympathetic neuron survival in vivo by silencing an ongoing p75NTR-mediated death signal. J Cell Biol 155:1275-1285.

Manthorpe M, Fagnani R, Skaper SD, Varon S (1986) An automated colorimetric microassay for neuronotrophic factors. Brain Res 390:191-198.

Maroney AC, Glicksman MA, Basma AN, Walton KM, Knight E, Jr., Murphy CA, Bartlett BA, Finn JP, Angeles T, Matsuda Y, Neff NT, Dionne CA (1998) Motoneuron apoptosis is blocked by CEP-1347 (KT 7515), a novel inhibitor of the JNK signaling pathway. J Neurosci 18:104-111.

Maroney AC, Finn JP, Bozyczko-Coyne D, O'Kane TM, Neff NT, Tolkovsky AM, Park DS, Yan CY, Troy CM, Greene LA (1999) CEP-1347 (KT7515), an inhibitor of JNK activation, rescues sympathetic neurons and neuronally differentiated PC12 cells from death evoked by three distinct insults. J Neurochem 73:1901-1912.

Maroney AC, Finn JP, Connors TJ, Durkin JT, Angeles T, Gessner G, Xu Z, Meyer SL, Savage MJ, Greene LA, Scott RW, Vaught JL (2001) Cep-1347 (KT7515), a semisynthetic inhibitor of the mixed lineage kinase family. J Biol Chem 276:25302-25308.

Martin DP, Schmidt RE, DiStefano PS, Lowry OH, Carter JG, Johnson EM, Jr. (1988) Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. J Cell Biol 106:829-844.

Martin LJ, Liu Z (2002) Injury-induced spinal motor neuron apoptosis is preceded by DNA single-strand breaks and is p53- and Bax-dependent. J Neurobiol 50:181-197.

Martin-Zanca D, Barbacid M, Parada LF (1990) Expression of the trk proto-oncogene is restricted to the sensory cranial and spinal ganglia of neural crest origin in mouse development. Genes Dev 4:683-694.

Martinou JC, Dubois-Dauphin M, Staple JK, Rodriguez I, Frankowski H, Missotten M, Albertini P, Talabot D, Catsicas S, Pietra C, et al. (1994) Overexpression of BCL-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia. Neuron 13:1017-1030.

Matsuda S, Yasukawa T, Homma Y, Ito Y, Niikura T, Hiraki T, Hirai S, Ohno S, Kita Y, Kawasumi M, Kouyama K, Yamamoto T, Kyriakis JM, Nishimoto I (2001) c-Jun N-terminal kinase (JNK)-interacting protein-1b/islet-brain-1 scaffolds Alzheimer's amyloid precursor protein with JNK. J Neurosci 21:6597-6607.

Mazzoni IE, Said FA, Aloyz R, Miller FD, Kaplan D (1999) Ras regulates sympathetic neuron survival by suppressing the p53-mediated cell death pathway. J Neurosci 19:9716-9727.

McQuillen PS, DeFreitas MF, Zada G, Shatz CJ (2002) A novel role for p75NTR in subplate growth cone complexity and visual thalamocortical innervation. J Neurosci 22:3580-3593.

Mechta-Grigoriou F, Gerald D, Yaniv M (2001) The mammalian Jun proteins: redundancy and specificity. Oncogene 20:2378-2389.

Merry DE, Korsmeyer SJ (1997) Bcl-2 gene family in the nervous system. Annu Rev Neurosci 20:245-267.

Meyer D, Liu A, Margolis B (1999) Interaction of c-Jun amino-terminal kinase interacting protein-1 with p190 rhoGEF and its localization in differentiated neurons. J Biol Chem 274:35113-35118.

Mihara M, Erster S, Zaika A, Petrenko O, Chittenden T, Pancoska P, Moll UM (2003) p53 has a direct apoptogenic role at the mitochondria. Mol Cell 11:577-590.

Miller FD, Pozniak CD, Walsh GS (2000) Neuronal life and death: an essential role for the p53 family. Cell Death Differ 7:880-888.

Miller FD, Kaplan DR (2001) Neurotrophin signalling pathways regulating neuronal apoptosis. Cell Mol Life Sci 58:1045-1053.

Mills AA, Zheng B, Wang XJ, Vogel H, Roop DR, Bradley A (1999) p63 is a p53 homologue required for limb and epidermal morphogenesis. Nature 398:708-713.

Mischel PS, Smith SG, Vining ER, Valletta JS, Mobley WC, Reichardt LF (2001) The extracellular domain of p75NTR is necessary to inhibit neurotrophin-3 signaling through TrkA. J Biol Chem 276:11294-11301.

Miyashita T, Reed JC (1995) Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 80:293-299.

Moroni MC, Hickman ES, Denchi EL, Caprara G, Colli E, Cecconi F, Muller H, Helin K (2001) Apaf-1 is a transcriptional target for E2F and p53. Nat Cell Biol 3:552-558.

Morris EJ, Keramaris E, Rideout HJ, Slack RS, Dyson NJ, Stefanis L, Park DS (2001) Cyclin-dependent kinases and P53 pathways are activated independently and mediate Bax activation in neurons after DNA damage. J Neurosci 21:5017-5026.

Morrison RS, Wenzel HJ, Kinoshita Y, Robbins CA, Donehower LA, Schwartzkroin PA (1996) Loss of the p53 tumor suppressor gene protects neurons from kainate-induced cell death. J Neurosci 16:1337-1345.

Morrison RS, Kinoshita Y (2000) The role of p53 in neuronal cell death. Cell Death Differ 7:868-879.

Mota M, Reeder M, Chernoff J, Bazenet CE (2001) Evidence for a role of mixed lineage kinases in neuronal apoptosis. J Neurosci 21:4949-4957.

Motoyama N, Wang F, Roth KA, Sawa H, Nakayama K, Negishi I, Senju S, Zhang Q, Fujii S, et al. (1995) Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. Science 267:1506-1510.

Mowla SJ, Pareek S, Farhadi HF, Petrecca K, Fawcett JP, Seidah NG, Morris SJ, Sossin WS, Murphy RA (1999) Differential sorting of nerve growth factor and brain-derived neurotrophic factor in hippocampal neurons. J Neurosci 19:2069-2080.

Mukai J, Hachiya T, Shoji-Hoshino S, Kimura MT, Nadano D, Suvanto P, Hanaoka T, Li Y, Irie S, Greene LA, Sato TA (2000) NADE, a p75NTR-associated cell death executor, is involved in signal transduction mediated by the common neurotrophin receptor p75NTR. J Biol Chem 275:17566-17570.

Murray SS, Perez P, Lee R, Hempstead BL, Chao MV (2004) A novel p75 neurotrophin receptor-related protein, NRH2, regulates nerve growth factor binding to the TrkA receptor. J Neurosci 24:2742-2749.

Nagata K, Puls A, Futter C, Aspenstrom P, Schaefer E, Nakata T, Hirokawa N, Hall A (1998) The MAP kinase kinase kinase MLK2 co-localizes with activated JNK along microtubules and associates with kinesin superfamily motor KIF3. Embo J 17:149-158.

Napieralski JA, Raghupathi R, McIntosh TK (1999) The tumor-suppressor gene, p53, is induced in injured brain regions following experimental traumatic brain injury. Brain Res Mol Brain Res 71:78-86.

Narita M, Shimizu S, Ito T, Chittenden T, Lutz RJ, Matsuda H, Tsujimoto Y (1998) Bax interacts with the permeability transition pore to induce permeability transition and cytochrome c release in isolated mitochondria. Proc Natl Acad Sci U S A 95:14681-14686.

Naumann T, Casademunt E, Hollerbach E, Hofmann J, Dechant G, Frotscher M, Barde YA (2002) Complete deletion of the neurotrophin receptor p75NTR leads to long-lasting increases in the number of basal forebrain cholinergic neurons. J Neurosci 22:2409-2418.

Negri S, Oberson A, Steinmann M, Sauser C, Nicod P, Waeber G, Schorderet DF, Bonny C (2000) cDNA cloning and mapping of a novel islet-brain/JNK-interacting protein. Genomics 64:324-330.

Nykjaer A, Lee R, Teng KK, Jansen P, Madsen P, Nielsen MS, Jacobsen C, Kliemannel M, Schwarz E, Willnow TE, Hempstead BL, Petersen CM (2004) Sortilin is essential for proNGF-induced neuronal cell death. Nature 427:843-848.

Okubo Y, Blakesley VA, Stannard B, Gutkind S, Le Roith D (1998) Insulin-like growth factor-I inhibits the stress-activated protein kinase/c-Jun N-terminal kinase. J Biol Chem 273:25961-25966.

Oppenheim RW (1991) Cell death during development of the nervous system. Annu Rev Neurosci 14:453-501.

Oren M (2003) Decision making by p53: life, death and cancer. Cell Death Differ 10:431-442.

Otten U, Goedert M, Schwab M, Thibault J (1979) Immunization of adult rats against 2.5 S NGF: effects on the peripheral sympathetic nervous system. Brain Res 176:79-90.

Palko ME, Coppola V, Tessarollo L (1999) Evidence for a role of truncated trkC receptor isoforms in mouse development. J Neurosci 19:775-782.

Palmada M, Kanwal S, Rutkoski NJ, Gustafson-Brown C, Johnson RS, Wisdom R, Carter BD (2002) c-jun is essential for sympathetic neuronal death induced by NGF withdrawal but not by p75 activation. J Cell Biol 158:453-461.

Park DS, Farinelli SE, Greene LA (1996) Inhibitors of cyclin-dependent kinases promote survival of post-mitotic neuronally differentiated PC12 cells and sympathetic neurons. J Biol Chem 271:8161-8169.

Park DS, Levine B, Ferrari G, Greene LA (1997) Cyclin dependent kinase inhibitors and dominant negative cyclin dependent kinase 4 and 6 promote survival of NGF-deprived sympathetic neurons. J Neurosci 17:8975-8983.

Park DS, Morris EJ, Padmanabhan J, Shelanski ML, Geller HM, Greene LA (1998) Cyclin-dependent kinases participate in death of neurons evoked by DNA-damaging agents. J Cell Biol 143:457-467.

Peterson DA, Dickinson-Anson HA, Leppert JT, Lee KF, Gage FH (1999) Central neuronal loss and behavioral impairment in mice lacking neurotrophin receptor p75. J Comp Neurol 404:1-20.

Pettmann B, Henderson CE (1998) Neuronal cell death. Neuron 20:633-647.

Philpott KL, McCarthy MJ, Klippel A, Rubin LL (1997) Activated phosphatidylinositol 3-kinase and Akt kinase promote survival of superior cervical neurons. J Cell Biol 139:809-815.

Pozniak CD, Radinovic S, Yang A, McKeon F, Kaplan DR, Miller FD (2000) An anti-apoptotic role for the p53 family member, p73, during developmental neuron death. Science 289:304-306.

Pozniak CD, Barnabe-Heider F, Rymar VV, Lee AF, Sadikot AF, Miller FD (2002) p73 is required for survival and maintenance of CNS neurons. J Neurosci 22:9800-9809.

Purves D, Lichtman JW (1985) Geometrical differences among homologous neurons in mammals. Science 228:298-302.

Purves D (1988) Body and Brain: A trophic theory of neural connections. Cambridge, Massachusetts: Harvard University Press.

Putcha GV, Deshmukh M, Johnson EM, Jr. (1999) BAX translocation is a critical event in neuronal apoptosis: regulation by neuroprotectants, BCL-2, and caspases. J Neurosci 19:7476-7485.

Putcha GV, Deshmukh M, Johnson EM, Jr. (2000) Inhibition of apoptotic signaling cascades causes loss of trophic factor dependence during neuronal maturation. J Cell Biol 149:1011-1018.

Putcha GV, Moulder KL, Golden JP, Bouillet P, Adams JA, Strasser A, Johnson EM (2001) Induction of BIM, a proapoptotic BH3-only BCL-2 family member, is critical for neuronal apoptosis. Neuron 29:615-628.

Putcha GV, Le S, Frank S, Besirli CG, Clark K, Chu B, Alix S, Youle RJ, LaMarche A, Maroney AC, Johnson EM, Jr. (2003) JNK-mediated BIM phosphorylation potentiates BAX-dependent apoptosis. Neuron 38:899-914.

Qin ZH, Chen RW, Wang Y, Nakai M, Chuang DM, Chase TN (1999) Nuclear factor kappaB nuclear translocation upregulates c-Myc and p53 expression during NMDA receptor-mediated apoptosis in rat striatum. J Neurosci 19:4023-4033.

Rabizadeh S, Oh J, Zhong LT, Yang J, Bitler CM, Butcher LL, Bredesen DE (1993) Induction of apoptosis by the low-affinity NGF receptor. Science 261:345-348.

Radeke MJ, Misko TP, Hsu C, Herzenberg LA, Shooter EM (1987) Gene transfer and molecular cloning of the rat nerve growth factor receptor. Nature 325:593-597.

Raff M (1998) Cell suicide for beginners. Nature 396:119-122.

Raivich G, Bohatschek M, Da Costa C, Iwata O, Galiano M, Hristova M, Nateri AS, Makwana M, Riera-Sans L, Wolfer DP, Lipp HP, Aguzzi A, Wagner EF, Behrens A (2004) The AP-1 transcription factor c-Jun is required for efficient axonal regeneration. Neuron 43:57-67.

Raoul C, Henderson CE, Pettmann B (1999) Programmed cell death of embryonic motoneurons triggered through the Fas death receptor. J Cell Biol 147:1049-1062.

Riccio A, Ahn S, Davenport CM, Blendy JA, Ginty DD (1999) Mediation by a CREB family transcription factor of NGF-dependent survival of sympathetic neurons. Science 286:2358-2361.

Robertson AG, Banfield MJ, Allen SJ, Dando JA, Mason GG, Tyler SJ, Bennett GS, Brain SD, Clarke AR, Naylor RL, Wilcock GK, Brady RL, Dawbarn D (2001) Identification and structure of the nerve growth factor binding site on TrkA. Biochem Biophys Res Commun 282:131-141.

Rodriguez-Viciana P, Warne PH, Dhand R, Vanhaesebroeck B, Gout I, Fry MJ, Waterfield MD, Downward J (1994) Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature 370:527-532.

Ross GM, Shamovsky IL, Lawrance G, Solc M, Dostaler SM, Weaver DF, Riopelle RJ (1998) Reciprocal modulation of TrkA and p75NTR affinity states is mediated by direct receptor interactions. Eur J Neurosci 10:890-898.

Roux PP, Colicos MA, Barker PA, Kennedy TE (1999) p75 neurotrophin receptor expression is induced in apoptotic neurons after seizure. J Neurosci 19:6887-6896.

Roux PP, Dorval G, Boudreau M, Angers-Loustau A, Morris SJ, Makkerh J, Barker PA (2002) K252a and CEP1347 are neuroprotective compounds that inhibit mixed-lineage kinase-3 and induce activation of Akt and ERK. J Biol Chem 277:49473-49480.

Ruit KG, Osborne PA, Schmidt RE, Johnson EM, Jr., Snider WD (1990) Nerve growth factor regulates sympathetic ganglion cell morphology and survival in the adult mouse. J Neurosci 10:2412-2419.

Ruit KG, Snider WD (1991) Administration or deprivation of nerve growth factor during development permanently alters neuronal geometry. J Comp Neurol 314:106-113.

Sah VP, Attardi LD, Mulligan GJ, Williams BO, Bronson RT, Jacks T (1995) A subset of p53-deficient embryos exhibit exencephaly. Nat Genet 10:175-180.

Sakhi S, Bruce A, Sun N, Tocco G, Baudry M, Schreiber SS (1994) p53 induction is associated with neuronal damage in the central nervous system. Proc Natl Acad Sci U S A 91:7525-7529.

Sakhi S, Gilmore W, Tran ND, Schreiber SS (1996) p53-deficient mice are protected against adrenalectomy-induced apoptosis. Neuroreport 8:233-235.

Sakhi S, Sun N, Wing LL, Mehta P, Schreiber SS (1996) Nuclear accumulation of p53 protein following kainic acid-induced seizures. Neuroreport 7:493-496.

Salehi AH, Roux PP, Kubu CJ, Zeindler C, Bhakar A, Tannis LL, Verdi JM, Barker PA (2000) NRAGE, a novel MAGE protein, interacts with the p75 neurotrophin receptor and facilitates nerve growth factor-dependent apoptosis. Neuron 27:279-288.

Salehi AH, Xanthoudakis S, Barker PA (2002) NRAGE, a p75 neurotrophin receptor-interacting protein, induces caspase activation and cell death through a JNK-dependent mitochondrial pathway. J Biol Chem 277:48043-48050.

Sarmiere PD, Freeman RS (2001) Analysis of the NF-kappa B and PI 3-kinase/Akt survival pathways in nerve growth factor-dependent neurons. Mol Cell Neurosci 18:320-331.

Schuler M, Bossy-Wetzel E, Goldstein JC, Fitzgerald P, Green DR (2000) p53 induces apoptosis by caspase activation through mitochondrial cytochrome c release. J Biol Chem 275:7337-7342.

Scorrano L, Korsmeyer SJ (2003) Mechanisms of cytochrome c release by proapoptotic BCL-2 family members. Biochem Biophys Res Commun 304:437-444.

Seidah NG, Benjannet S, Pareek S, Chretien M, Murphy RA (1996) Cellular processing of the neurotrophin precursors of NT3 and BDNF by the mammalian proprotein convertases. FEBS Lett 379:247-250.

Sendtner M, Kreutzberg GW, Thoenen H (1990) Ciliary neurotrophic factor prevents the degeneration of motor neurons after axotomy. Nature 345:440-441.

Sendtner M, Holtmann B, Kolbeck R, Thoenen H, Barde YA (1992) Brain-derived neurotrophic factor prevents the death of motoneurons in newborn rats after nerve section. Nature 360:757-759.

Senger DL, Campenot RB (1997) Rapid retrograde tyrosine phosphorylation of trkA and other proteins in rat sympathetic neurons in compartmented cultures. J Cell Biol 138:411-421.

Serber Z, Lai HC, Yang A, Ou HD, Sigal MS, Kelly AE, Darimont BD, Duijf PH, Van Bokhoven H, McKeon F, Dotsch V (2002) A C-terminal inhibitory domain controls the activity of p63 by an intramolecular mechanism. Mol Cell Biol 22:8601-8611.

Shen S, Wiemelt AP, McMorris FA, Barres BA (1999) Retinal ganglion cells lose trophic responsiveness after axotomy. Neuron 23:285-295.

Sherr CJ, Weber JD (2000) The ARF/p53 pathway. Curr Opin Genet Dev 10:94-99.

Shimada A, Kato S, Enjo K, Osada M, Ikawa Y, Kohno K, Obinata M, Kanamaru R, Ikawa S, Ishioka C (1999) The transcriptional activities of p53 and its homologue p51/p63: similarities and differences. Cancer Res 59:2781-2786.

Shindler KS, Yunker AM, Cahn R, Zha J, Korsmeyer SJ, Roth KA (1998) Trophic support promotes survival of bcl-x-deficient telencephalic cells in vitro. Cell Death Differ 5:901-910.

