Signaling pathways implicated in p75 neurotrophin receptor-mediated neuronal survival and death

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Dedicated to my father

Nothing will come of nothing.

Dare mighty things.

- *Shakespeare*

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CONTRIBUTIONS OF CO-AUTHORS

This thesis has a manuscript-based structure. According to the faculty regulations, manuscripts co-authored by the candidate and others must be accompanied with an explicit statement as to who contributed to such work and to what extent. Copyright waivers from the co-authors and the publishers appear in the appendix.

Chapter 2

My contribution to the manuscript entitled "p75 neurotrophin receptor expression is induced in apoptotic neurons after seizure" involved performing all experiments, with the exception of the *in situ* hybridizations, which were performed by Dr. Michael Colicos. Dr. Philip Barker, Dr. Timothy Kennedy, and I contributed to the writing of the manuscript.

Chapter 3

I performed all experiments that contributed to the manuscript entitled "The p75 neurotrophin receptor (p75NTR) activates Akt (protein kinase B) through a phosphatidylinositol 3-kinase-dependent pathway". Ms. Asha Bhakar provided technical assistance with the primary cortical cultures. Both Dr. Philip Barker and I contributed to the writing of the manuscript.

Chapter 4

I performed all experiments for the manuscript entitled "K252a and CEP1347 mediate survival by activating PI3-K and MEK", and Mathieu Boudreau provided the MLK3 recombinant adenovirus. Both Dr. Philip Barker and I contributed to the writing of the manuscript.

ABSTRACT

The neurotrophins are growth factors involved in the development, maintenance, survival, and death of the nervous system. The signal transducing systems that mediate the diverse biological functions of the neurotrophins are initiated by their interactions with two categories of cell surface receptors, the Trk family of tyrosine kinases, and the p75 neurotrophin receptor (p75NTR). In contrast to the rapid progress made in elucidating the mechanism of action of the Trk receptors, the physiological roles of p75NTR are uncertain, but two general functions have been ascribed to p75NTR. First, p75NTR can positively or negatively modulate Trk receptor signaling, and second, p75NTR can autonomously activate signaling cascades that results in cellular apoptosis. The signaling pathways employed by p75NTR to mediate its effects are unclear, but p75NTR was found to activate the NF-κB and JNK pathways, and to interact with several adaptor proteins, such as NRAGE, NADE, NRIF, TRAF proteins, and FAP-1.

Nervous system injuries represent interesting models to study p75NTR because several types of injury induce p75NTR expression. In the first part of this thesis, we have used the pilocarpine model of seizure in the rat and found that this type of injury induces neuronal apoptosis and p75NTR expression. Apoptosis was tightly linked with p75NTR expression, suggesting that p75NTR may promote apoptotic cell death after seizure, and consistent with this, we have found that p75NTR can promote JNK activation and apoptosis *in vitro*. In the second study, we discovered that p75NTR can also facilitate survival under some cellular circumstances. The survival-promoting effect of p75NTR was accompanied with PI3-K-dependent Akt activation, and correlated with a reduction in cytosolic protein tyrosine phosphatase activity, suggesting that p75NTR may regulate a tyrosine phosphatase involved in the regulation of Akt and survival. In the last study, we have found that the related neuroprotective compounds, K252a and CEP1347, are potent MLK3 inhibitors, yet they simultaneously activated Akt and ERK, and survival through MLK3-independent mechanisms. These findings suggested that K252a and CEP1347 may act on a novel target responsible for their survival-promoting activities.

Taken together, the data in this thesis adds to our understanding of the physiological functions of p75NTR, and contributes to our knowledge of the cellular machinery that control neuronal cell survival and death.

RÉSUMÉ

Les neurotrophines sont des facteurs de croissance impliqués dans le développement, la maintenance, la survie, et la mort du système nerveux. Les récepteurs tyrosine kinase de la famille Trk, ainsi que le récepteur de neurotrophines p75 (p75NTR), représentent les deux classes de récepteurs des neurotrophines. p75NTR peut moduler l'activité des récepteurs Trk, mais peut également signaler de façon indépendante. La fonction et les mécanismes exacts utilisés par p75NTR sont encore inconnus, mais il fut démontré que l'activation de p75NTR mène à l'engagement des voies de signalisation NF-κB et JNK, et à l'interaction du récepteur avec les protéines cytosoliques NRAGE, NADE, NRIF, les protéines TRAF, et FAP-1.