Skaper SD, Floreani M, Negro A, Facci L, Giusti P (1998) Neurotrophins rescue cerebellar granule neurons from oxidative stress-mediated apoptotic death: selective involvement of phosphatidylinositol 3-kinase and the mitogen-activated protein kinase pathway. J Neurochem 70:1859-1868.

Slack RS, Belliveau DJ, Rosenberg M, Atwal J, Lochmuller H, Aloyz R, Haghighi A, Lach B, Seth P, Cooper E, Miller FD (1996) Adenovirus-mediated gene transfer of the tumor suppressor, p53, induces apoptosis in postmitotic neurons. J Cell Biol 135:1085-1096.

Smeyne RJ, Klein R, Schnapp A, Long LK, Bryant S, Lewin A, Lira SA, Barbacid M (1994) Severe sensory and sympathetic neuropathies in mice carrying a disrupted Trk/NGF receptor gene. Nature 368:246-249.

Snider WD, Elliott JL, Yan Q (1992) Axotomy-induced neuronal death during development. J Neurobiol 23:1231-1246.

Snider WD (1994) Functions of the neurotrophins during nervous system development: what the knockouts are teaching us. Cell 77:627-638.

Soilu-Hanninen M, Ekert P, Bucci T, Syroid D, Bartlett PF, Kilpatrick TJ (1999) Nerve growth factor signaling through p75 induces apoptosis in Schwann cells via a Bcl-2-independent pathway. J Neurosci 19:4828-4838.

Soppet D, Escandon E, Maragos J, Middlemas DS, Reid SW, Blair J, Burton LE, Stanton BR, Kaplan DR, Hunter T, et al. (1991) The neurotrophic factors brain-derived neurotrophic factor and neurotrophin-3 are ligands for the trkB tyrosine kinase receptor. Cell 65:895-903.

Stockinger W, Brandes C, Fasching D, Hermann M, Gotthardt M, Herz J, Schneider WJ, Nimpf J (2000) The reelin receptor ApoER2 recruits JNK-interacting proteins-1 and -2. J Biol Chem 275:25625-25632.

Stucky CL, Koltzenburg M (1997) The low-affinity neurotrophin receptor p75 regulates the function but not the selective survival of specific subpopulations of sensory neurons. J Neurosci 17:4398-4405.

Syroid DE, Maycox PJ, Soilu-Hanninen M, Petratos S, Bucci T, Burrola P, Murray S, Cheema S, Lee KF, Lemke G, Kilpatrick TJ (2000) Induction of postnatal schwann cell death by the low-affinity neurotrophin receptor in vitro and after axotomy. J Neurosci 20:5741-5747.

Tan Z, Sankar R, Shin D, Sun N, Liu H, Wasterlain CG, Schreiber SS (2002) Differential induction of p53 in immature and adult rat brain following lithium-pilocarpine status epilepticus. Brain Res 928:187-193.

Tandrup T, Woolf CJ, Coggeshall RE (2000) Delayed loss of small dorsal root ganglion cells after transection of the rat sciatic nerve. J Comp Neurol 422:172-180.

Tapley P, Lamballe F, Barbacid M (1992) K252a is a selective inhibitor of the tyrosine protein kinase activity of the trk family of oncogenes and neurotrophin receptors. Oncogene 7:371-381.

Tessarollo L, Vogel KS, Palko ME, Reid SW, Parada LF (1994) Targeted mutation in the neurotrophin-3 gene results in loss of muscle sensory neurons. Proc Natl Acad Sci U S A 91:11844-11848.

Tessarollo L, Tsoulfas P, Donovan MJ, Palko ME, Blair-Flynn J, Hempstead BL, Parada LF (1997) Targeted deletion of all isoforms of the trkC gene suggests the use of alternate receptors by its ligand neurotrophin-3 in neuronal development and implicates trkC in normal cardiogenesis. Proc Natl Acad Sci U S A 94:14776-14781.

Thanos CD, Bowie JU (1999) p53 Family members p63 and p73 are SAM domain-containing proteins. Protein Sci 8:1708-1710.

Thoenen H, Barde YA (1980) Physiology of nerve growth factor. Physiol Rev 60:1284-1335.

Thompson NA, Haefliger JA, Senn A, Tawadros T, Magara F, Ledermann B, Nicod P, Waeber G (2001) Islet-brain1/JNK-interacting protein-1 is required for early embryogenesis in mice. J Biol Chem 276:27745-27748.

Toma JG, El-Bizri H, Barnabe-Heider F, Aloyz R, Miller FD (2000) Evidence that helix-loop-helix proteins collaborate with retinoblastoma tumor suppressor protein to regulate cortical neurogenesis. J Neurosci 20:7648-7656.

Tong JX, Vogelbaum MA, Drzymala RE, Rich KM (1997) Radiation-induced apoptosis in dorsal root ganglion neurons. J Neurocytol 26:771-777.

Tournier C, Hess P, Yang DD, Xu J, Turner TK, Nimnual A, Bar-Sagi D, Jones SN, Flavell RA, Davis RJ (2000) Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. Science 288:870-874.

Tournier C, Dong C, Turner TK, Jones SN, Flavell RA, Davis RJ (2001) MKK7 is an essential component of the JNK signal transduction pathway activated by proinflammatory cytokines. Genes Dev 15:1419-1426.

Tsoulfas P, Soppet D, Escandon E, Tessarollo L, Mendoza-Ramirez JL, Rosenthal A, Nikolics K, Parada LF (1993) The rat trkC locus encodes multiple neurogenic receptors that exhibit differential response to neurotrophin-3 in PC12 cells. Neuron 10:975-990.

Tsui-Pierchala BA, Ginty DD (1999) Characterization of an NGF-P-TrkA retrograde-signaling complex and age-dependent regulation of TrkA phosphorylation in sympathetic neurons. J Neurosci 19:8207-8218.

Vaillant AR, Mazzoni I, Tudan C, Boudreau M, Kaplan DR, Miller FD (1999) Depolarization and neurotrophins converge on the phosphatidylinositol 3-kinase-Akt pathway to synergistically regulate neuronal survival. J Cell Biol 146:955-966.

Vanderluit JL, McPhail LT, Fernandes KJ, McBride CB, Huguenot C, Roy S, Robertson GS, Nicholson DW, Tetzlaff W (2000) Caspase-3 is activated following axotomy of neonatal facial motoneurons and caspase-3 gene deletion delays axotomy-induced cell death in rodents. Eur J Neurosci 12:3469-3480.

Verdi JM, Ip N, Yancopoulos GD, Anderson DJ (1994) Expression of trk in MAH cells lacking the p75 low-affinity nerve growth factor receptor is sufficient to permit nerve growth factor-induced differentiation to postmitotic neurons. Proc Natl Acad Sci U S A 91:3949-3953.

Verhey KJ, Meyer D, Deehan R, Blenis J, Schnapp BJ, Rapoport TA, Margolis B (2001) Cargo of kinesin identified as JIP scaffolding proteins and associated signaling molecules. J Cell Biol 152:959-970.

Virdee K, Bannister AJ, Hunt SP, Tolkovsky AM (1997) Comparison between the timing of JNK activation, c-Jun phosphorylation, and onset of death commitment in sympathetic neurones. J Neurochem 69:550-561.

Vogel KS, Parada LF (1998) Sympathetic neuron survival and proliferation are prolonged by loss of p53 and neurofibromin. Mol Cell Neurosci 11:19-28.

Vogelbaum MA, Tong JX, Rich KM (1998) Developmental regulation of apoptosis in dorsal root ganglion neurons. J Neurosci 18:8928-8935.

Vogelstein B, Lane D, Levine AJ (2000) Surfing the p53 network. Nature 408:307-310.

von Bartheld CS, Kinoshita Y, Prevette D, Yin QW, Oppenheim RW, Bothwell M (1994) Positive and negative effects of neurotrophins on the isthmo-optic nucleus in chick embryos. Neuron 12:639-654.

von Schack D, Casademunt E, Schweigreiter R, Meyer M, Bibel M, Dechant G (2001) Complete ablation of the neurotrophin receptor p75NTR causes defects both in the nervous and the vascular system. Nat Neurosci 4:977-978.

Wallach D, Boldin M, Goncharov T, Goltsev Y, Mett I, Malinin N, Adar R, Kovalenko A, Varfolomeev E (1996) Exploring cell death mechanisms by analyzing signaling cascades of the TNF/NGF receptor family. Behring Inst Mitt:144-155.

Walsh GS, Krol KM, Kawaja MD (1999) Absence of the p75 neurotrophin receptor alters the pattern of sympathosensory sprouting in the trigeminal ganglia of mice overexpressing nerve growth factor. J Neurosci 19:258-273.

Wang Y, Qin ZH, Nakai M, Chen RW, Chuang DM, Chase TN (1999) Co-stimulation of cyclic-AMP-linked metabotropic glutamate receptors in rat striatum attenuates excitotoxin-induced nuclear factor-kappaB activation and apoptosis. Neuroscience 94:1153-1162.

Watanabe H, Ohta S, Kumon Y, Sakaki S, Sakanaka M (1999) Increase in p53 protein expression following cortical infarction in the spontaneously hypertensive rat. Brain Res 837:38-45.

Watson FL, Heerssen HM, Moheban DB, Lin MZ, Sauvageot CM, Bhattacharyya A, Pomeroy SL, Segal RA (1999) Rapid nuclear responses to target-derived neurotrophins require retrograde transport of ligand-receptor complex. J Neurosci 19:7889-7900.

Watson FL, Heerssen HM, Bhattacharyya A, Klesse L, Lin MZ, Segal RA (2001) Neurotrophins use the Erk5 pathway to mediate a retrograde survival response. Nat Neurosci 4:981-988.

Weston CR, Wong A, Hall JP, Goad ME, Flavell RA, Davis RJ (2004) The c-Jun NH2-terminal kinase is essential for epidermal growth factor expression during epidermal morphogenesis. Proc Natl Acad Sci U S A 101:14114-14119.

White FA, Keller-Peck CR, Knudson CM, Korsmeyer SJ, Snider WD (1998) Widespread elimination of naturally occurring neuronal death in Bax-deficient mice. J Neurosci 18:1428-1439.

Whitfield J, Neame SJ, Paquet L, Bernard O, Ham J (2001) Dominant-negative c-Jun promotes neuronal survival by reducing BIM expression and inhibiting mitochondrial cytochrome c release. Neuron 29:629-643.

Whitmarsh AJ, Cavanagh J, Tournier C, Yasuda J, Davis RJ (1998) A mammalian scaffold complex that selectively mediates MAP kinase activation. Science 281:1671-1674.

Whitmarsh AJ, Davis RJ (1998) Structural organization of MAP-kinase signaling modules by scaffold proteins in yeast and mammals. Trends Biochem Sci 23:481-485.

Whitmarsh AJ, Kuan CY, Kennedy NJ, Kelkar N, Haydar TF, Mordes JP, Appel M, Rossini AA, Jones SN, Flavell RA, Rakic P, Davis RJ (2001) Requirement of the JIP1 scaffold protein for stress-induced JNK activation. Genes Dev 15:2421-2432.

Wiese S, Digby MR, Gunnersen JM, Gotz R, Pei G, Holtmann B, Lowenthal J, Sendtner M (1999a) The anti-apoptotic protein ITA is essential for NGF-mediated survival of embryonic chick neurons. Nat Neurosci 2:978-983.

Wiese S, Metzger F, Holtmann B, Sendtner M (1999b) The role of p75NTR in modulating neurotrophin survival effects in developing motoneurons. Eur J Neurosci 11:1668-1676.

Wolf DE, McKinnon CA, Daou MC, Stephens RM, Kaplan DR, Ross AH (1995) Interaction with TrkA immobilizes gp75 in the high affinity nerve growth factor receptor complex. J Biol Chem 270:2133-2138.

Wood KA, Youle RJ (1994) Apoptosis and free radicals. Ann N Y Acad Sci 738:400-407.

Wyatt S, Davies AM (1995) Regulation of nerve growth factor receptor gene expression in sympathetic neurons during development. J Cell Biol 130:1435-1446.

Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 270:1326-1331.

Xiang H, Hochman DW, Saya H, Fujiwara T, Schwartzkroin PA, Morrison RS (1996) Evidence for p53-mediated modulation of neuronal viability. J Neurosci 16:6753-6765.

Xiang H, Kinoshita Y, Knudson CM, Korsmeyer SJ, Schwartzkroin PA, Morrison RS (1998) Bax involvement in p53-mediated neuronal cell death. J Neurosci 18:1363-1373.

Xu Z, Maroney AC, Dobrzanski P, Kukekov NV, Greene LA (2001) The MLK family mediates c-Jun N-terminal kinase activation in neuronal apoptosis. Mol Cell Biol 21:4713-4724.

Xu Z, Kukekov NV, Greene LA (2003) POSH acts as a scaffold for a multiprotein complex that mediates JNK activation in apoptosis. Embo J 22:252-261.

Yang E, Korsmeyer SJ (1996) Molecular thanatopsis: a discourse on the BCL2 family and cell death. Blood 88:386-401.

Yang DD, Kuan CY, Whitmarsh AJ, Rincon M, Zheng TS, Davis RJ, Rakic P, Flavell RA (1997) Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. Nature 389:865-870.

Yang A, Kaghad M, Wang Y, Gillett E, Fleming MD, Dotsch V, Andrews NC, Caput D, McKeon F (1998) p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. Mol Cell 2:305-316.

Yang A, Schweitzer R, Sun D, Kaghad M, Walker N, Bronson RT, Tabin C, Sharpe A, Caput D, Crum C, McKeon F (1999) p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. Nature 398:714-718.

Yang A, Walker N, Bronson R, Kaghad M, Oosterwegel M, Bonnin J, Vagner C, Bonnet H, Dikkes P, Sharpe A, McKeon F, Caput D (2000) p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours. Nature 404:99-103.

Yano H, Chao MV (2000) Neurotrophin receptor structure and interactions. Pharm Acta Helv 74:253-260.

Yao R, Cooper GM (1995) Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. Science 267:2003-2006.

Yasuda J, Whitmarsh AJ, Cavanagh J, Sharma M, Davis RJ (1999) The JIP group of mitogen-activated protein kinase scaffold proteins. Mol Cell Biol 19:7245-7254.

Ye X, Mehlen P, Rabizadeh S, VanArsdale T, Zhang H, Shin H, Wang JJ, Leo E, Zapata J, Hauser CA, Reed JC, Bredesen DE (1999) TRAF family proteins interact with the common neurotrophin receptor and modulate apoptosis induction. J Biol Chem 274:30202-30208.

Ye H, Kuruvilla R, Zweifel LS, Ginty DD (2003) Evidence in support of signaling endosome-based retrograde survival of sympathetic neurons. Neuron 39:57-68.

Yeiser EC, Rutkoski NJ, Naito A, Inoue J, Carter BD (2004) Neurotrophin signaling through the p75 receptor is deficient in traf6-/- mice. J Neurosci 24:10521-10529.

Yeo TT, Chua-Couzens J, Butcher LL, Bredesen DE, Cooper JD, Valletta JS, Mobley WC, Longo FM (1997) Absence of p75NTR causes increased basal forebrain cholinergic neuron size, choline acetyltransferase activity, and target innervation. J Neurosci 17:7594-7605.

Yip HK, Rich KM, Lampe PA, Johnson EM, Jr. (1984) The effects of nerve growth factor and its antiserum on the postnatal development and survival after injury of sensory neurons in rat dorsal root ganglia. J Neurosci 4:2986-2992.

Yoon SO, Casaccia-Bonnefil P, Carter B, Chao MV (1998) Competitive signaling between TrkA and p75 nerve growth factor receptors determines cell survival. J Neurosci 18:3273-3281.

Yoshida H, Kong YY, Yoshida R, Elia AJ, Hakem A, Hakem R, Penninger JM, Mak TW (1998) Apaf1 is required for mitochondrial pathways of apoptosis and brain development. Cell 94:739-750.

Yuan J, Yankner BA (2000) Apoptosis in the nervous system. Nature 407:802-809.

Yuan J, Lipinski M, Degterev A (2003) Diversity in the mechanisms of neuronal cell death. Neuron 40:401-413.

Zaika AI, Slade N, Erster SH, Sansome C, Joseph TW, Pearl M, Chalas E, Moll UM (2002) DeltaNp73, a dominant-negative inhibitor of wild-type p53 and TAp73, is up-regulated in human tumors. J Exp Med 196:765-780.

Zhu J, Jiang J, Zhou W, Chen X (1998) The potential tumor suppressor p73 differentially regulates cellular p53 target genes. Cancer Res 58:5061-5065.

Zou H, Henzel WJ, Liu X, Lutschg A, Wang X (1997) Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell 90:405-413.

APPENDIX A

Majdan, M., **Walsh, G.S.,** Aloyz, R., Miller, F.D. (2001) TrkA mediates developmental sympathetic neuron survival in vivo by silencing an ongoing p75NTR-mediated death signal. *Journal of Cell Biology* 155:1275-85.

JCB Article

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

Marta Majdan, Gregory S. Walsh, Raquel Aloyz, and Freda D. Miller

Center for Neuronal Survival, Montreal Neurological Institute, McGill University, Montreal, Canada H3A 2B4

Developmental 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$^{-/-}$ and TrkA$^{-/-}$ mice indicate that the coincident absence of p75NTR substantially rescues TrkA$^{-/-}$ 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$^{-/-}$ sympathetic neurons die during the late neonatal and early postnatal periods (Smeyne et al., 1994; Fagan et al., 1996); (b) in p75NTR$^{-/-}$ 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$^{-/-}$ 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$^{-/-}$ and NT-3$^{-/-}$ 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.

Address correspondence to Freda D. Miller, Ph.D., Professor, Center for Neuronal Survival, Montreal Neurological Institute, McGill University, 3801 rue University, Montreal, Quebec, Canada H3A 2B4. Tel.: (514) 398-4261. Fax: (514) 398-1319. E-mail: mdfm@musica.mcgill.ca

M. Majdan and G.S. Walsh contributed equally to this study.

*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-Bonnefil 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 $p75NTR^{-/-}$ and $TrkA^{-/-}$ mice demonstrated that the coincident absence of p75NTR substantially rescued the sympathetic neuron apoptosis observed in $TrkA^{-/-}$ 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 $p75NTR^{-/-}$ 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 the ganglion. To distinguish between these possibilities, we assessed apoptosis and proliferation in $pT5NTR^{-/-}$ versus wild-type SCG.