Les blessures du système nerveux représentent des modèles intéressants pour l'étude de p75NTR parce que plusieurs types de blessures induisent l'expression de p75NTR. Dans la première partie de cette thèse, nous avons démontré que les crises épileptiques chez le rat induites par l'injection de pilocarpine peuvent mener à la mort neuronale par apoptose et à l'expression de p75NTR. Nous avons trouvé une très forte association entre l'apoptose neuronale et l'expression de p75NTR, suggérant que p75NTR est impliqué dans la mort apoptotique suite aux traumas tel les crises épileptiques. Nous avons confirmé ces résultats en démontrant que p75NTR peut activer à la fois la voie JNK et l'apoptose *in vitro*. Dans la seconde étude, nous avons découvert que p75NTR peut également favoriser la survie cellulaire, en activant la kinase Akt, suivant une voie cellulaire impliquant l'enzyme PI3-K. Nous avons trouvé que p75NTR réduit l'activité de protéines tyrosine phosphatases cytosoliques, suggérant que p75NTR contrôle une tyrosine phosphatase impliquée dans la régulation d'Akt et de la survie. Finalement, nous avons découvert dans la dernière étude que les composés K252a et CEP1347 sont tous les deux des inhibiteurs de MLK3, mais que ces composés sont également capable d'activer Akt, ERK, et la survie, en utilisant un mécanisme indépendant de MLK3. Ces résultats démontrent que K252a et CEP1347 agissent sur une cible inconnue pour induire leurs activités neuroprotectrices.

En conclusions, les résultats dans cette thèse apportent une meilleure connaissance des rôles physiologiques de p75NTR, et contribues à notre savoir sur les machineries cellulaires impliquées dans la survie et la mort des neurones.

LIST OF ABBREVIATIONS

Akt	Protein kinase B, or protein kinase related to A and C
ARMS	Ankyrin-rich membrane spanning
Bcl-2	B cell lymphoma-2 protein
BDNF	Brain-derived neurotrophic factor
BH3	Bcl-2 homology-3
CARD	Caspase recruitment domain
CDK	Cyclin-dependent kinase
CHK	Csk-homologous kinase
CNS	Central nervous system
CRD	Cysteine-rich domain
CRIB	Cdc42/Rac1 interactive binding
CRNF	Cysteine-rich neurotrophic factor
DD	Death domain
DISC	Death-inducing signaling complex
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglia
E1	Embryonic day one
ERK	Extracellular signal-regulated kinase
FADD	Fas-associated death domain-containing protein
FAP-1	Fas-associated phosphatase-1
FRS2	Fibroblast growth factor receptor substrate-2
GAP	GTPase-activating protein
Gab-1	Grb-associated binder-1
GSK-3	Glycogen synthase kinase-3
GTP	Guanine triphosphate
IKK1	IκB kinase-1
IRS	Insulin receptor substrate
JNK	c-Jun amino-terminal kinase
kb	Kilobase pair
KCl	Potassium chloride
Kd	Dissociation constant
kDa	KiloDalton
MAPK	Mitogen-activated protein kinase
MEKK	MAPK kinase kinase
MHD	MAGE homology domain
MLK	Mixed lineage kinase
mRNA	Messenger ribonucleic acid
NADE	Neurotrophin receptor-associated death effector
NES	Nuclear export signal
NF-κB	Nuclear factor-kappaB
NGF	Nerve growth factor
NLS	Nuclear localization signal
NMR	Nuclear magnetic resonance

NRIF	Neurotrophin receptor-interacting factor
NT-3, NT-4/5	Neurotrophin-3, neurotrophin-4/5
NRAGE	Neurotrophin receptor-interacting Mage homolog
p75NTR	p75 neurotrophin receptor
PC12	Pheochromocytoma cell line
PDK1	3-phosphoinositide-dependent kinase-1
PDZ	Domain identified as conserved elements in postsynaptic density protein PSD-95, Disc-large tumor suppressor Dlg, Zonula occludens protein ZO-1.
PI3-K	Phosphatidylinositol 3-kinase
PKC	protein kinase C
PLCγ	Phospholipase Cgamma
PNS	Peripheral nervous system
PTPase	Protein tyrosine phosphatase
rAPS	Rat adaptor molecule containing PH and SH2 domains
Rb	Retinoblastoma protein
RIP-2	Receptor-interacting protein-2
RSK	Ribosomal S6 kinase
SC-1	Schwann cell factor-1
SE	Status epilepticus
TNF	Tumor necrosis factor
TRADD	TNF receptor-associated death domain containing protein
TRAF	TNF receptor-associated factor
Trk	Tropomyosin-related kinase
TUNEL	Terminal transferase-mediated dUTP nicked DNA end-labeling
ZLK	Leucine zipper-bearing kinase

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.0 GENERAL INTRODUCTION

Development and maintenance of the nervous system is orchestrated by a complex interplay between diffusible cues and their corresponding cell surface receptors. The best characterized mammalian neuronal differentiation factors are the neurotrophins. The neurotrophins were initially identified as target-derived neuron survival factors (Purves et al., 1988; Oppenheim, 1991), but are now recognized to mediate a wide range of responses that include the regulation of neuroblast and neural crest cell proliferation, regulation of neurite outgrowth, modulation of synapse properties, maintenance of survival and induction of apoptosis (Lewin and Barde, 1996; Bibel and Barde, 2000; Huang and Reichardt, 2001).

In addition to their normal physiological roles, neurotrophins have also been implicated in neurological disorders, including Alzheimer's disease (Higgins and Mufson, 1989; Mufson et al., 1989; Phillips et al., 1991; Strada et al., 1992), Parkinson's disease (Hyman et al., 1991; Spina et al., 1992), epilepsy (Gall and Isackson, 1989; Isackson et al., 1991), and cancers of the central nervous system (CNS)(Kogner et al., 1993; Segal et al., 1994; Hoehner et al., 1995; Ryden et al., 1996). It is likely that elucidation of the molecular mechanisms that control neurotrophin function will reveal targets for pharmacological intervention that will have substantial benefit in treating human nervous system diseases.

The signal transduction systems that mediate the diverse biological functions of the neurotrophins are initiated through interactions with two categories of cell surface receptors, the Trk (tropomyosin-related kinase) tyrosine kinase receptors and the p75 neurotrophin receptor (p75NTR). These receptors share no sequence similarity in either ligand-binding or cytoplasmic domains, and activate distinct neurotrophin-dependent signaling pathways. In many instances, Trk and p75NTR not only activate autonomous pathways but also collaborate to mediate effects of the neurotrophins.

1.1 THE NEUROTROPHINS

Nerve growth factor (NGF), the prototypic neurotrophin, is the best characterized member of this family of growth factors that in mammals also includes brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-3, and NT-4/5 (Snider, 1994). With the exception of NT-4/5, which is not detected in avian species, the neurotrophin sequences are highly conserved in vertebrates. The *nt-6* and *nt-7* genes encode neurotrophins that have been identified only in fish, and do not appear to have mammalian orthologues (Gotz et al., 1994; Nilsson et al., 1998).

All four mammalian neurotrophin genes encode glycosylated neurotrophin precursors (31-35 kDa) which are cleaved to give rise to mature processed neurotrophins (13.2-15.9 kDa)(Figure 1.1). Neurotrophin precursors contain an amino-terminal signal peptide followed by well conserved pro-regions that are processed by convertases, including Furin and Convertases 1 and 2 (PC1 and PC2) which are present in the trans-Golgi network and in dense-core secretory granules (Hosaka et al., 1991; Molloy et al., 1994; Dubois et al., 1995; Malide et al., 1995). When fully processed, neurotrophins exist as noncovalently bound homodimers in which the six conserved cysteine residues present in mature forms of all neurotrophins give rise to a cystine knot, a step-like arrangement of three disulfide bridges which is also present in other secreted proteins such as transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF)(McDonald and Hendrickson, 1993). NGF and NT-3 are released only through the constitutive secretory pathway, but in neurons, BDNF is secreted in an activity-dependent manner that is likely to be important for regulating synaptic plasticity (Ghosh et al., 1994; Mowla et al., 1999; Farhadi et al., 2000).