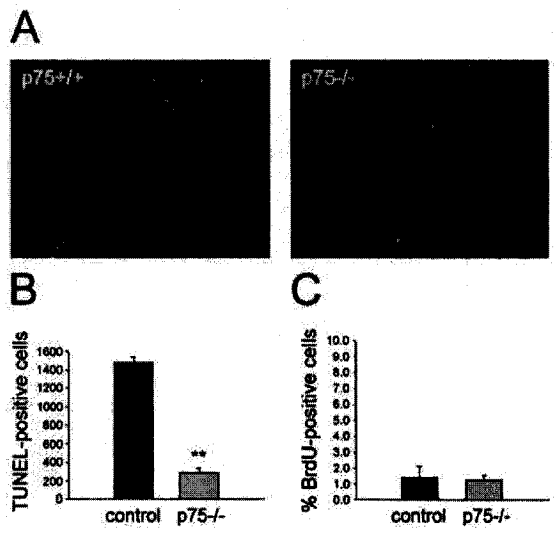


Figure 1. **The increase in sympathetic neuron number in the neonatal $p75NTR^{-/-}$ SCG is due to reduced apoptosis, not increased proliferation.** (A) Fluorescence photomicrographs of TUNEL analysis of representative sections through the P2 SCG of $p75NTR^{+/+}$ and $p75NTR^{-/-}$ animals. (B) Quantitation of TUNEL analysis similar to that seen in A. Numbers represent the total mean number of apoptotic nuclei in the SCG of $p75NTR^{+/+}$ (control) versus $p75NTR^{-/-}$ ($p75^{-/-}$) animals. (**$P < 0.0005$, $n = 3$). (C) Percentage of BrdU-positive cells with neuronal morphology in the $p75NTR^{+/+}$ (control) versus $p75NTR^{-/-}$ ($p75^{-/-}$) 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.

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 ganglia. As predicted, many TUNEL-positive nuclei were detected within the wild-type SCG; 1,472 ± 60 per ganglion ($n = 3$). In contrast, the $p75NTR^{-/-}$ 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 $p75NTR^{-/-}$ versus $p75NTR^{+/+}$ ganglia. To examine the extent of ongoing cell division, $p75NTR^{+/+}$ and $p75NTR^{-/-}$ 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 $p75NTR^{+/+}$ and $p75NTR^{-/-}$ ganglia (1.43 ± 0.7%, $n = 3$ and 1.25 ± 0.3%, $n = 3$, 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

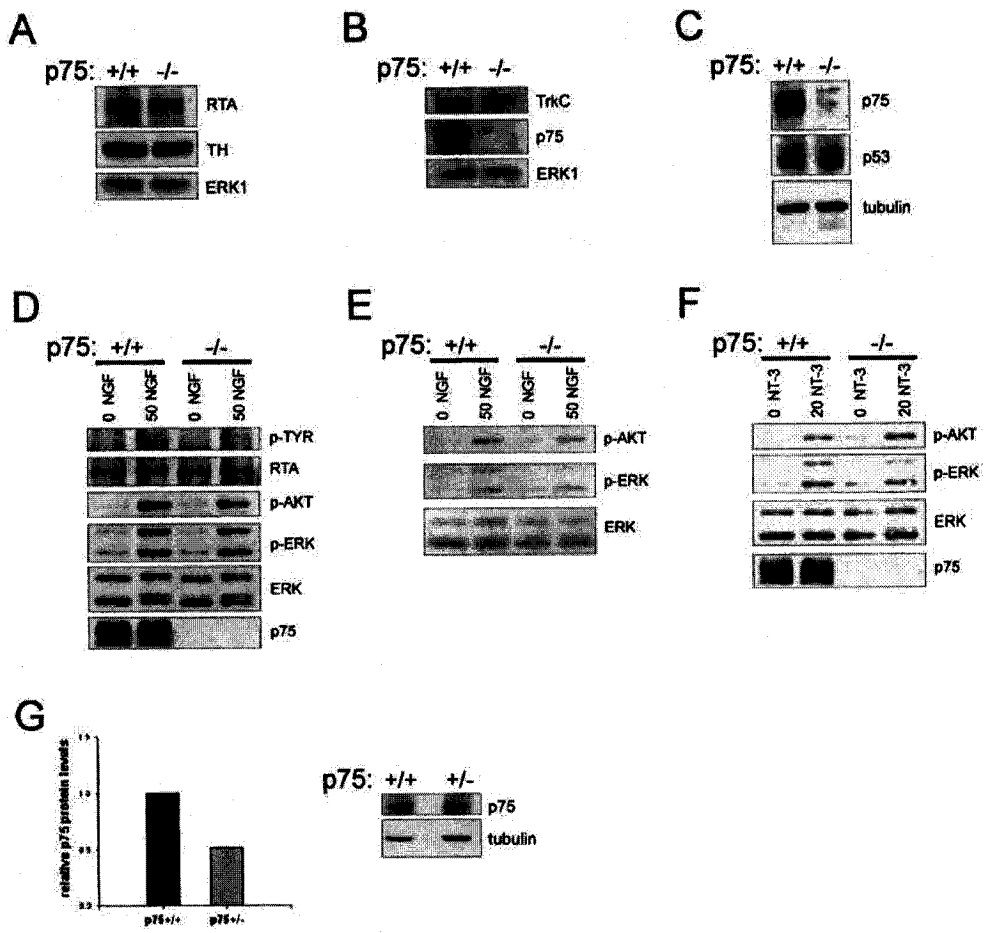


Figure 2. **Levels of Trk receptors, Trk receptor activation, and downstream survival signaling in p75NTR$^{-/-}$ SCG neurons.** (A) Western blot analysis of equal amounts of protein from p75NTR$^{-/-}$ versus p75NTR$^{+/+}$ SCG at P7, probed for TrkA (RTA), tyrosine hydroxylase (TH) and ERK1. (B) Western blot analysis of lysates of P7 p75NTR$^{-/-}$ versus p75NTR$^{+/+}$ 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$^{-/-}$ versus p75NTR$^{+/+}$ ganglia probed for p75NTR (p75), p53, or for total tubulin. (D) Western blot analysis of equal amounts of protein from cultured p75NTR$^{-/-}$ versus p75NTR$^{+/+}$ 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$^{-/-}$ versus p75NTR$^{+/+}$ 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$^{+/+}$ versus p75NTR$^{+/-}$ 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$^{-/-}$ sympathetic neurons

Three potential explanations for the deficit in apoptosis observed in p75NTR$^{-/-}$ 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 signaling 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$^{-/-}$ ganglia and cultured p75NTR$^{-/-}$ neurons. Initially, we examined levels of TrkA and TrkC in p75NTR$^{-/-}$ 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$^{-/-}$ 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$^{-/-}$ SCG is not due to an increase in Trk receptor levels.

We also compared a number of additional proteins in the p75NTR$^{-/-}$ and p75NTR$^{+/+}$ 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$^{-/-}$ animals examined in this study (Von Schack et al., 2001). No p75NTR protein corresponding to this smaller variant was detectable either in the p75NTR$^{-/-}$ ganglia or in cultured p75NTR$^{-/-}$ 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$^{-/-}$ and p75NTR$^{+/+}$ 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$^{-/-}$ ganglia (Fig. 2 C).

Because examination of the p75NTR$^{-/-}$ 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$^{-/-}$ and p75NTR$^{+/+}$ 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$^{+/+}$ and p75NTR$^{-/-}$ 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$^{-/-}$ and p75NTR$^{+/+}$ 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$^{-/-}$ sympathetic neurons have

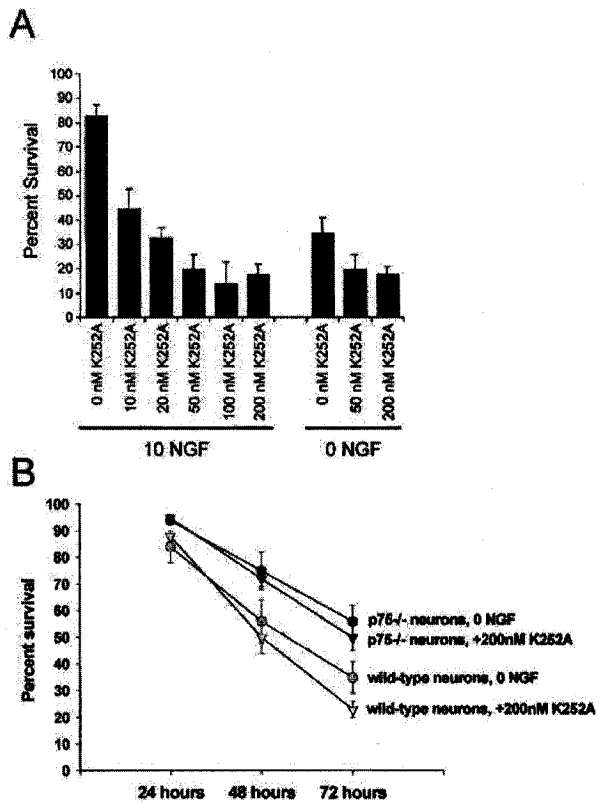


Figure 3. **Cultured p75NTR$^{-/-}$ neurons show enhanced survival in the absence of all Trk signaling.** (A) Percentage survival of mouse sympathetic neurons switched for 72 h into varying concentrations of K252A in the presence or absence of 10 ng/ml NGF. Results are normalized so that the number of 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$^{-/-}$ versus p75NTR$^{+/+}$ (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 ± 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$^{-/-}$ and p75NTR$^{+/+}$ 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 reprobed 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

$p75NTR^{+/+}$ and $p75NTR^{-/-}$ 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 $p75NTR^{-/-}$ neurons show enhanced survival in the absence of all Trk signaling

Together, these data suggest that the enhanced survival observed in $p75NTR^{-/-}$ neurons is not due to enhanced Trk signaling. However, to formally rule out this possibility, we cultured $p75NTR^{-/-}$ 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 $p75NTR^{+/+}$ versus $p75NTR^{-/-}$ 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 $p75NTR^{-/-}$ neurons died significantly more slowly in the absence of NGF (Fig. 3 B). Specifically, 24 h after NGF withdrawal, 94 ± 1% of $p75NTR^{-/-}$ neurons were still alive, compared with 84 ± 6% of wild-type neurons ($P = 0.07$). By 48 h, 75 ± 7% of $p75NTR^{-/-}$ neurons were still alive, compared with 56 ± 8% of control neurons ($P = 0.06$). By 72 h, 56 ± 6% of $p75NTR^{-/-}$ 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 $p75NTR^{-/-}$ neurons. Specifically, 24 h after NGF withdrawal, 95 ± 1% of $p75NTR^{-/-}$ neurons were still alive, compared with 88 ± 2% of controls ($P < 0.05$), and by 48 h 72 ± 3% of $p75NTR^{-/-}$ neurons were alive, versus 50 ± 6% of the wild-type neurons ($P < 0.05$). By 72 h, 50 ± 5% of the $p75NTR^{-/-}$ 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 $TrkA^{-/-}$ sympathetic neuron apoptosis in vivo

Although the data presented here on $p75NTR^{-/-}$ 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 $p75NTR^{-/-}$ and $TrkA^{-/-}$ animals; sympathetic neurons undergo a rapid apoptotic death in $TrkA^{-/-}$ 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 $TrkA^{-/-}$ phenotype. If, in contrast, p75NTR mediates neuronal apoptosis in a Trk-independent fashion, then the severe neuronal loss seen in $TrkA^{-/-}$ SCG would be at least partially rescued in the absence of p75NTR.

To perform these studies, we initially confirmed the $p75NTR^{-/-}$ phenotype in the mixed C129/C57BL6 background that resulted from these crosses. $TrkA^{+/-}$ and

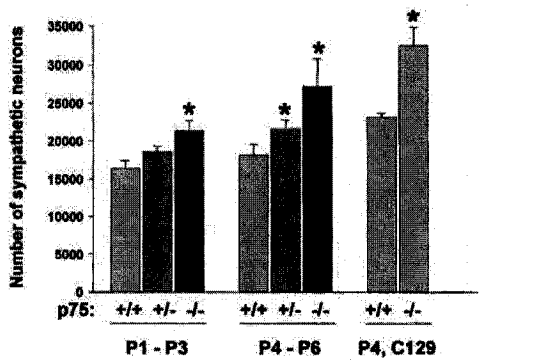


Figure 4. **Sympathetic neuron number is increased in the developing C129/C57Bl6 SCG as an inverse function of p75NTR gene dosage.** Sympathetic neuron number in the SCG of $p75NTR^{+/+}$, $p75NTR^{+/-}$, and $p75NTR^{-/-}$ animals at ages P1–P3 and P4–P6. Results represent mean ± standard error ($n = 3$–7 for each genotype). At P1–P3, $p75NTR^{+/+}$ and $p75NTR^{-/-}$ SCG numbers are significantly different (*$P < 0.05$), and at P4–P6, $p75NTR^{+/+}$ neuron counts are significantly different from the $p75NTR^{+/-}$ and $p75NTR^{-/-}$ counts (*$P < 0.05$ for both groups). For comparison, neuronal numbers from the P4 SCG of $p75NTR^{+/+}$ and $p75NTR^{-/-}$ C129 animals are shown (Bamji et al., 1998).

p75NTR$^{-/-}$ animals were crossed, and then their TrkA$^{+/-}$, p75NTR$^{+/-}$ progeny were bred to produce animals for analysis. The SCGs were analyzed from the resultant TrkA$^{+/+}$, p75NTR$^{+/+}$, p75NTR$^{+/-}$, and p75NTR$^{-/-}$ animals; ganglia were grouped by age, P1–P3 and P4–P6. This analysis revealed an increase in the numbers of neurons in the p75NTR$^{-/-}$ versus p75NTR$^{+/+}$ 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$^{+/-}$ 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$^{+/+}$ and p75NTR$^{+/-}$ animals at P7 (Fig. 2 G). This analysis revealed that p75NTR levels were lower in the p75NTR$^{+/-}$ animals, correlating with the observed increase in neuronal survival.

We then characterized neuronal numbers in the SCG of the p75NTR$^{+/+}$, TrkA$^{+/+}$, TrkA$^{+/-}$, and TrkA$^{-/-}$ animals over this same time frame. This analysis revealed that, as previously reported (Fagan et al., 1996), there was a dramatic decrease in the number of SCG neurons at P1–P3 in the TrkA$^{-/-}$ animals relative to their TrkA$^{+/+}$ littermates (6,108 ± 411, n = 3 versus 16,390 ± 1,003, n = 5). By P4–P6, the neuronal loss in the TrkA$^{-/-}$ 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$^{-/-}$ ganglia at P1–P3 and P4–P6, respectively.

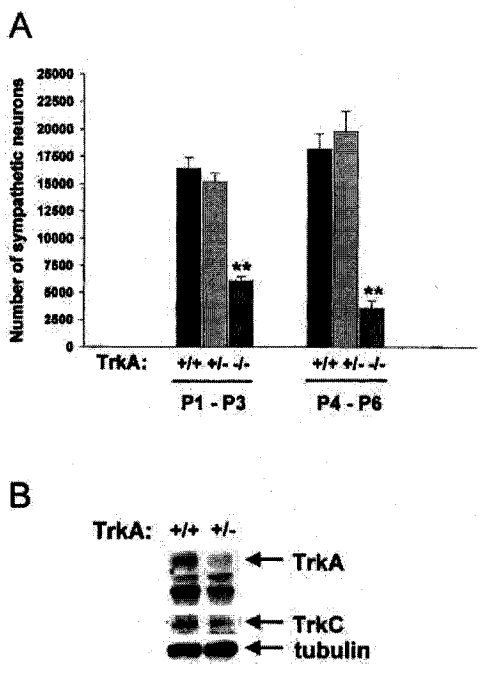


Figure 5. **Analysis of sympathetic neuron number in TrkA$^{+/+}$, TrkA$^{+/-}$, and TrkA$^{-/-}$ SCG.** (A) Sympathetic neuron number in the SCG of TrkA$^{+/+}$, TrkA$^{+/-}$, and TrkA$^{-/-}$ animals at ages P1–P3 and P4–P6 in the C129/C57BL6 background. Results represent mean ± standard error (n =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

Interestingly, quantitation of sympathetic neurons in the TrkA$^{+/-}$ SCG revealed no significant difference between numbers in TrkA$^{+/+}$ and TrkA$^{+/-}$ ganglia at these two time points (P1–P3: 15,176 ± 796, n = 6; P4–P6: 19,777 ± 1880, n = 3 for the TrkA$^{+/-}$ 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$^{+/+}$ and TrkA$^{+/-}$ 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$^{-/-}$ SCG. At P1–P3, the concomitant absence of p75NTR almost completely rescued sympathetic neuron numbers in the TrkA$^{-/-}$, p75NTR$^{-/-}$ SCG; 13,665 ± 730 neurons (n = 3) versus 16,390 ± 1,003 neurons (n = 5) in the TrkA$^{+/+}$, p75NTR$^{+/+}$ SCG (Fig. 6 A). Moreover, at this age, even the loss of one p75NTR allele was enough to significantly increase neuronal survival; the TrkA$^{-/-}$, p75NTR$^{+/-}$ SCG contained 11,000 neurons, whereas the TrkA$^{-/-}$, p75NTR$^{+/+}$ 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$^{-/-}$, p75NTR$^{+/+}$ SCG (Fig. 6 A). Similarly, a rescue was still observed in the TrkA$^{-/-}$, p75NTR$^{+/-}$ ganglia at P4–P6, although this was of a lesser magnitude than that observed in the p75NTR$^{-/-}$ 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$^{-/-}$ animals, and most died within the first three postnatal days. Moreover, cresyl violet–stained sections of P4 wild-type and TrkA$^{-/-}$, p75NTR$^{-/-}$ 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$^{-/-}$ cells (Deckwerth et al., 1996). To ensure that these smaller cells were in fact neurons, we immunostained sections from P2 TrkA$^{-/-}$, p75NTR$^{-/-}$ 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-

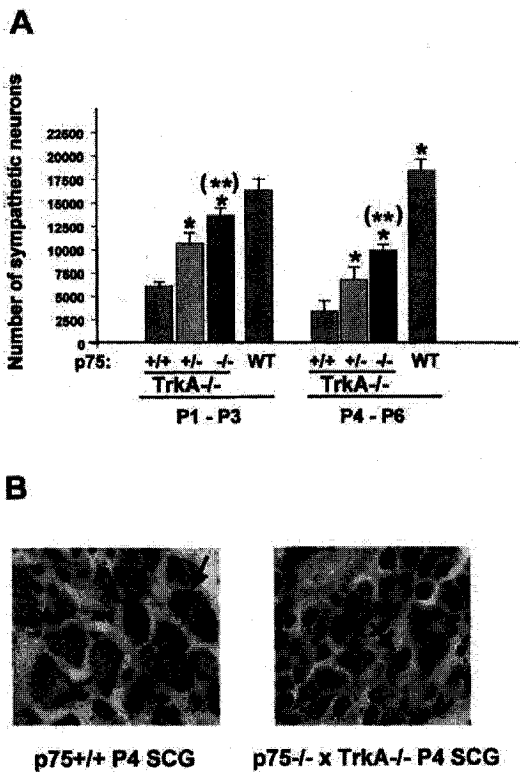


Figure 6. **The absence of p75NTR rescues the sympathetic neuron apoptosis observed in the neonatal TrkA$^{-/-}$ SCG.** (A) Sympathetic neuron number in TrkA$^{-/-}$, p75NTR$^{+/+}$, p75NTR$^{+/-}$, and p75NTR$^{-/-}$ SCG at P1–P3 and P4–P6. For comparison, TrkA$^{+/+}$, p75NTR$^{+/+}$ (WT) SCG counts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

stained sections were similar: in two different p75NTR$^{-/-}$, TrkA$^{-/-}$ SCGs, 790 and 1032 cresyl violet–stained neurons were present in representative sections, and 930 and 808 βIII 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, βIII-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.