NGF was shown to be synthesized and secreted by sympathetic and sensory target organs (reviewed in Korsching, 1993). From these sources, it is captured in nerve terminals by receptor-mediated endocytosis and is transported through axons to neuronal cell bodies where it acts to promote survival and differentiation. Target organs that synthesize NGF become innervated by the axons of the responsive neurons, and neurotrophins are also expressed in the regions being invaded by sensory neurons, providing trophic support to

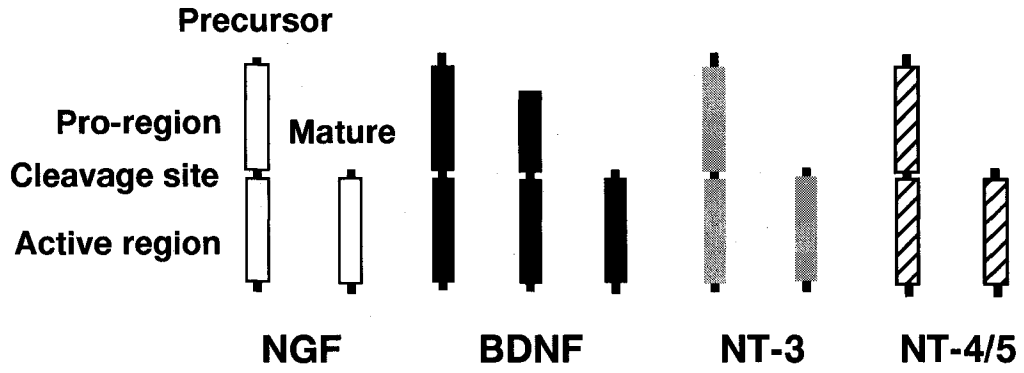

Figure 1.1. Schematic of the neurotrophin isoforms. The four mammalian neurotrophin genes encode neurotrophin precursors, which are cleaved by specific convertases to release mature neurotrophins. The BDNF precursor has two cleavage sites, leading to a neurotrophin and an incompletely digested neurotrophin precursor.

neurons as they reach their final target (Farinas et al., 1996, 1998; Ringstedt et al., 1999; Huang and Reichardt, 2001). Following injury, Schwann cells, fibroblasts, and activated mast cells have also been shown to synthesize NGF (reviewed in Levi-Montalcini et al., 1996). Finally, neurons also synthesize neurotrophins. While paracrine and autocrine mechanisms have been described for the action of BDNF in the DRG, it may also be transported anterogradely and act trans-synaptically on targets of the central afferents of these neurons within the brain (von Bartheld et al., 1996; Altar et al., 1997; Fawcett et al., 1998; Brady et al., 1999).

1.2 THE TRK RECEPTORS

TrkA was originally characterized as a transforming oncogene in which tropomyosin was fused to an unknown tyrosine kinase (Kaplan et al., 1991a; Klein et al., 1991a). The corresponding protooncogene was shown to be a member of a highly related family of transmembrane tyrosine kinases which were expressed in discrete neuronal populations and which bound and were activated by specific neurotrophins, with TrkA preferentially binding NGF, TrkB preferring BDNF and NT-4/5, and TrkC interacting with NT-3 (Klein et al., 1990, 1991b; Cordon-Cardo et al., 1991; Kaplan et al., 1991a, 1991b; Lamballe et al., 1991; Soppet et al., 1991; Squinto et al., 1991; Ip et al., 1993b). In the absence of p75NTR, high concentrations of NT-4/5 activate TrkA and likewise, NT-3 can activate TrkA and TrkB. Under these circumstances, NT-3 and NT-4/5 are classified as non-preferred Trk receptor ligands (Figure 1.2)(Segal and Greenberg, 1996).