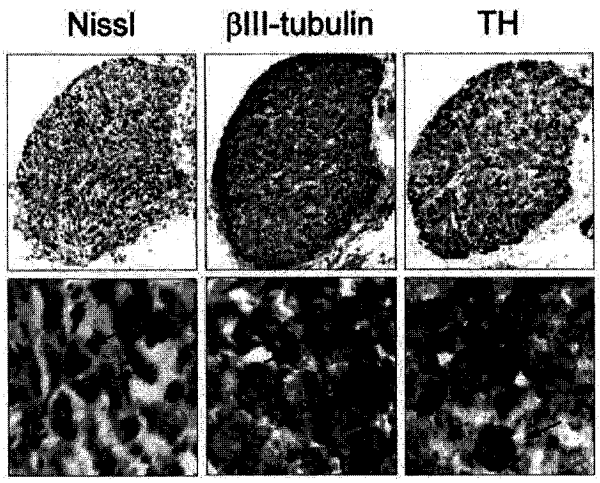


Figure 7. **p75NTR$^{-/-}$, TrkA$^{-/-}$ sympathetic neurons express neuron-specific βIII-tubulin and tyrosine hydroxylase.** Photomicrographs of alternate sections taken from a p75NTR$^{-/-}$, TrkA$^{-/-}$ ganglia and then either Nissl-stained or immunostained for the neuron-specific protein βIII-tubulin 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$^{-/-}$ 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$^{-/-}$ sympathetic neurons. Third, downstream NGF or NT-3–mediated survival signaling is similar in p75NTR$^{+/+}$ and p75NTR$^{-/-}$ sympathetic neurons. Fourth, pharmacological studies demonstrate that p75NTR$^{-/-}$ sympathetic neurons are deficient in apoptosis after NGF withdrawal, even when all Trk signaling is inhibited. Fifth, crosses of the p75NTR$^{-/-}$ and TrkA$^{-/-}$ animals demonstrate that the loss of sympathetic neurons seen in neonatal TrkA$^{-/-}$ 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$^{-/-}$ 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$^{+/+}$ and p75NTR$^{-/-}$ 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$^{-/-}$ 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$^{-/-}$ sympathetic neurons are delayed in their apoptosis in the absence of all Trk receptor signaling, and the absence of p75NTR$^{-/-}$ substantially rescues neonatal TrkA$^{-/-}$ 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$^{-/-}$ 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$^{-/-}$ 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$^{-/-}$ SCG, suggesting that p75NTR may contribute to the activation of this same apoptotic pathway in vivo.

Previous work on p75NTR$^{-/-}$ 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$^{-/-}$ and p75NTR$^{+/+}$ neurons equally well. In contrast, Lee et al. (1994b) reported that acutely dissociated sympathetic neurons from p75NTR$^{-/-}$ 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 $^{-/-}$ 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$^{-/-}$, TrkA$^{-/-}$ 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$^{-/-}$ 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$^{-/-}$ 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$^{-/-}$ animals were crossed with TrkA$^{+/-}$ animals, their progeny were genotyped using PCR, and the p75NTR$^{+/-}$, TrkA$^{+/-}$ 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-μm–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 ± 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 βIII-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 βIII-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 (Biowhittaker, 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 μg/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^2 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$^{-/-}$ and p75NTR$^{+/+}$ 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.)

We would like to thank Anne Aumont for excellent technical assistance, and David Kaplan and the members of the Miller laboratory for their advice and input during the course of this work.

M. Majdan and G. Walsh were funded by Medical Research Council/ Canadian Institutes of Health Research (CIHR) studentships, and F.D. Miller is a CIHR Senior Scientist. This work was supported by an operating grant from the CIHR.

Submitted: 3 October 2001
Accepted: 6 November 2001

References

Agerman, K., C. Baudet, B. Fundin, C. Wilson, and P. Ernfors. 2000. Attenuation of a caspase-3 dependent cell death in NT4- and p75NTR-deficient sensory neurons. *Mol. Cell. Neurosci.* 16:258–268.

Aloyz, R.S., S.X. Bamji, C.D. Pozniak, J.G. Toma, J. Atwal, D.R. Kaplan, and F.D. Miller. 1998. p53 is essential for developmental neuron death as regulated by the TrkA and p75NTR neurotrophin receptors. *J. Cell Biol.* 143: 1691–1703.

Bamji, S.X., M. Majdan, C.D. Pozniak, D.J. Belliveau, R. Aloyz, J. Kohn, C.G. Causing, and F.D. Miller. 1998. The p75NTR neurotrophin receptor mediates neuronal apoptosis and is essential for naturally occurring sympathetic neuron death. *J. Cell Biol.* 140:911–923.

Belliveau, D.J., I. Krivko, J. Kohn, C. Lachance, C. Pozniak, D. Rusakov, D. Kaplan, and F.D. Miller. 1997. NGF and neurotrophin-3 both activate TrkA on sympathetic neurons but differentially regulate survival and neuritogenesis. *J. Cell Biol.* 136:375–388.

Benedetti, M., A. Levi, and M.V. Chao. 1993. Differential expression of nerve growth factor receptors leads to altered binding affinity and neurotrophin responsiveness. *Proc. Natl. Acad. Sci. USA.* 90:7859–7863.

Bibel, M., E. Hoppe, and Y.-A. Barde. 1999. Biochemical and functional interactions between the neurotrophin receptors trk and p75NTR. *EMBO J.* 18: 616–622.

Bredesen, D.E., X. Ye, A. Tasinato, S. Sperandio, J.J. Wang, N. Assa-Munt, and S. Rabizadeh. 1998. P75NTR and the concept of cellular dependence: seeing how the other half die. *Cell Death Differ.* 5:365–371.

Brennan, C., K. Rivas-Plata, and S.C. Landis. 1999. The p75NTR neurotrophin receptor influences NT-3 responsiveness of sympathetic neurons in vivo. *Nat. Neurosci.* 2:699–705.

Burstein, D.E., and L.A. Greene. 1982. Nerve growth factor has both mitogenic and antimitogenic activities. *Dev. Biol.* 94:477–482.

Casaccia-Bonnefil, P., B.D. Carter, R.T. Dobrowsky, and M.V. Chao. 1996. Death of oligodendrocytes mediated by the interaction of nerve growth factors with its receptor p75. *Nature.* 383:716–719.

Causing, C.G., A. Gloster, R. Aloyz, S.X. Bamji, E. Chang, J. Fawcett, G. Kuchel, and F.D. Miller. 1997. Synaptic innervation density is regulated by neuron-derived BDNF. *Neuron.* 18:257–267.

Clary, D.O., G. Weskamp, L.R. Austin, and L.F. Reichardt. 1994. TrkA cross-linking mimics neuronal responses to nerve growth factor. *Mol. Biol. Cell.* 5:549–563.

Coggeshall, R.E. 1984. An empirical method for converting nucleolar counts to neuronal numbers. *J. Neurosci. Meth.* 12:125–132.

Cordon-Cardo, C., P. Tapley, S.Q. Jing, V. Nanduri, E. O'Rourke, F. Lamballe, K. Kovary, R. Klein, K.R. Jones, L.F. Reichardt, and M. Barbacid. 1991. The trk tyrosine protein kinase mediates the mitogenic properties of nerve growth factor and neurotrophin-3. *Cell.* 66:173–183.

Davies, A.M., K.F. Lee, and R. Jaenisch. 1993. p75-deficient trigeminal sensory neurons have an altered response to NGF but not to other neurotrophins. *Neuron.* 11:565–574.

Davey, F., and A.M. Davies. 1998. TrkB signalling inhibits p75NTR-mediated apoptosis induced by nerve growth factor in embryonic proprioceptive neurons. *Curr. Biol.* 8:915–918.

Deckwerth, T.L., J.L. Elliott, C.M. Knudson, E.M. Johnson, Jr., W.D. Snider, and S.J. Korsmeyer. 1996. Bax is required for neuronal death after trophic factor deprivation and during development. *Neuron.* 17:401–411.

Fagan, A.M., H. Zhang, S. Landis, R.J. Smeyne, I. Silos-Santiago, and M. Barbacid. 1996. TrkA, but not TrkC, receptors are essential for survival of sympathetic neurons in vivo. *J. Neurosci.* 16:6208–6218.

Frade, J.M., A. Rodriguez-Tebar, and Y.-A. Barde. 1996. Induction of cell death by endogenous nerve growth factor through its p75 receptor. *Nature.* 383: 166–168.

Ghosh, A., and M. Greenberg. 1995. Distinct roles for bFGF and NT-3 in the regulation of cortical neurogenesis. *Neuron.* 15:89–103.

Hempstead, B.L., D. Martin-Zanca, D.R. Kaplan, L.F. Parada, and M.V. Chao. 1991. High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor. *Nature.* 350:678–683.

Ip, N.Y., T.N. Stitt, P. Tapley, R. Klein, D.J. Glass, J. Fandl, L.A. Greene, M. Barbacid, and G.D. Yancopoulos. 1993. Similarities and differences in the way neurotrophins interact with the Trk receptors in neuronal and nonneuronal cells. *Neuron.* 10:137–149.

Johnson, D., A. Lanahan, C.R. Buck, A. Sehgal, C. Morgan, E. Mercer, M. Bothwell, and M. Chao. 1986. Expression and structure of the human NGF receptor. *Cell.* 47:545–554.

Jost, C.A., M.C. Marin, and W.G. Kaelin, Jr. 1997. P73 is a human p53 related protein that can induce apoptosis. *Nature.* 389:191–194.

Kaghad, M., H. Bonnet, A. Yang, L. Creacier, J.C. Biscan, A. Valent, A. Minty, P. Chalon, J.M. Lellas, X. Dumpont, et al. 1997. Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. *Cell.* 90:809–819.

Kaplan, D.R., B.L. Hempstead, D. Martin-Zanca, M.V. Chao, and L.F. Parada. 1991a. The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. *Science.* 252:554–558.

Kaplan, D.R., D. Martin-Zanca, and L.F. Parada. 1991b. Tyrosine phosphorylation and tyrosine kinase activity of the trk proto-oncogene product induced by NGF. *Nature.* 35:158–160.

Kaplan, D.R., and F.D. Miller. 2000. Neurotrophin signal transduction in the nervous system. *Curr. Opin. Neurobiol.* 10:381–391.

Klein, R., S. Jing, V. Nanduri, E. O'Rourke, and M. Barbacid. 1991. The trk proto-oncogene encodes a receptor for nerve growth factor. *Cell.* 65:189–197.

Kohn, J., R.S. Aloyz, J.G. Toma, M. Haak-Frendscho, and F.D. Miller. 1999. Functionally antagonistic interactions between the TrkA and p75NTR neurotrophin receptors regulate sympathetic neuron growth and target innervation. *J. Neurosci.* 19:5393–5408.

Lee, K.F., E. Li, L.J. Huber, S.C. Landis, A.H. Sharpe, M.V. Chao, and R. Jaenisch. 1992. Targeted mutation of the gene encoding the low affinity NGF receptor p75NTR leads to deficitis in the peripheral sensory nervous system. *Cell.* 69:737–749.

Lee, K.F., K. Bachman, S. Landis, and R. Jaenisch. 1994a. Dependence on p75NTR for innervation of some sympathetic targets. *Science.* 263:1447–1449.

Lee, K.F., A.M. Davies, and R. Jaenisch. 1994b. P75NTR-deficient embryonic dorsal root sensory and neonatal sympathetic neurons display a decreased sensitivity to NGF. *Development.* 12:1027–1033.

Leibrock, J., F. Lottspeich, A. Hohn, M. Hofer, B. Hengerer, P. Masiakowski, H. Thoenen, and Y.-A. Barde. 1989. Molecular cloning and expression of brain-derived neurotrophic factor. *Nature.* 341:149–152.

Levi-Montalcini, R. 1987. The nerve growth factor 35 years later. *Science.* 237: 1154–1162.

Majdan, M., and F.D. Miller. 1999. Neuronal life and death decisions: functional antagonism between the Trk and p75 neurotrophin receptors. *Int. J. Dev. Neurosci.* 17:153–161.

Mazzoni, I.E., F.A. Said, R. Aloyz, F.D. Miller, and D. Kaplan. 1999. Ras regulates sympathetic neuron survival by suppressing the p53-mediated cell death pathway. *J. Neurosci.* 19:9716–9727.

Mischel, P.S., S.G. Smith, E.R. Vining, J.S. Valletta, W.C. Mobley, and L.F. Reichardt. 2001. The extracellular domain of p75NTR is necessary to inhibit neurotrophin-3 signaling through TrkA. *J. Biol. Chem.* 276:11294–11301.

Miller, F.D., C.D. Pozniak, and G.S. Walsh. 2000. Neuronal life and death: an essential role for the p53 family. *Cell Death Differ.* 7:880–888.

Oppenheim, R.S. 1991. Cell death during development of the nervous system. *Annu. Rev. Neurosci.* 14:453–501.

Park, D.S., S.E. Farinelli, and L.A. Greene. 1996. Inhibitors of cyclin-dependent kinases promote survival of post-mitotic neuronally differentiated PC12 cells and sympathetic neurons. *J. Biol. Chem.* 271:8161–8170.

Park, D.S., B. Levine, G. Ferrari, and L.A. Greene. 1997. Cyclin dependent kinase inhibitor and dominant negative cyclin dependent kinase 4 and 6 promote survival of NGF-deprived sympathetic neurons. *J. Neurosci.* 17:8975–8983.

Pozniak, C.D., S. Radinovic, A. Yang, F. McKeon, D.R. Kaplan, and F.D. Miller. 2000. An anti-apoptotic role for the p53 family member, p73, during devel-

opmental neuron death. *Science.* 289:304–306.

Radeke, M.J., T.P. Misko, C. Hsu, L.A. Herzenberg, and E.M. Shooter. 1987. Gene transfer and molecular cloning of the rat nerve growth factor receptor. *Nature.* 325:593–596.

Raff, M. 1998. Cell suicide for beginners. *Nature.* 396:119–122.

Raoul, C., C.E. Henderson, and B. Pettmann. 1999. Programmed cell death of embryonic motoneurons triggered through the Fas death receptor. *J. Cell Biol.* 147:1049–1062.

Sherr, C.J., and J.D. Weber. 2000. The ARF/p53 pathway. *Curr. Opin. Genet. Dev.* 10:94–99.

Slack, R.S., D.J. Belliveau, M. Rosenberg, J. Atwal, H. Lochmuller, R. Aloyz, A. Haghighi, B. Lach, P. Seth, E. Cooper, and F.D. Miller. 1996. Adenovirus-mediated gene transfer of the tumor suppressor, p53, induces apoptosis in postmitotic neurons. *J. Cell Biol.* 135:1–12.

Smeyne, R.J., R. Klein, A. Schnapp, L.K. Long, S. Bryant, A. Lewin, S. Lira, and M. Barbacid. 1994. Severe sensory and sympathetic neuropathies in mice carrying a disrupted Trk/NGF receptor gene. *Nature.* 368:246–249.

Soilu-Hanninen, M., P. Ekert, T. Bucci, D. Syroid, P.F. Bartlett, and T.J. Kilpatrick. 1999. Nerve growth factor signaling through p75NTR induces apoptosis in Schwann cells via a Bcl2-independent pathway. *J. Neurosci.* 19: 4828–4838.

Tapley, P., F. Lamballe, and M. Barbacid. 1992. K252a is a selective inhibitor of the tyrosine kinase activity of the trk family of oncogenes and neurotrophin receptors. *Oncogene.* 7:371–381.

Thoenen, H., and Y.-A. Barde. 1980. Physiology of nerve growth factor. *Physiol. Rev.* 60:1284–1335.

Vaillant, A.R., I. Mazzoni, C. Tudan, M. Boudreau, D.R. Kaplan, and F.D. Miller. 1999. Depolarization and neurotrophins converge on the phosphatidylinositol 3-kinase–akt pathway to synergistically regulate neuronal survival. *J. Cell Biol.* 146:955–966.

Verdi, J.M., and D.J. Anderson. 1994. Neurotrophins regulate sequential changes in neurotrophin receptor expression by sympathetic neuroblasts. *Neuron.* 13: 1359–1372.

Von Schack, D., E. Casademunt, R. Schweigreiter, M. Meyer, M. Bibel, and G. Dechant. 2001. Complete ablation of the neurotrophin receptor p75NTR causes defects both in the nervous and vascular system. *Nat. Neurosci.* 4:977–978.

Yang, A., M. Kaghad, Y. Wang, E. Gillett, M.D. Fleming, V. Dotsch, N.C. Andrews, D. Caput, and F. McKeon. 1998. p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Mol. Cell.* 2:305–316.

Yoon, S.O., P. Cassacia-Bonnefil, B. Carter, and M.V. Chao. 1998. Competitive signaling between TrkA and p75 nerve growth factor receptors determines cell survival. *J. Neurosci.* 18:3273–3281.

APPENDIX B

Walsh, G.S., Orike, N., Kaplan, D.R., Miller, F.D. (2004) The invulnerability of adult neurons: A critical role for p73. *Journal of Neuroscience* 24:9638-47.

Cellular/Molecular

The Invulnerability of Adult Neurons: A Critical Role for p73

Gregory S. Walsh,[1,2]* Nina Orike,[2]* David R. Kaplan,[1,2] and Freda D. Miller[1,2]

[1]Developmental Biology and Cancer Research, Hospital for Sick Children, Departments of Medical Genetics and Physiology, University of Toronto, Toronto, Ontario, Canada M5G 1X8, and [2]Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, Quebec, Canada H3A 2B4

Here, we investigated the intracellular mechanisms that underlie the relative invulnerability of adult versus developing dorsal root ganglion (DRG) sensory neurons. In culture, adult neurons were resistant to stimuli that caused apoptosis of their neonatal counterparts. In both adult and neonatal neurons, death stimuli induced the apoptotic c-Jun N-terminal protein kinase (JNK) pathway, but JNK activation only caused death of neonatal neurons, indicating that adult neurons have a downstream block to apoptosis. Expression of the dominant-inhibitory p53 family member, ΔNp73, rescued JNK-induced apoptosis of neonatal neurons, suggesting that it might participate in the downstream apoptotic block in adult neurons. To test this possibility, we examined adult DRG neurons cultured from p73+/− mice. Adult p73+/− DRG neurons were more vulnerable to apoptotic stimuli than their p73+/+ counterparts, and invulnerability could be restored to the p73+/− neurons by increased expression of ΔNp73. Moreover, although DRG neuron development was normal in p73+/− animals *in vivo*, axotomy caused death of adult p73+/− but not p73+/+ DRG neurons. Thus, one way adult neurons become invulnerable is to enhance endogenous survival pathways, and one critical component of these survival pathways is p73.

Key words: p73; p53 tumor suppressor; sensory neurons; dorsal root ganglion; sciatic nerve injury; nerve growth factor; neuronal apoptosis; JNK; PI3-kinase; DNA damage

Introduction

A fundamental question is how developing neurons, which are programmed to undergo apoptosis unless they establish the appropriate connectivity, become resistant to apoptotic stimuli as they mature, a transition that ensures their survival for the remainder of an animal's lifetime. This transition is perhaps best characterized in the peripheral nervous system. During the developmental cell death period, peripheral neurons are absolutely dependent on trophic factors produced by their targets, and those developing neurons that fail to compete successfully for sufficient target support die by apoptosis. Moreover, any insult that interrupts the supply of target-derived growth factors causes apoptosis of newly connected developing neurons. For example, axotomy or target removal leads to rapid and extensive cell death in both central and peripheral neurons (for review, see Snider et al., 1992), and exogenous replacement of target-derived growth factors rescues these axotomized neurons (Hendry and Campbell, 1976; Yip et al., 1984). This contrasts to the situation in adulthood, where peripheral neurons no longer depend on target-derived growth factors for survival (Angeletti et al., 1971; Bjerre et al., 1975; Goedert et al., 1978; Otten et al., 1979), and axotomy results only in delayed and restricted neuronal loss (Tandrup et al., 2000; Ma et al., 2001). The mechanisms that underlie the relative invulnerability of adult neurons *in vivo* are not well understood.

Culture studies have confirmed that the enhanced invulnerability of adult peripheral neurons is a cell-intrinsic phenomenon. For example, acutely isolated adult sympathetic or sensory neurons do not require exogenous neurotrophins for survival in culture (Lazarus et al., 1976; Chun and Patterson, 1977; Lindsay, 1988), a finding that might be partially explained by endogenous neurotrophin synthesis in older neurons (Acheson et al., 1995). However, these older neurons are also less sensitive to apoptosis induced by ionizing radiation (Tong et al., 1997), and removal of exogenous neurotrophins activates the same initial apoptotic signaling events in neonatal sympathetic neurons as in neurons that have been "aged" 3 weeks in culture (Easton et al., 1997; Vogelbaum et al., 1998), suggesting the existence of additional cell-intrinsic mechanisms that confer apoptotic resistance on the older neurons.

What might these cell-intrinsic mechanisms be? Potential differences might include an impairment in apoptotic signaling pathways and/or an enhancement in intrinsic neuronal survival pathways that might "block" apoptosis. Potential insights into the specifics of such pathways derive from extensive work on naturally occurring cell death in NGF-dependent sympathetic neurons. During development, the ultimate survival of any given sympathetic neuron is a function of survival signals deriving from the tyrosine receptor kinase A (TrkA)/NGF receptor and apoptotic signals deriving from the p75 panneurotrophin receptor (Bamji et al., 1998; Majdan et al., 2001) (for previous see Miller and

Received April 6, 2004; revised Sept. 1, 2004; accepted Sept. 14, 2004.

This work was supported by grants from the Canadian Institutes of Health Research (CIHR). D.R.K. is a Canada Research Chair, F.D.M. is a Senior Scientist of CIHR, and G.S.W. was supported by a studentship from the Hospital for Sick Children Research Institute. We thank Yew Meng Heng, Julia Hwang, and Howard Rosenberg of the Electron Microscopy Laboratory, Hospital for Sick Children, for their excellent technical assistance.

*G.S.W. and N.O. contributed equally to this work.

Correspondence should be addressed to Dr. Freda D. Miller, Developmental Biology, Hospital for Sick Children, Room Black 3203A, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8. E-mail: fredam@sickkids.ca.

N. Orike's present address: Renovis Inc., 2 Corporate Drive South, San Francisco, CA 94080.

DOI:10.1523/JNEUROSCI.1299-04.2004

Kaplan, 2001). TrkA mediates survival primarily via a phosphatidylinositol 3 (PI3)-kinase–Akt-dependent pathway, whereas activation of a c-Jun N-terminal protein kinase (JNK)-Bax apoptotic pathway occurs after either p75NTR activation or NGF withdrawal. Interestingly, work with sympathetic neurons that have been aged for 3 weeks in culture and that lose their NGF dependence show that after NGF withdrawal, one of the major downstream apoptotic events, translocation of Bax to the mitochondria, no longer occurs (Easton et al., 1997; Putcha et al., 2000). These findings suggest that in the mature, resistant neurons, either an essential link between the cell surface and Bax is no longer present and/or a potent survival signal inhibits the apoptotic pathway at this level.

In this regard, we identified previously a key apoptotic checkpoint in developing sympathetic neurons that involves the p53 tumor suppressor family. Specifically, we have shown that p53 functions as a proapoptotic molecule during developmental sympathetic neuron death (Aloyz et al., 1998) and that a second family member, ΔNp73, is an essential prosurvival protein during the same developmental window (Pozniak et al., 2000). The p73 gene encodes two classes of protein products: full-length isoforms that are structurally homologous to p53 (TAp73α, TAp73β) and N-terminal truncated isoforms that lack the transactivation domain (ΔNp73α, ΔNp73β) (for review, see Irwin and Kaelin, 2001). In developing sympathetic neurons, NGF potently upregulates the prosurvival ΔNp73 isoform, and ΔNp73 maintains sympathetic neuron survival both *in vivo* and in culture by p53-dependent and -independent mechanisms (Pozniak et al., 2000; Lee et al., 2004).

In the CNS, ΔNp73 variants are also the predominant isoforms, acting as potent prosurvival proteins in cultured cortical neurons exposed to a variety of apoptotic insults (Pozniak et al., 2002). In this regard, we have shown recently that p73−/− animals display a prolonged and steady loss of cortical neurons from the first to the eighth postnatal week *in vivo*, suggesting that p73 is important for the maintenance of mature cortical neurons (Pozniak et al., 2002). On the basis of these findings, we hypothesized that ΔNp73 may well provide a key prosurvival mechanism for adult neurons. In this study, we tested this hypothesis and show that ΔNp73 is an essential component of a cell-intrinsic pathway that is required for the survival of adult sensory neurons both in culture and *in vivo* after axonal injury.

Materials and Methods

Animals. Mice deficient in p73 have been described previously (Pozniak et al., 2000, 2002; Yang et al., 2000). These mice were maintained through heterozygote breeding pairs in a C3H background, and genotyping was performed as described previously (Pozniak et al., 2000).

Primary neuronal cultures. Newborn dorsal root ganglia (DRGs) were removed rapidly, placed in F12 media (Invitrogen, San Diego, CA), and digested in 0.125% collagenase in F12 media for 20 min followed by 10 min in 0.25% trypsin, all at 37°C. For adult (6 weeks of age) animals, DRGs were dissected and digested in 0.125% collagenase in F12 media for 1.5 hr followed by 30 min in 0.25% trypsin. Ganglia were washed in F12 media and then gently triturated through a glass-polished pipette, and the cell suspension was centrifuged through a 15% BSA solution (Sigma, St. Louis, MO). The cell pellet was resuspended in Ultraculture media containing 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 ng/ml NGF, 5 μM cytosine arabinoside, and 20 μM fluorodeoxyuridine. Neurons were maintained for 24 hr and then treated as follows. For NGF deprivation, cultures were rinsed with Ultraculture, followed by the addition of Ultraculture containing goat anti-NGF antibodies (Abs) (Sigma). For cell survival experiments, cultures were rinsed with Ultraculture, followed by the addition of Ultraculture containing 50 ng/ml NGF alone or in combination with either 50 μM LY294002 (4-Morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one (LY294002) or 10 μM camptothecin. For cultures treated with anti-NGF, LY294002, or camptothecin, the number of phase-bright neurons within a defined area was counted on the day of treatment and at 24 hr intervals thereafter. Cells with round, phase-bright bodies and intact neurites were counted as surviving neurons. Survival is expressed as a percentage of the original number of neurons. Approximately 200 neurons were counted for each experiment. Statistical differences between conditions were examined with a Student's *t* test.

Cultures of sympathetic neurons were prepared from newborn p73+/+ and p73+/− mice. Superior cervical ganglia from individual mice were dissected and triturated as described previously (Majdan et al., 2001). Neurons were then plated on collagen-coated 96-well culture dishes (Falcon Plastics, Franklin Lakes, NJ) in Ultraculture media containing 2 mM glutamine, 100 U/ml penicillin, 100 gm/ml streptomycin, 3% FBS (Invitrogen), and 50 ng/ml mouse 2.5 S NGF prepared from mouse salivary gland (Cedarlane, Hornby, Ontario, Canada). One day after plating, neurons were fed with the same media containing 0.5% cytosine arabinoside (Sigma). For cell survival experiments, cultures were rinsed with Ultraculture, followed by the addition of Ultraculture containing 50 ng/ml NGF alone or in combination with either 50 μM LY294002 or 10 μM camptothecin. Two days later, cultures were rinsed and fixed in 4% paraformaldehyde and stained with Hoechst. Apoptosis was assessed by counting the number of neurons that displayed evidence of condensed apoptotic nuclei. Statistical differences between values were examined with a Student's *t* test.

Adenovirus infections. For JNK activation experiments, semiconfluent human embryonic kidney (HEK) 293A cells were rinsed with DMEM and infected with 20 multiplicities of infection (MOI) of adenovirus expressing wild-type mixed-lineage kinase 3 (MLK3)/green fluorescent protein (GFP) (Roux et al., 2002) or GFP alone. Alternatively, DRG cultures were rinsed with Ultraculture 24 hr after plating, followed by the addition of Ultraculture containing 50 ng/ml NGF and 100 MOI of adenovirus expressing wild-type MLK3/GFP or GFP alone. After 24 hr, cell lysates were prepared, and the level of JNK phosphorylation was assessed by immunoblotting (see below). For survival experiments with DRG neurons, cultures were rinsed with Ultraculture 24 hr after plating, followed by the addition of Ultraculture containing 50 ng/ml NGF and 100 MOI of adenovirus expressing wild-type MLK3/GFP or GFP alone. Two days later, cultures were switched to media containing 50 ng/ml NGF alone for an additional 3 d. Cultures were then rinsed and fixed in 4% paraformaldehyde and stained with Hoechst. Apoptosis was assessed by counting 200 infected neurons and determining the number of infected neurons displaying evidence of condensed apoptotic nuclei. Statistical differences between values were examined with a Student's *t* test. For the ΔNp73 rescue experiments, cultures of adult p73+/− neurons were rinsed with Ultraculture 24 hr after plating, followed by the addition of Ultraculture containing 50 ng/ml NGF and 100 MOI of adenovirus expressing ΔNp73β/GFP (Pozniak et al., 2000) or GFP alone. Two days later, cultures were switched to media either lacking NGF or containing NGF with LY294002 or camptothecin. Apoptosis was assessed as for the MLK3 experiments. For double-infection experiments with sympathetic neurons, cultures were rinsed with Ultraculture 4 d after plating, followed by the addition of Ultraculture containing 10 ng/ml NGF, 50 mM KCl, and 100 MOI of adenovirus expressing wild-type MLK3/GFP either alone or in combination with 100 MOI of ΔNp73β/GFP or 100 MOI of GFP. One day later, cultures were washed free of virus and replaced with Ultraculture media containing 10 ng/ml NGF. Two days later, cultures were then rinsed and fixed in 4% paraformaldehyde and stained with Hoechst, and apoptosis was assessed as described above.

Reverse transcription-PCR. Whole DRGs were dissected from neonatal and adult (6 weeks of age) wild-type mice and quickly frozen. Total RNA was isolated from tissues using the Trizol reagent and was reverse transcribed using M-MuLV reverse transcriptase (Fermentas, Burlington, Ontario, Canada). PCR was performed using site-specific primers. Primer sequences were as follows: p73 core, 5′-TGCCCCGGCTAGCCACCTCATCC-3′ and 5′-CCTCGTCCCCGTGGCGTCTCTTCT-3′; TAp73, 5′-GAGCACCTGTGGAGTTCTCTAGAG-3′ and 5′-GGTATT-

ACAC-3′ and 5′-GGGGCAGCGCTTAACAATGT-3′; p53, 5′-AACCGCCGACCTATCCTTACCATC-3′ and 5′-AGGCCCCACTTTCTTGACCATTGT-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-ACGGCAAGTTCAATGGCACAGTCA-3′ and 5′-GCTTTCCAGAGGGGCCATCCACAG-3′. Expected PCR product sizes were as follows: p73 core, 434 bp; TAp73, 297 bp; ΔNp73, 429 bp; p53, 432 bp; GAPDH, 425 bp.

Surgical procedures. Sciatic nerve resections were performed on adult (6 weeks of age) p73 heterozygous mice and their wild-type littermates. Adult animals were anesthetized with an intraperitoneal injection of a ketamine/xylazine mixture (100 mg/kg per 10 mg/kg). The right sciatic nerve was exposed at midthigh level, and a 5 mm segment of the nerve was resected. The wound was sutured, and the animals were allowed to recover for 1 month.

Immunohistochemistry. Mice were terminally anesthetized with sodium pentobarbital and then perfused transcardially with a solution of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The L4 DRGs were removed, postfixed for 2 hr in the same fixative, and then immersed for 3 d in 30% phosphate-buffered sucrose. Ganglia were embedded in OCT compound, frozen in isopentane, and cryosectioned at 10 μm onto chrom-alum gelatin-coated slides. Sections were stained with cresyl violet or incubated for 1 hr in 0.1 M PBS containing 10% normal goat serum and 0.25% Triton X-100, followed by incubation with primary antibody overnight at 4°C. Primary antibodies were mouse anti-neurofilament 200 (Sigma) and rabbit anti-calcitonin gene-related peptide (CGRP) (Chemicon, Temecula, CA). After rinsing in PBS, sections were incubated with either Alexa 594-conjugated anti-mouse or Alexa 350-conjugated anti-rabbit secondary antibodies (Molecular Probes, Eugene, OR) for 2 hr at room temperature. Sections were washed in PBS, mounted, and viewed under a Zeiss (Thornwood, NY) fluorescence microscope with 20× magnification. For isolectin B4 (IB4) staining, after the blocking step, sections were incubated for 2 hr with FITC-labeled IB4 lectin (Sigma) in 0.1 M PBS containing 0.25% Triton X-100. Sections were then rinsed and coverslipped with geltol (Immunon, Shandon, Pittsburgh, PA) and analyzed with a Zeiss Axioplan microscope using 20× objective and the Northern Eclipse computer-based image analysis software (Empix Imaging, Ontario, Canada).

Electron microscopy. The L4 dorsal (sensory) spinal roots from adult p73 heterozygous mice and their wild-type littermates were examined for the number of nerve fibers in plastic-embedded material. Dorsal root specimens were fixed in 4% paraformaldehyde and 1% gluteraldehyde in 0.1 M phosphate buffer. They were then postfixed in aqueous 1% OsO4 and dehydrated through a graded series of acetone followed by infiltration and embedding in the Epon-Araldite epoxy resin (Canemco, Quebec, Canada). Semithin sections were cut and stained with Toluidene Blue for light microscopy examination. Specimen blocks with appropriate cross sections were selected for ultrathin sectioning. Sections ~80 nm thick were cut with a diamond knife and stained with uranyl acetate and lead citrate for transmission electron microscopy (TEM) imaging.

The sections were examined in a JEM-1230 TEM (JEOL, Peabody, MA) operating at 80 kV. Digital electron micrographs were recorded directly using CCD camera with a computer system (AMT Advantage CCD ORCA camera; AMT, Deben UK, Suffolk, UK). Images with offset overlapping edges covering the entire area of the dorsal root were systematically recorded. The total number of nerve fibers (both myelinated and unmyelinated) in the L4 dorsal roots of heterozygous and wild-type mice was determined from electron micrographs at 5000×. Statistical differences between wild-type and heterozygous values were examined with a Student's *t* test.

Western blot analysis. HEK293A cells or DRG neurons were rinsed briefly in cold TBS and then lysed in TBS lysis buffer (137 mM NaCl, 20 mM Tris, pH 8.0, 1% v/v NP-40, and 10% v/v glycerol) supplemented with Mini Complete protease inhibitor mixture (Roche Diagnostics, Mannheim, Germany) and 1.5 mM sodium vanadate. Lysates were scraped into Eppendorf tubes (Eppendorf Scientific, Westbury, NY) and rocked for 10 min at 4°C. Samples were then cleared by centrifugation. Protein concentration was determined by the BCA assay (Pierce, Rockford, IL) using BSA as a standard. Samples were diluted in sample buffer and placed in a 95°C heat block for 5 min. Equal amounts of protein were separated on 10% SDS-PAGE gels and transferred onto nitrocellulose membranes. For all antibodies, blots were blocked in 3% skim milk in TBS overnight at 4°C. Primary antibodies used included anti-JNK1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-JNK (Promega, Madison, WI), anti-MLK3 (Santa Cruz Biotechnology), anti-GFP (Clontech, Cambridge, UK). For Western blots, secondary antibodies used were HRP-conjugated anti-mouse (1:10,000) and anti-rabbit (1:10,000) polyclonal Abs (Roche Diagnostics). All incubations were performed in 3% skim milk in TBS plus 0.1% Tween 20 (Sigma). For detection, blots were washed with TBS, and antibody localization was visualized using the ECL chemiluminescence kit (Amersham, Buckinghamshire, UK).

Results

Adult sensory neurons are resistant to stimuli that induce apoptosis of neonatal sensory neurons

To study the intracellular mechanisms that promote the survival of adult neurons, *in vitro* studies have relied primarily on embryonic neurons that have been aged in culture (Easton et al., 1997; Vogelbaum et al., 1998; Putcha et al., 2000), despite the possibility that neurons may develop differently in culture than *in vivo*. To overcome this limitation, we examined instead the survival requirements of sensory neurons acutely isolated from adult (6 weeks of age) rat DRGs and directly compared them to cultured neurons isolated from neonatal (postnatal day 0) DRGs. In all of these experiments, neurons were isolated from lumbar DRGs, and low-density cultures were established and maintained in serum-free culture conditions containing few non-neuronal cells. Under these conditions, the adult cultures were comprised of phase-bright neurons of varying sizes, consistent with neuronal heterogeneity in the DRG.