1.2.1 Structure of the Trk receptors

All Trk receptors are large, Type I transmembrane proteins, members of the receptor tyrosine kinase superfamily (Martin-Zanca et al., 1989; and reviewed in Ip and Yancopoulos, 1994; Barbacid, 1995). The extracellular domains of the Trk receptors contain two cysteine-rich regions (domains 1 and 3) flanking leucine-rich repeats (domain 2), followed by two immunoglobulin (IgG)-like domains in the juxtamembrane region (domains 4 and 5; Figure 1.3)(Windisch et al., 1995). TrkA binding studies and deletion studies on TrkA, B and C indicate that domain 5 binds neurotrophins with almost wild-type affinity (Urfer et al., 1995, 1998; Ultsch et al., 1999). The second leucine-rich domain may also have a modulatory, perhaps indirect, role in ligand interaction (Windisch et al., 1995). Co-crystallization of NGF with domain 5 of TrkA has shown distinct interactions with both the β-sheets that form the core of the NGF homodimer, and with the ligand's amino-terminus (Wiesmann et al., 1999). Structural analysis of unliganded domain 5 of TrkA, B and C suggests that the neurotrophin's core β-sheets are responsible for most binding energy, but that binding specificity originates from interactions with the neurotrophin's distinct amino-terminus.