Initially, we confirmed the previously reported finding (Lindsay, 1988; Vogelbaum et al., 1998) that adult DRG neurons no longer require neurotrophins for their survival. Adult or neonatal DRG neurons were cultured in the presence of NGF for 1 d and then switched into media lacking NGF but with the addition of function-blocking anti-NGF antibodies. Quantitation of surviving, phase-bright neurons 2 d later revealed that, whereas only 40 ± 9% of neonatal neurons were still alive, the large majority of adult neurons (77 ± 11%) survived the NGF withdrawal (Fig. 1*A*). We then asked whether this enhanced survival of adult DRG neurons in the absence of NGF reflected a more general resistance of adult neurons to apoptotic stimuli. We first asked whether adult DRG neurons were more resistant to apoptosis caused by inhibition of the PI3-kinase survival pathway; established cultures of neonatal or adult neurons were switched into media containing NGF plus 50 μM of the pharmacological PI3-kinase inhibitor LY294002. Western blot analysis demonstrated the specificity of this concentration of LY294002 to inhibit the phosphorylation of the PI3-kinase target Akt in these neurons (data not shown), as we published previously for sympathetic neurons (Vaillant et al., 1999). We then quantitated survival; after 2 d of PI3-kinase inhibition, only 25 ± 14% of neonatal DRG neurons remained alive, whereas the majority (62 ± 6%) of adult neurons survived (Fig. 1*B*). As a second, more robust, apoptotic insult, we treated neurons with the DNA damaging agent camptothecin, which causes p53-dependent neuronal apoptosis (Xiang et al., 1998). Established cultures of adult or neonatal DRG neurons were treated with 10 μM camptothecin, and survival was quantitated 2 d later. This analysis demonstrated that, surprisingly, adult DRG neurons were relatively resistant to this apoptotic stimulus; after 2 d, 72 ± 8% of adult neurons survived versus 26 ± 7% of neonatal neurons (Fig. 1*C*).

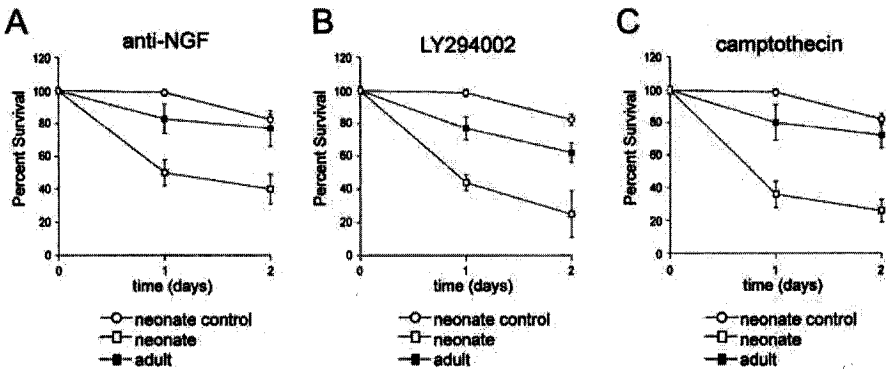


Figure 1. Adult sensory neurons are more resistant to apoptotic insults than their neonatal counterparts. *A–C*, Adult (black squares) or neonatal (open squares) sensory neurons of the L4 DRG were cultured for 24 hr in NGF and then cultured for an additional 2 d after NGF withdrawal and in the presence of anti-NGF antibodies (*A*), in the presence of NGF but with the addition of 50 μM LY294002, an inhibitor of PI3-kinase (*B*), or in the presence of NGF but with the addition of 10 μM camptothecin (*C*), a DNA-damaging agent. As controls, sister cultures were maintained in NGF over the same time period (open circles). Phase-bright neurons with intact neurites in multiple identified fields were counted at 0, 1, and 2 d for a total of at least 200 neurons per experiment, and the amount of survival was plotted relative to the initial number of neurons in the same fields at the time the treatment was initiated (percentage survival). Results are the cumulative results of at least three independent experiments, and the error bars represent SEM (*t* test; $p < 0.005$; comparison at 2 d time point for all experiments).

Activation of the JNK pathway is sufficient to cause apoptosis of neonatal, but not adult, neurons, and JNK-mediated neonatal neuron apoptosis can be rescued by ΔNp73

A generalized apoptotic resistance in adult neurons could result from an impairment in apoptotic signaling pathways and/or enhanced adult survival pathways. To distinguish these two possibilities, we examined one common component of the apoptotic pathways induced by a variety of stimuli, activation of the N-terminal jun-kinase. Specifically, adult or neonatal DRG neurons were established and then either withdrawn from NGF or treated with 50 μM LY294002 or 10 μM camptothecin for 24 hr. Western blot analysis revealed that JNK phosphorylation was increased in neonatal DRG neurons in all of these conditions (Fig. 2*A*). An increase inJNK phosphorylation was also observed in adult DRG neurons treated in the same way (Fig. 2*B*), although the magnitude of the increase was smaller. Thus, apoptotic pathways upstream of JNK were intact in both populations, but adult neurons apparently had a downstream block to apoptosis.

To directly test this hypothesis, we exogenously activated JNK and asked whether this was, on its own, sufficient to cause apoptosis of neonatal versus adult neurons. To perform these experiments, we used a bicistronic recombinant adenovirus that coexpressed GFP and wild-type MLK3, an upstream kinase that efficiently activates JNK when overexpressed in peripheral neurons (Maroney et al., 1999). Confirmation of the efficacy of this adenovirus was obtained by transducing HEK293A cells or neonatal DRG cultures and assaying for expression of MLK3 and activation of JNK by Western blot analysis (Fig. 2*C*). We then infected established neonatal or adult neurons with adenoviruses expressing MLK3/GFP or GFP alone and scored GFP-positive neurons for apoptotic nuclei as indicated by Hoechst staining. This analysis revealed that GFP alone had no effect on neuronal survival. However, overexpression of MLK3 was sufficient to cause the apoptosis of the majority of neonatal, but not adult, neurons (Fig. 2*D*), suggesting that adult neurons have a relative impairment in the apoptotic pathways downstream of JNK.

One protein that acts downstream of JNK in neonatal sympathetic neurons, and that plays an essential prosurvival role, is the dominant-inhibitory p53 family member ΔNp73 (Pozniak et al., 2000; Lee et al., 2004). To determine whether enhanced ΔNp73 levels or function could explain the downstream block to JNK-mediated apoptosis in adult neurons, we overexpressed ΔNp73 in neonatal neurons and asked whether this could rescue MLK3-mediated apoptosis. To perform this experiment, we turned to neonatal sympathetic neurons, which apoptose in response to JNK pathway activation (Aloyz et al., 1998; Eilers et al., 1998) and can be reliably double infected by recombinant adenovirus. Neonatal neurons were infected with recombinant adenoviruses expressing GFP, MLK3, and/or ΔNp73, and neuronal survival was quantitated by assessing nuclear morphology with Hoechst 3 d after infection. These experiments demonstrated that, as seen with neonatal sensory neurons (Fig. 2*D*), overexpression of MLK3 in neonatal sympathetic neurons was sufficient to induce apoptosis in the presence of NGF (Fig. 2*E*). The magnitude of this apoptotic effect was unaffected by coinfection with a control, GFP-expressing adenovirus. In contrast, when neurons were coinfected with adenoviruses expressing MLK3 and ΔNp73, infected sympathetic neurons were rescued from apoptosis (Fig. 2*E*). Thus, enhanced levels of ΔNp73 were sufficient to block neonatal neuron apoptosis in response to JNK pathway activation, suggesting that upregulation of ΔNp73 function would be sufficient to uncouple extracellular apoptotic stimuli from an apoptotic response in adult neurons.

p73 is required for the relative invulnerability of cultured adult DRG neurons

These studies indicated that enhanced ΔNp73 levels and/or function would be sufficient to confer relative invulnerability to apoptotic stimuli on adult neurons. We therefore asked whether ΔNp73 was necessary for the invulnerability of adult sensory neurons. To do this, we initially characterized the expression of full-length (TAp73) versus N-terminal truncated (ΔNp73) isoforms in dorsal root ganglia isolated from adult mice. Reverse transcription (RT)-PCR analysis with specific primers demonstrated that mRNAs encoding both TAp73 and ΔNp73 isoforms were expressed in adult DRGs as they are in the developing brain (Fig. 3*A*,*B*) (Pozniak et al., 2002). A similar analysis demonstrated that these isoforms are also both expressed in the neonatal DRG (Fig. 3*A*) (data not shown). The specificity of this analysis was demonstrated by showing that these RT-PCR products were not present in the p73−/− brain (Fig. 3*B*).

We next asked whether p73 was required for the relative resistance of adult sensory neurons to death-inducing stimuli. To perform these experiments, we chose to directly compare adult DRG neurons from p73+/+ and p73+/− mice. To ensure that p73 heterozygosity did not developmentally influence sensory neuron repertoire, we characterized 6-week-old p73+/+ versus p73+/− L4 DRGs [most p73−/− animals die at postnatal day 10 (P10)]. Sectioning and Nissl-staining of ganglia from 6-week-old p73+/+ and p73+/− mice revealed that they were similar in size and morphology (Fig. 4*A*). Immunostaining of these sections for neurofilament 200 kD (NF200), which marks predominantly large diameter myelinated neurons, CGRP, which marks predominantly small diameter peptidergic nociceptors, and IB4, which marks small diameter nonpeptidergic nociceptors, demonstrated that the sensory neuron repertoire was also similar (Fig.

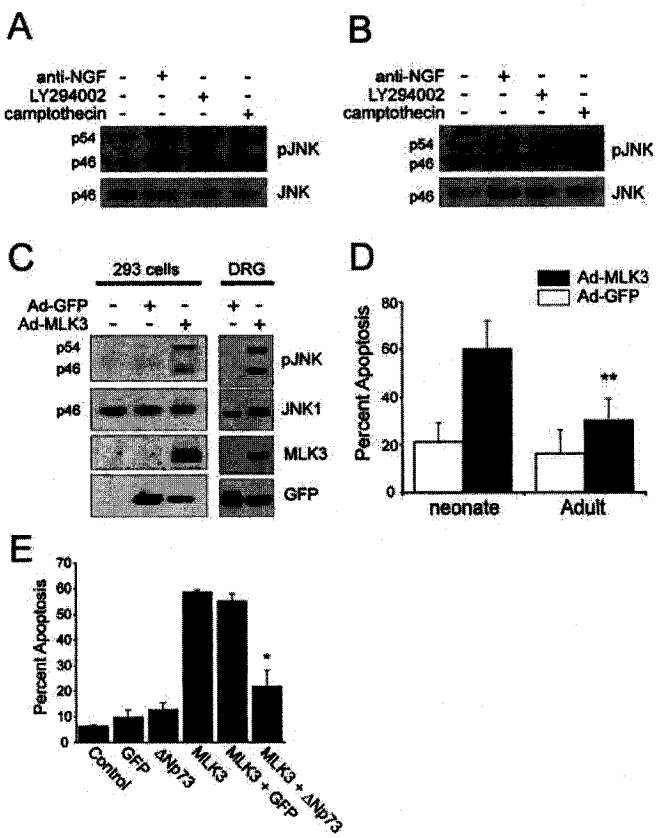


Figure 2. JNK activation occurs in both adult and neonatal sensory neurons, but exogenous JNK activation preferentially causes apoptosis of neonatal neurons. *A*, *B*, Western blot analysis with an antibody specific for the phosphorylated, activated form of JNK (p-JNK) in lysates of neonatal (*A*) or adult (*B*) neurons that were established in NGF for 1 d and then either withdrawn from NGF (anti-NGF) or treated with 50 μM LY294002 or 10 μM camptothecin for 24 hr. Equal amounts of protein were present in all lanes, as determined by reprobing the same blots for total JNK protein. The numbers to the left of the blots refer to the molecular weights of the two bands, which correspond to p46 and p54 isoforms of JNK. *C*, Western blot analysis for the activated, phosphorylated form of JNK (pJNK) in equal amounts of protein from lysates of HEK293 cells (left panel) or neonatal DRG cultures (right panel) that were infected with replication-deficient recombinant adenoviruses expressing either GFP alone (Ad-GFP) or both GFP and the upstream kinase MLK3 (Ad-MLK3). Uninfected cells were also examined as an additional control. Blots were reprobed with anti-JNK to demonstrate that equal amounts of protein were present as well as for MLK3 and GFP. Note that overexpression of MLK3 causes activation of both p46 and p54 isoforms of JNK. *D*, Quantitation of the percentage of GFP-positive infected neurons with apoptotic nuclei, as determined by Hoechst staining 3 d after infection. Neonatal or adult sensory neurons were established, infected with adenoviruses expressing either GFP alone or GFP plus MLK3, and apoptosis was quantitated 3 d later. Note that the majority of neonatal neurons become apoptotic in response to MLK3 overexpression (*t* test; $^{**}p < 0.05$). Error bars represent SD. *E*, MLK3-induced neonatal neuron death is rescued by ectopic expression of ΔNp73. Quantitation of the percentage of GFP-positive infected neonatal sympathetic neurons with apoptotic nuclei, as determined by Hoechst staining 3 d after infection. Neonatal sympathetic neurons were established, infected with adenoviruses expressing GFP, MLK3/GFP, and/or ΔNp73/GFP, and then apoptosis was quantitated 3 d later. Note that ΔNp73 rescued MLK3-mediated death (ANOVA; $^{*}p < 0.001$). Error bars represent SD.

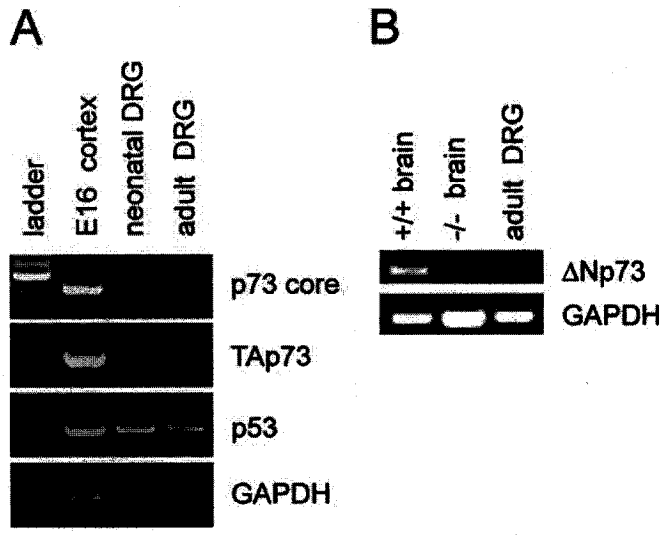


Figure 3. Expression of p73 in L4 DRG. RT-PCR analysis for p73 mRNA in total RNA isolated from the neonatal (P1 DRG) and adult (6-week-old DRG) mouse dorsal root ganglia. Total RNA from the E16 cortex or from the total brain (P1) of p73+/+ versus p73−/− animals were all used as controls. Primers were specific to the core region that is shared among all p73 isoforms (p73 core) (*A*), to the full-length TA-containing isoforms (TAp73) (*A*), to the N-terminal truncated isoforms (ΔNp73) (*B*), or as a positive control to p53. RT-PCR for GAPDH was used to demonstrate that RNA was present in all samples. Note that all p73 isoforms are expressed in the developing brain, as we reported previously (Pozniak et al., 2000, 2002), and are not present in the p73−/− brain RNA samples.

4*B*). Confirmation that the actual number of neurons was similar in p73+/+ versus p73+/− L4 DRGs was obtained by quantitating axons in dorsal root transverse sections using electron microscopy, a method that provides a reliable index of neuronal number in the L4 DRG (Coggeshall et al., 1997). This analysis revealed that p73+/+ and p73+/− dorsal roots contain 3411 ± 170 ($n = 3$) and 3301 ± 179 ($n = 3$) unmyelinated and 2376 ± 104 ($n = 3$) and 2335 ± 131 ($n = 3$) myelinated axons, respectively, numbers that were statistically similar between the genotypes (Fig. 4*C*,*D*).

Having demonstrated that p73 heterozygosity had no impact on the repertoire or number of DRG neurons *in vivo*, we then asked whether the decreased levels of p73 in p73+/− neurons made them more vulnerable to apoptotic insults in culture. Adult DRG neurons were isolated from p73+/+ and p73+/− mice, established in the presence of NGF for 24 hr, treated with the DNA damaging agent camptothecin (10 μM), and the number of phase-bright neurons counted over the ensuing 2 d. As seen with adult rat DRG neurons (Fig. 1*B*), the survival of adult p73+/+ mouse DRG neurons was only slightly diminished (80 ± 7% surviving neurons) by camptothecin at 48 hr (Fig. 5*A*). In contrast, p73+/− neurons showed a significantly enhanced sensitivity to camptothecin treatment relative to the p73+/+ neurons (Fig. 5*A*), with only 54 ± 6% surviving at 48 hr. A similar difference was seen when we inhibited the PI3-kinase survival pathway in these two populations of neurons using 50 μM LY294002. At 48 hr, survival was 72 ± 7 versus 40 ± 6% for p73+/+ versus p73+/− neurons, respectively (Fig. 5*B*). Thus, adult DRG neurons from p73+/− heterozygous mice are more vulnerable to apoptotic stimuli than their wild-type counterparts.

Although these data argued that p73 was essential for the relative invulnerability of adult neurons, and that even a small decrease in levels was sufficient to make them more sensitive to apoptotic stimuli, it was still formally possible that the p73+/− neurons differed from their wild-type counterparts in some other parameter. To address this possibility, we performed a rescue experiment. If the increased sensitivity of p73+/− adult neurons to apoptotic stimuli was attributable to a decrease in levels of ΔNp73, then we should be able to rescue this phenotype by exogenously increasing expression of ΔNp73. To perform this rescue experiment, established cultures of adult p73+/− DRGs were infected with recombinant adenoviruses expressing ΔNp73/GFP or GFP alone, and 1 d later, neurons were either withdrawn from NGF or treated with 10 μM camptothecin or 50 μM

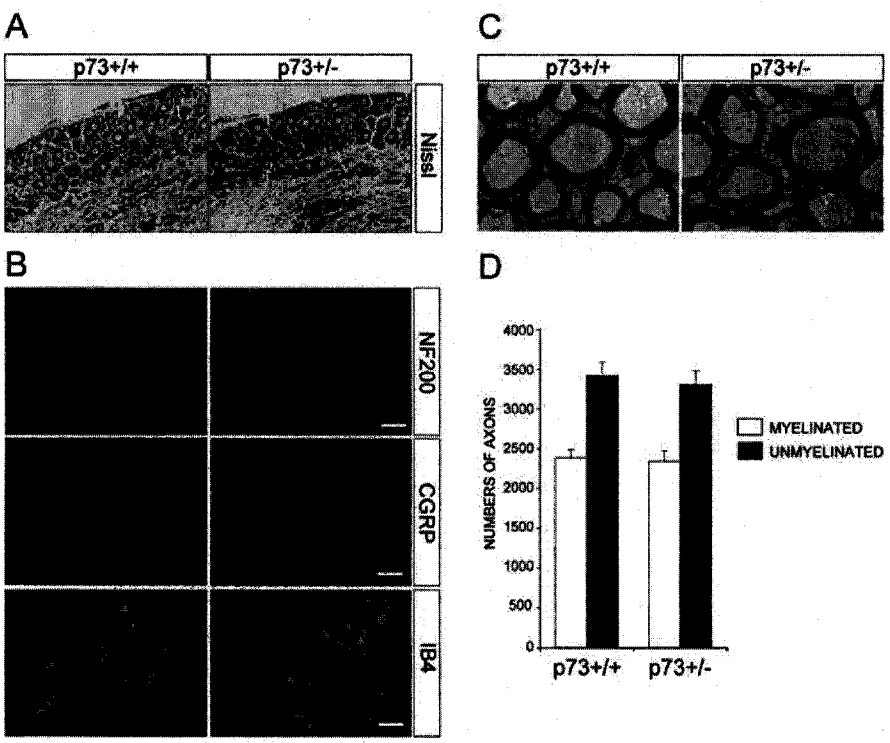


Figure 4. Deletion of one p73 allele does not affect the development of sensory neurons of the L4 DRG. *A*, Cresyl violet-stained sections of the L4 DRG from p73+/+ versus p73+/− adult littermates. Scale bar, 100 μm. *B*, Immunocytochemical analysis of sections through the p73+/+ and p73+/− L4 DRG with antibodies specific to NF200 (top panels), which marks predominantly large-diameter myelinated neurons, CGRP (middle panels), which marks predominantly small-diameter peptidergic nociceptors, and IB4 (bottom panels), which marks small-diameter nonpeptidergic nociceptors. Scale bars, 50 μm. *C*, Electron micrographs of transverse sections through the L4 dorsal roots of adult p73+/+ versus p73+/− animals. Scale bars, 2 μm. *D*, Quantitation of sections similar to those shown in *C* for the number of myelinated and unmyelinated axons in the L4 dorsal roots of p73+/+ and p73+/− animals.

LY294002. Quantitation of the number of GFP-positive, phase-bright neurons 48 hr later revealed that the overexpression of ΔNp73, but not GFP, rescued the enhanced susceptibility of p73+/− neurons (Fig. 5*C*), arguing that ΔNp73 was essential for making adult DRG neurons resistant to apoptotic stimuli, at least in culture.

One other population of neurons that requires ΔNp73 for their survival is neonatal sympathetic neurons (Pozniak et al., 2000; Lee et al., 2004). Moreover, exogenously increased expression of ΔNp73 is sufficient to rescue sympathetic neurons from apoptosis induced by NGF withdrawal (Pozniak et al., 2000; Lee et al., 2004). We therefore asked whether p73+/− neonatal sympathetic neurons were also more sensitive to apoptotic insults than their wild-type counterparts. Neurons were cultured from neonatal p73+/− versus p73+/+ littermates, these neurons were then exposed to either 10 μM camptothecin or 50 μM LY294002, and apoptosis was quantified 48 hr later by assessing nuclear morphology. As shown previously (Crowder and Freeman, 1998; Park et al., 1998; Vaillant et al., 1999), both of these apoptotic stimuli were sufficient to cause sympathetic neuron apoptosis in the presence of NGF (Fig. 5*D*). However, unlike adult DRG neurons, apoptosis of p73+/− sympathetic neurons was not enhanced relative to p73+/+ sympathetic neurons (Fig. 5*D*).

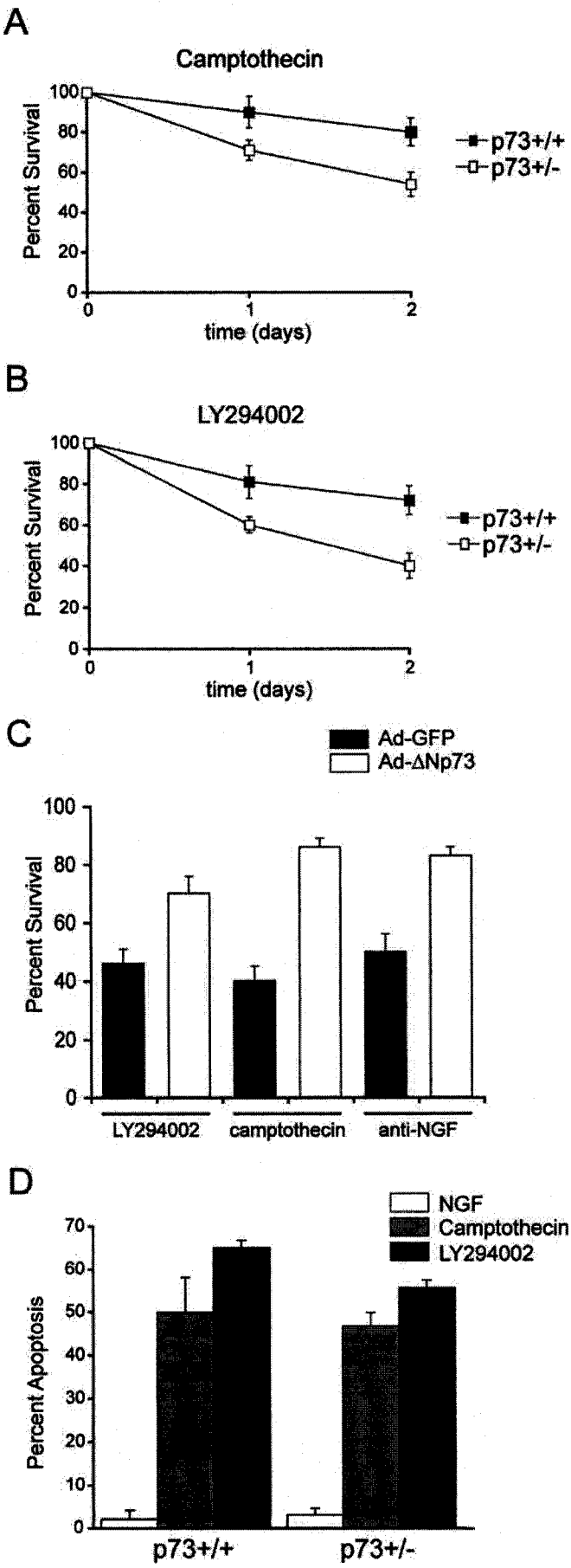
p73 is necessary for the survival of adult DRG neurons after peripheral nerve injury *in vivo*

The anatomical analysis of the L4 DRG presented above demonstrated that the development of sensory neurons is normal in the p73+/− animals, but our culture data implied that adult neurons might be impaired in their survival after injury. To directly test this idea, we resected the sciatic nerve of 6-week-old p73+/+ versus p73+/− animals and, 1 month later, asked whether sensory neuron survival was affected by counting myelinated and unmyelinated axons in electron micrographs of the L4 DRG dorsal roots. This analysis revealed that, as previously reported in rats (Coggeshall et al., 1997; Tandrup et al., 2000), the amount of axonal loss in wild-type mice (p73+/+ littermates) was minimal 1 month after this type of axonal injury (Fig. 6*A*,*B*). The number of small, unmyelinated axons was reduced from 3411 ± 170 to 3061 ± 309 ($n = 3$) (a decrease of ~10%), whereas the number of large, myelinated axons was unaffected (2376 ± 105, $n = 3$, vs 2314 ± 132, $n = 3$). In contrast, although the number of myelinated axons was unaffected after axotomy of p73+/− mice (2335 ± 130, $n = 3$, vs 2292 ± 154, $n = 3$), there was a substantive and significant 30% loss of unmyelinated axons in p73+/− animals (2144 ± 209, $n = 3$, vs 3061 ± 309, $n = 4$, p73+/− vs p73+/+, respectively). Thus, a decrease in the levels of p73 is sufficient to cause enhanced neuronal vulnerability both *in vivo* and *in vitro*, arguing that this molecule plays a key role in rendering adult neurons relatively invulnerable to injury.

Discussion

In this study, we present data indicating that the relative invulnerability of adult neurons is attributable to a cell-intrinsic enhancement of neuronal survival pathways, and that a key component of these survival pathways is ΔNp73. More specifically, our data support three major conclusions. First, we provide evidence that adult sensory neurons are much more resistant to a variety of apoptotic stimuli than are their neonatal counterparts, and that this resistance is mediated downstream of JNK. Second, we show that increased expression of ΔNp73 is sufficient to rescue JNK-mediated apoptosis of neonatal neurons, and that the loss of one allele of p73 renders adult sensory neurons more vulnerable to apoptotic stimuli in culture. This enhanced vulnerability is attributable to decreased ΔNp73 because the invulnerability of adult sensory neurons can be restored by exogenous expression of ΔNp73 in p73+/− neurons. Third, we demonstrate that these findings are relevant *in vivo*; the loss of even one p73 allele, while having no overt effects on developing sensory neurons, enhanced the death of small unmyelinated DRG neurons after axonal injury in adults. Thus, p73 represents one key mechanism used by neurons to ensure that they survive the lifetime of the animal.

We demonstrated previously that the ΔNp73 isoforms are highly potent survival proteins, inhibiting the death of cultured sympathetic and cortical neurons as mediated by a variety of stimuli, including NGF withdrawal and DNA damage (Pozniak et al., 2000, 2002). Moreover, we previously demonstrated enhanced death of p73−/− postnatal sympathetic and cortical neu-



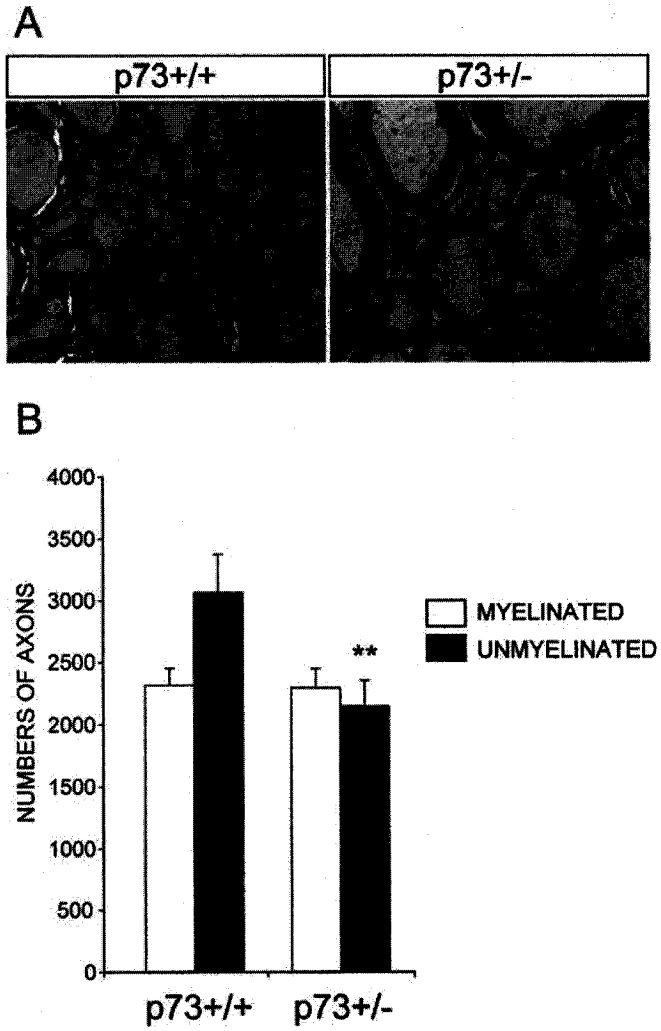


Figure 6. The absence of one p73 allele leads to enhanced death of small diameter, unmyelinated adult sensory neurons after axonal injury. *A*, Electron micrographs of transverse sections through the L4 dorsal roots of p73+/− versus p73+/+ animals 1 month after sciatic nerve resection. Scale bar, 2 μm. *B*, Quantitation of the number of myelinated and unmyelinated axons present in cross sections similar to those shown in *A* (*t* test; $^{**}p < 0.005$). Error bars represent SD.

←

Figure 5. *A*, *B*, p73+/− adult sensory neurons are more vulnerable to apoptotic stimuli. Adult sensory neurons cultured from p73+/+ versus p73+/− animals were established in NGF for 1 d and then were maintained in NGF but with the addition of 10 μM camptothecin, a DNA-damaging agent (*A*) or 50 μM LY294002, an inhibitor of PI3-kinase (*B*). Phase-bright neurons with intact neurites in multiple identified fields were counted at 0, 1, and 2 d, and the amount of survival plotted relative to the initial number of neurons in the same fields at the time the treatment was initiated (percentage survival). Results are the cumulative results of at least three independent experiments, and the error bars represent SEM (*t* test; $p < 0.005$; comparison between groups at 2 d time point). *C*, The enhanced vulnerability of p73+/− adult sensory neurons can be rescued by expression of ΔNp73. Adult p73+/− neurons were established in NGF, infected with recombinant adenoviruses expressing either GFP or ΔNp73β and GFP, and then either withdrawn from NGF (anti-NGF) or maintained in the presence of NGF with the addition of 50 μM LY294002 or 10 μM camptothecin. Two days later, the survival of GFP-positive neurons was quantitated and expressed as a percentage of the total number of GFP-positive neurons. Error bars represent SEM. *D*, p73+/− and p73+/+ neonatal sympathetic neurons are equally sensitive to apoptotic stimuli. Neonatal sympathetic neurons cultured from p73+/+ versus p73+/− animals were established in NGF for 5 d and then maintained in either 10 ng/ml NGF or 10 ng/ml NGF with the addition of either 10 μM camptothecin or 50 μM LY294002. The number of apoptotic nuclei was quantified as determined by Hoechst staining 48 hr after treatment. Error bars represent SEM.

rons *in vivo*, indicating that this gene was essential for the survival of at least some populations of developing neurons (Pozniak et al., 2000, 2002; Lee et al., 2004). However, the first indication that p73 might be important for survival of mature neurons was our finding of ongoing loss of cortical neurons between 3 and 8 weeks postnatally in p73−/− animals. Although there are many potential explanations for such a finding, this observation suggested that p73 might play a previously unsuspected role in determining the maintenance of adult neurons. In that regard, the data documented here demonstrate such a cell-intrinsic role for p73 in peripheral neurons, and our previous *in vivo* work on the p73−/− cortex suggests that this role may well generalize to CNS neurons.

How does p73 determine the long-term survival of adult neurons? We propose that it is the ΔNp73 isoforms that are important for this function because (1) ΔNp73 isoforms are the only isoforms detectable in the brain at the protein level, (2) as discussed above, ΔNp73 is a very potent survival protein when overexpressed, whereas overexpression of TAp73 causes neuronal apoptosis (Lo et al., 2003), and (3) as shown here, ΔNp73 rescues the enhanced vulnerability of p73+/− adult sensory neurons. We also propose that one of the ways that ΔNp73 determines survival of mature neurons is by functionally antagonizing p53. p53 has been shown to play a key role in mediating the apoptosis of mature neurons *in vivo* in response to a wide variety of stimuli, including excitotoxicity, DNA damage (for review, see Miller et al., 2000; Morrison and Kinoshita, 2000), and, with regard to the current study, axonal injury (Martin and Liu, 2002). Support for this model derives from the fact that ΔNp73 rescues sympathetic neurons from apoptosis induced by p53 overexpression (Pozniak et al., 2000), and from our recent finding that deletion of even one p53 allele is sufficient to partially rescue the enhanced sympathetic neuron apoptosis seen in developing p73−/− animals (Lee et al., 2004). Together, we believe these findings make a compelling argument that p53 is one of the targets of ΔNp73. However, it is also clear that ΔNp73 has additional targets (Lee et al., 2004), and we propose that these additional targets include other full-length, proapoptotic p53 family members such as p63, the third p53 family member (Yang et al., 1998, 1999), which is predominantly expressed in the nervous system as a full-length TAp63 isoform (Jacobs et al., 2004). In that regard, we propose that it is ultimately the balance of full-length, proapoptotic p53/TAp63 family members relative to the prosurvival N-terminal truncated ΔNp73/ΔNp63 isoforms that determines the life versus death of any given neuron.

How does such a p53 family-dependent checkpoint integrate with our current knowledge of adult neuron survival and apoptosis? Although most of what we know about neuronal survival and death pathways derives from studies of developing neurons, studies on peripheral neurons aged in culture have provided some insight. In particular, aged, NGF-independent sympathetic neurons still activate the JNK pathway after NGF withdrawal, but they do not translocate Bax in the mitochondrion, and Bcl2+/− neurons are just as resistant to NGF withdrawal as Bcl2+/+ neurons, implying that a major block to apoptosis occurs between JNK and Bax (Greenlund et al., 1995; Putcha et al., 2000). Is there any evidence that the p53 family might act at such an upstream point? Although p53 is thought to mediate at least some of its proapoptotic actions at the transcriptional level, for example by enhanced transcription of Bax (Miyashita and Reed, 1995), there is also evidence that it can promote apoptosis directly at the level of the mitochondrion in a cell-free system (Schuler et al., 2000; Mihara et al., 2003). Moreover, we demonstrate here that ΔNp73 can rescue apoptosis induced by JNK pathway activation, and our recent studies in sympathetic neurons demonstrate that ΔNp73 can block the mitochondrial apoptotic transition after NGF withdrawal, potentially by inhibiting full-length p53 family members and/or binding directly to JNK itself (Lee et al., 2004). Thus, all of these data are consistent with the hypothesis that adult neurons have a major block to apoptosis downstream of JNK but upstream of the mitochondrion, and that this major checkpoint involves ΔNp73. It is likely that additional downstream checkpoints also exist, potentially involving the Bcl2 and/or inhibitor of apoptosis protein families (Wiese et al., 1999; Yuan and Yankner, 2000). In that regard, if one way that adult neurons become resistant to apoptosis is by enhancing survival pathways, it makes sense to have multiple checkpoints at different levels to ensure that mature neurons are only eliminated when they have received multiple and continuous apoptotic insults.

Our ongoing studies on the role of p73 in the nervous system indicate that in addition to the essential role in maintenance of mature neurons that is demonstrated here, ΔNp73 is also important for the survival of a number of populations of developing neurons, including neonatal sympathetic and cortical neurons (Pozniak et al., 2000, 2002; Lee et al., 2004). Moreover, p73−/− mice display reduced numbers of caudal CNS neurons at birth, including facial motor neurons and neurons of the deep cerebellar nuclei (Pozniak et al., 2002), implying that these neuronal populations might also have enhanced apoptosis during their embryonic developmental death period in the absence of p73. However, whereas our findings argue that p73 is important for neuronal survival both in development and in adulthood, data presented here suggest that the requirement for p73 somehow differs in developing versus adult neurons; neonatal sympathetic neurons are not sensitive to the loss of a single p73 allele, whereas adult sensory neurons are sensitive to the loss. One potential explanation for this difference is that developing versus adult neurons differ in how they regulate ΔNp73 levels. For example, when neonatal sympathetic neurons are withdrawn from NGF, ΔNp73 levels fall dramatically (Pozniak et al., 2000), a response that is necessary to ensure the death of neurons that do not compete successfully for target-derived NGF *in vivo*. Disruption of the essential PI3-kinase survival pathway and/or DNA damage may lead to a similar loss of ΔNp73 expression in neonatal sympathetic neurons, thereby making p73+/− and p73−/− neurons equally vulnerable to apoptotic stimuli. In contrast, if adult neurons maintain higher basal levels of ΔNp73 through a cell-intrinsic mechanism that is essentially impervious to extrinsic cues, these neurons would be relatively invulnerable to apoptotic stimuli. However, a decrease in basal ΔNp73 levels as a function of decreased p73 gene dosage would then be predicted to enhance neuronal vulnerability to these same apoptotic insults.

One of the major implications of this study derives from our finding that the lack of one p73 allele is sufficient to have a major impact on neuronal survival after injury. Although the finding that relatively small potential alterations in protein levels would influence neuronal survival is a prediction of a "rheostat" model as we proposed, it is nonetheless surprising and has broad implications with regard to a genetic propensity for injury-induced damage to the adult nervous system and even with regard to neurodegeneration. In particular, if the levels of ΔNp73 serve as a "buffer," a small decrease in levels might well be sufficient to enhance the probability that an individual neuron would apoptose in response to an extrinsic cue, something that would ultimately result in an increased rate of neuronal loss over time (Clarke et al., 2000). This is precisely what is seen in the culture

studies reported here, where the rate of cell death in response to apoptotic stimuli is increased in p73+/− neurons. Moreover, a similar increase in rate might be responsible for the axotomy results reported here, because the loss of small diameter unmyelinated sensory neurons seen in the p73+/− animals at 1 month is approximately similar in magnitude to the loss that is seen in rats after 8 months (Tandrup et al., 2000). Such a genetically determined increase in the rate of death would be particularly problematic in situations of acute injury, such as during ischemic stroke and potentially even in the aging brain, where the DNA damage response, which is regulated by the p53 family, is now known to be elevated (Lu et al., 2004).

References

Acheson A, Conover JC, Fandl JP, DeChiara TM, Russell M, Thadani A, Squinto SP, Yancopoulos GD, Lindsay RM (1995) A BDNF autocrine loop in adult sensory neurons prevents cell death. Nature 374:450–453.

Aloyz RS, Bamji SX, Pozniak CD, Toma JG, Atwal J, Kaplan DR, Miller FD (1998) p53 is essential for developmental neuron death as regulated by the TrkA and p75 neurotrophin receptors. J Cell Biol 143:1691–1703.

Angeletti PU, Levi-Montalcini R, Caramia F (1971) Analysis of the effects of the antiserum to the nerve growth factor in adult mice. Brain Res 27:343–355.

Bamji SX, Majdan M, Pozniak CD, Belliveau DJ, Aloyz R, Kohn J, Causing CG, Miller FD (1998) The p75 neurotrophin receptor mediates neuronal apoptosis and is essential for naturally occurring sympathetic neuron death. J Cell Biol 140:911–923.

Bjerre B, Bjorklund A, Mobley W, Rosengren E (1975) Short- and long-term effects of nerve growth factor on the sympathetic nervous system in the adult mouse. Brain Res 94:263–277.

Chun LL, Patterson PH (1977) Role of nerve growth factor in the development of rat sympathetic neurons *in vitro*. I. Survival, growth, and differentiation of catecholamine production. J Cell Biol 75:694–704.

Clarke G, Collins RA, Leavitt BR, Andrews DF, Hayden MR, Lumsden CJ, McInnes RR (2000) A one-hit model of cell death in inherited neuronal degenerations. Nature 406:195–199.

Coggeshall RE, Lekan HA, Doubell TP, Allchorne A, Woolf CJ (1997) Central changes in primary afferent fibers following peripheral nerve lesions. Neuroscience 77:1115–1122.

Crowder RJ, Freeman RS (1998) Phosphatidylinositol 3-kinase and Akt protein kinase are necessary and sufficient for the survival of nerve growth factor-dependent sympathetic neurons. J Neurosci 18:2933–2943.

Easton RM, Deckwerth TL, Parsadanian AS, Johnson Jr EM (1997) Analysis of the mechanism of loss of trophic factor dependence associated with neuronal maturation: a phenotype indistinguishable from Bax deletion. J Neurosci 17:9656–9666.

Eilers A, Whitfield J, Babij C, Rubin LL, Ham J (1998) Role of the Jun kinase pathway in the regulation of c-Jun expression and apoptosis in sympathetic neurons. J Neurosci 18:1713–1724.

Goedert M, Otten U, Thoenen H (1978) Biochemical effects of antibodies against nerve growth factor on developing and differentiated sympathetic ganglia. Brain Res 148:264–268.

Greenlund LJ, Korsmeyer SJ, Johnson Jr EM (1995) Role of BCL-2 in the survival and function of developing and mature sympathetic neurons. Neuron 15:649–661.

Hendry IA, Campbell J (1976) Morphometric analysis of rat superior cervical ganglion after axotomy and nerve growth factor treatment. J Neurocytol 5:351–360.

Irwin MS, Kaelin WG (2001) p53 family update: p73 and p63 develop their own identities. Cell Growth Differ 12:337–349.

Jacobs WB, Govoni G, Ho D, Mills AA, Miller FD, Kaplan DR (2004) p63 is an essential proapoptotic molecule during developmental neuronal death. Soc Neurosci Abstr, in press.

Lazarus KJ, Bradshaw RA, West NR, Bunge P (1976) Adaptive survival or rat sympathetic neurons cultured without supporting cells or exogenous nerve growth factor. Brain Res 113:159–164.

Lee AF, Ho DK, Zanassi P, Walsh GS, Kaplan DR, Miller FD (2004) Evidence that ΔNp73 promotes neuronal survival by p53-dependent and independent mechanisms. J Neurosci 24:9174–9184.

[illegible] (1988) [illegible] (NGF, BDNF) [illegible]

regeneration but are not required for survival of adult sensory neurons. J Neurosci 8:2394–2405.

Lo WD, Akhmametyeva EM, Zhu L, Friesen PD, Chang LS (2003) Induction of apoptosis by the p53-related p73 and partial inhibition by the baculovirus-encoded p35 in neuronal cells. Brain Res Mol Brain Res 113:1–12.

Lu T, Pan Y, Kao SY, Li C, Kohane I, Chan J, Yankner BA (2004) Gene regulation and DNA damage in the ageing human brain. Nature 429:883–891.

Ma J, Novikov LN, Wiberg M, Kellerth JO (2001) Delayed loss of spinal motoneurons after peripheral nerve injury in adult rats: a quantitative morphological study. Exp Brain Res 139:216–223.

Majdan M, Walsh GS, Aloyz R, Miller FD (2001) TrkA mediates developmental sympathetic neuron survival *in vivo* by silencing an ongoing p75NTR-mediated death signal. J Cell Biol 155:1275–1285.

Maroney AC, Finn JP, Bozyczko-Coyne D, O'Kane TM, Neff NT, Tolkovsky AM, Park DS, Yan CY, Troy CM, Greene LA (1999) CEP-1347 (KT7515), an inhibitor of JNK activation, rescues sympathetic neurons and neuronally differentiated PC12 cells from death evoked by three distinct insults. J Neurochem 73:1901–1912.

Martin LJ, Liu Z (2002) Injury-induced spinal motor neuron apoptosis is preceded by DNA single-strand breaks and is p53- and Bax-dependent. J Neurobiol 50:181–197.

Mihara M, Erster S, Zaika A, Petrenko O, Chittenden T, Pancoska P, Moll UM (2003) p53 has a direct apoptogenic role at the mitochondria. Mol Cell 11:577–590.

Miller FD, Kaplan DR (2001) Neurotrophin signalling pathways regulating neuronal apoptosis. Cell Mol Life Sci 58:1045–1053.

Miller FD, Pozniak CD, Walsh GS (2000) Neuronal life and death: an essential role for the p53 family. Cell Death Differ 7:880–888.

Miyashita T, Reed JC (1995) Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 80:293–299.

Morrison RS, Kinoshita Y (2000) The role of p53 in neuronal cell death. Cell Death Differ 7:868–879.

Otten U, Goedert M, Schwab M, Thibault J (1979) Immunization of adult rats against 2.5 S NGF: effects on the peripheral sympathetic nervous system. Brain Res 176:79–90.

Park DS, Morris EJ, Padmanabhan J, Shelanski ML, Geller HM, Greene LA (1998) Cyclin-dependent kinases participate in death of neurons evoked by DNA-damaging agents. J Cell Biol 1998 143:457–467.

Pozniak CD, Radinovic S, Yang A, McKeon F, Kaplan DR, Miller FD (2000) An anti-apoptotic role for the p53 family member, p73, during developmental neuron death. Science 289:304–306.

Pozniak CD, Barnabe-Heider F, Rymar VV, Lee AF, Sadikot AF, Miller FD (2002) p73 is required for survival and maintenance of CNS neurons. J Neurosci 22:9800–9809.

Putcha GV, Deshmukh M, Johnson Jr EM (2000) Inhibition of apoptotic signaling cascades causes loss of trophic factor dependence during neuronal maturation. J Cell Biol 149:1011–1018.

Roux PP, Dorval G, Boudreau M, Angers-Loustau A, Morris SJ, Makkerh J, Barker PA (2002) K252a and CEP1347 are neuroprotective compounds that inhibit mixed-lineage kinase-3 and induce activation of Akt and ERK. J Biol Chem 277:49473–49480.

Schuler M, Bossy-Wetzel E, Goldstein JC, Fitzgerald P, Green DR (2000) p53 induces apoptosis by caspase activation through mitochondrial cytochrome c release. J Biol Chem 275:7337–7342.

Snider WD, Elliott JL, Yan Q (1992) Axotomy-induced neuronal death during development. J Neurobiol 23:1231–1246.

Tandrup T, Woolf CJ, Coggeshall RE (2000) Delayed loss of small dorsal root ganglion cells after transection of the rat sciatic nerve. J Comp Neurol 422:172–180.

Tong JX, Vogelbaum MA, Drzymala RE, Rich KM (1997) Radiation-induced apoptosis in dorsal root ganglion neurons. J Neurocytol 26:771–777.

Vaillant AR, Mazzoni I, Tudan C, Boudreau M, Kaplan DR, Miller FD (1999) Depolarization and neurotrophins converge on the phosphatidylinositol 3-kinase-Akt pathway to synergistically regulate neuronal survival. J Cell Biol 146:955–966.

Vogelbaum MA, Tong JX, Rich KM (1998) Developmental regulation of [illegible]

Wiese S, Digby MR, Gunnersen JM, Gotz R, Pei G, Holtmann B, Lowenthal J, Sendtner M (1999) The anti-apoptotic protein ITA is essential for NGF-mediated survival of embryonic chick neurons. Nat Neurosci 2:978–983.

Xiang H, Kinoshita Y, Knudson CM, Korsmeyer SJ, Schwartzkroin PA, Morrison RS (1998) Bax involvement in p53-mediated neuronal cell death. J Neurosci 18:1363–1373.

Yang A, Kaghad M, Wang Y, Gillett E, Fleming MD, Dotsch V, Andrews NC, Caput D, McKeon F (1998) p63, a p53 homolog at 3q27–29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. Mol Cell 2:305–316.

Yang A, Schweitzer R, Sun D, Kaghad M, Walker N, Bronson RT, Tabin C, Sharpe A, Caput D, Crum C, McKeon F (1999) p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. Nature 398:714–718.

Yang A, Walker N, Bronson R, Kaghad M, Oosterwegel M, Bonnin J, Vagner C, Bonnet H, Dikkes P, Sharpe A, McKeon F, Caput D (2000) p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours. Nature 404:99–103.

Yip HK, Rich KM, Lampe PA, Johnson Jr EM (1984) The effects of nerve growth factor and its antiserum on the postnatal development and survival after injury of sensory neurons in rat dorsal root ganglia. J Neurosci 4:2986–2992.

Yuan J, Yankner BA (2000) Apoptosis in the nervous system. Nature 407: 802–809.

APPENDIX C

Ethics certificates for research involving animal subjects, microorganisms, living cells, other biohazards and/or radioactive material.

McGill University

University Biohazards Committee

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

No project should be commenced without prior approval of an application to use biohazardous materials. Submit this application to the Chair, Biohazards Committee, one month before starting new projects or expiry of a previously approved application.

1. PRINCIPAL INVESTIGATOR: Dr Freda Miller

ADDRESS: 3801 University Street, Room BT-112 — TELEPHONE: 398-4261

Montreal, Quebec, H3A 2B4 — FAX NUMBER: 398-6547

DEPARTMENT: Brain Tumor Research Centre — E-MAIL: mdfm@musica.mcgill.ca

PROJECT TITLE: Signalling Pathways Regulating Neuronal Apoptosis

2. FUNDING SOURCE: MRC NSERC NIH FCAR FRSQ

INTERNAL OTHER X (specify) CIHR (external)

Grant No.: 225082 — Beginning date: April 1st, 2001 — End date: April 1st 2005

3. Indicate if this is

Renewal use application: procedures have been previously approved and no alterations have been made to the protocol.
Approval End Date

New funding source: project previously reviewed and approved under an application to another agency.

Agency CIHR — Approval End Date : April 1st, 2005

New project: project not previously reviewed or procedures and/or microorganism altered from previously approved application.

CERTIFICATION STATEMENT: The Biohazards Committee approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in the "Laboratory Biosafety Guidelines" prepared by Health Canada and the MRC, and in the "McGill Laboratory Biosafety Manual".
Containment Level (circle 1): 1 (2) 3

Principal Investigator or course director: signature — date: day 03 month Oct year 2001

Chairperson, Biohazards Committee: LILIAS J. BRIEDIS, Dept. of Microbiology & Immunology, McGill University, 3775 University Street, Montreal, Quebec, Canada H3A 2B4 — signature — date: 11-10-2001

Approved period: beginning 11-10-2001 ending 01-04-2005

* as defined in the "McGill Laboratory Biosafety Manual"

1st REVISION, JANUARY 1996

RESEARCH PERSONNEL: (attach additional sheets if required)

Name	Department	Check appropriate classification: Investigator	Technician & Research Assistant	Student: Undergraduate	Student: Graduate	Fellow
Dr Freda Miller	BTRC	X				
Anne Aumont	BTRC		X			
Annie Sylvestre	BTRC		X			
Greg Walsh	BTRC				X	
Anna Lee	BTRC				X	
Fanie Barnabe-Heider	BTRC				X	
Catherine Menard	BTRC				X	
Mahnaz Akhvan	BTRC		X			
Jean Toma	BTRC					X
Mathieu Fortier	BTRC				X	
Paul Hein	BTRC				X	
Yeghat Wang	BTRC		X			
Amelie Rioux-Tache	BTRC		X			
Laura Craig	BTRC				X	
Karun Singh	BTRC				X	
Patrizia Zanassi	BTRC					X
Nina Orike	BTRC					X
Nao Kobayashi	BTRC					X
Karl Fernandes	BTRC					X
Annie Paquin	BTRC			X		
Ian Mckenzie	BTRC				X	

EMERGENCY: Person(s) designated to handle emergencies

Name: Dr Freda Miller — Phone No: work: 398-4261 — home: 482-2186

Name: Greg Walsh — Phone No: work: 398-3334 — home: 288-6271

6. Briefly describe:

i) the biohazardous material involved (e.g. bacteria, viruses, human tissues) & designated biosafety risk group

The biohazard material involved is the non-replicating *adenovirus*; the risk group is #2.

ii) the procedures involving biohazards:

The adenovirus is used in 2 different types of experiments:

1- microinjections of the virus into transgenic mice

2- infection with the virus in primary cultures and cell line

In both types of experiments the virus is used as a vector to insert certain specific genetic material, of a primarily neural origin into neurons. The virus is attenuated, and the genetic material they carry may cause a phenotypic effect but is not contagious. All work is done in Class II laminar flow hoods.

iii) the protocol for decontaminating spills

- **For a small spill, the area is covered with and wiped with paper towels that have been soaked in bleach (1% bleach, for at least 10 minutes.)**
- **The work area is wiped down before and after work with 70% ethanol.**
- **If a large spill (>500ml) occurs then it is allowed to settle for 30 minutes, before being decontaminated, as above.**
- **If the virus gets on skin, then the area is disinfected at once (medical help sought if necessary)**
- **Any contaminated clothing or materials are autoclaved.**

7. Does the protocol present conditions (e.g. handling of large volumes or high concentrations of pathogens) which could increase the hazards of the infectious agent(s)?

NO

8. Do the specific procedures to be employed involving genetically engineered organisms have a history of safe use?

YES

9. What precautions are being taken to reduce production of infectious droplets and aerosols?

- **The animals and cell culture experiments will be performed in class II laminar flow hoods.**
- **Whenever work is done, there will always be an autoclaved beaker of 1% bleach at hand to decontaminate small items (e.g. pipette tips, Pasteur pipettes); and spray containers of 1% bleach and 70% ethanol are in close proximity to the work.**
- **Personnel working with the virus will wear lab coats and gloves.**

10. List the biological safety cabinets to be used.

Building	Room No.	Manufacturer	Model No.	Serial No.	Date Certified
BTRC	BT-106	Forma Scientific	1286	17719-312	April, 2001
BTRC	BT-106	Forma Scientific	1286	17719-311	April, 2001
BTRC	BT-106	Forma Scientific	1286	19727-301	April, 2001

#7358 D level

McGill University
Animal Use Protocol – Research

Guidelines for completing the form are available at www.mcgill.ca/fgsr/rgo/animal/

Protocol #: 3793
Investigator #: 790
Approval End Date: JUNE 30, 2002
Facility Committee: MNI

☐ Pilot ☐ New Application ☒ Renewal of Protocol # 3793

Title (must match the title of the funding source application): Molecular Mechanisms of neuronal Differenciation

1. Investigator Data:

Principal Investigator:	Dr. Freda Miller	Office #:	398-4261
Department:	Brain Tumor Research Center	Fax#:	398-6547
Address:	BT-109, Montreal Neurological Institute	Email:	mdfm@musica.mcgill.ca

2. Emergency Contacts: Two people must be designated to handle emergencies.

Name:	Freda Miller	Work #:	398-4261	Emergency #:	482-2186
Name:	Annie Sylvestre	Work #:	398-3334	Emergency #:	529-0741

3. Funding Source:

External ☒
Source (s): MRC, partially funding from Christopher Reeves Paralysis Foundation.
Peer Reviewed: ☒ YES ☐ NO**
Status : ☐ Awarded ☐ Pending
Funding period: June 20, 2001 to June 20, 2002

Internal ☐
Source (s):
Peer Reviewed: ☐ YES ☐ NO**
Status: ☐ Awarded ☐ Pending
Funding period:

ACTION	✓	DATE
P.I.	✓	
FACC	✓	
RGO	✓	
VET	✓	
DB		

Approved

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed . e.g. Projects funded from industrial sources. Peer Review Forms are available at www.mcgill.ca/fgsr/rgo/animal/

Proposed Start Date of Animal Use (d/m/y):	June 20, 2001	or ongoing ☐
Expected Date of Completion of Animal Use (d/m/y):	June 20, 2002	or ongoing ☐

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator: [signature] Date: SEPT. 10, 2001

Approval Signatures:

Chair, Facility Animal Care Committee:	[signature]	Date: 10 Sep 2001
University Veterinarian:	[signature]	Date: 9/13/01
Chair, Ethics Subcommittee(as per UACC policy):	[signature]	Date: 9/26/01
Approved Period for Animal Use	Beginning: July 1, 2001	Ending: JUNE 30, 2002

☐ This protocol has been approved with the modifications noted in Section 13.

(NO ADMIN. APPROVAL PRIOR TO ETHICS)

SEP 13 2001