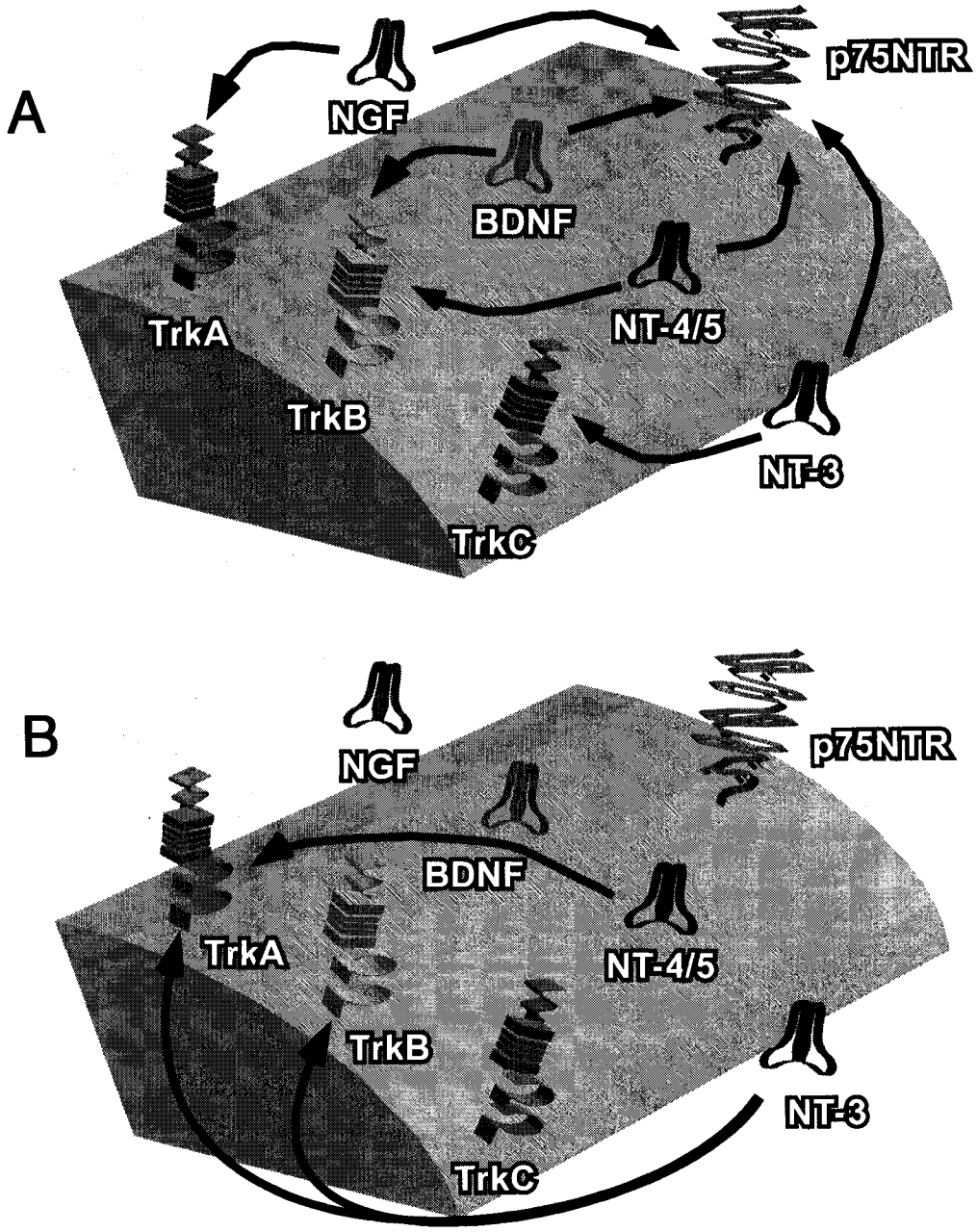

Figure 1.2. Specificities of neurotrophin binding to p75NTR and Trk receptors. (A) The neurotrophins can be classified as Trk receptor preferred ligands showing high affinity binding (NGF for TrkA, BDNF and NT-4/5 for TrkB, and NT-3 for TrkC), (B) non-preferred ligands showing lower binding affinity (NT-3 and NT-4/5 for TrkA, NT-3 for TrkB), and non-ligands that do not bind or activate the corresponding Trk receptor. On the other hand, p75NTR can bind all neurotrophins with approximately similar affinities.

The intracellular kinase domains of the Trk receptors are very similar (~80% amino acid identity between TrkA, B, and C) and binding of neurotrophin homodimer causes receptor dimerization, autophosphorylation on tyrosine (Y) residues within the activation loop (Y670, Y674 and Y675 according to human TrkA nomenclature), followed by phosphorylation of seven additional intracellular tyrosine residues (Jing et al., 1992; Heldin, 1995; Cunningham et al., 1997). Phosphorylated tyrosines within TrkA act as docking sites for signaling molecules which regulate cell growth and survival, including Ras, phophatidylinositol 3-kinase (PI3-K), and phospholipase Cγ (PLCγ) pathways (Segal and Greenberg, 1996; Kaplan and Miller, 2000).

Sequencing of the *Caenorrhabditis elegans* and *Drosophila melanogaster* genomes has indicated that insects and nematodes lack neurotrophins and their receptors. However, *Lymnaea stagnalis*, an invertebrate with a complex nervous system, contains a transmembrane tyrosine kinase domain which has intracellular signaling modules and extracellular features characteristic of the Trks (van Kesteren et al., 1998). This observation has led to the suggestion that the appearance of Trk receptors may have been prerequisite for development of complex nervous systems (Jaaro et al., 2001).

1.2.1.1 Trk receptor isoforms

Alternatively spliced isoforms of each of the Trks have been described (Figure 1.3). TrkA and TrkB receptors have alternative spliced products lacking 6 to 9 amino acids in the extracellular juxtamembrane region. TrkB variants lacking this region bind BDNF normally but show decreased affinity for NT-3 and NT-4/5 (Strohmaier et al., 1996). Similarly, TrkA isoforms lacking this sequence interact normally with NGF but show reduced NT-3 binding (Clary and Reichardt, 1994). Thus, alternative splicing of the Trk receptors affect the specificity of neuronal responsiveness to neurotrophins.

TrkB and TrkC genes also give rise to receptor isoforms with altered cytoplasmic domains. TrkC receptors can be produced with amino acid inserts (14, 25, or 39 residues) in their kinase domain which render them incapable of signaling (Tsouflas et al., 1993;

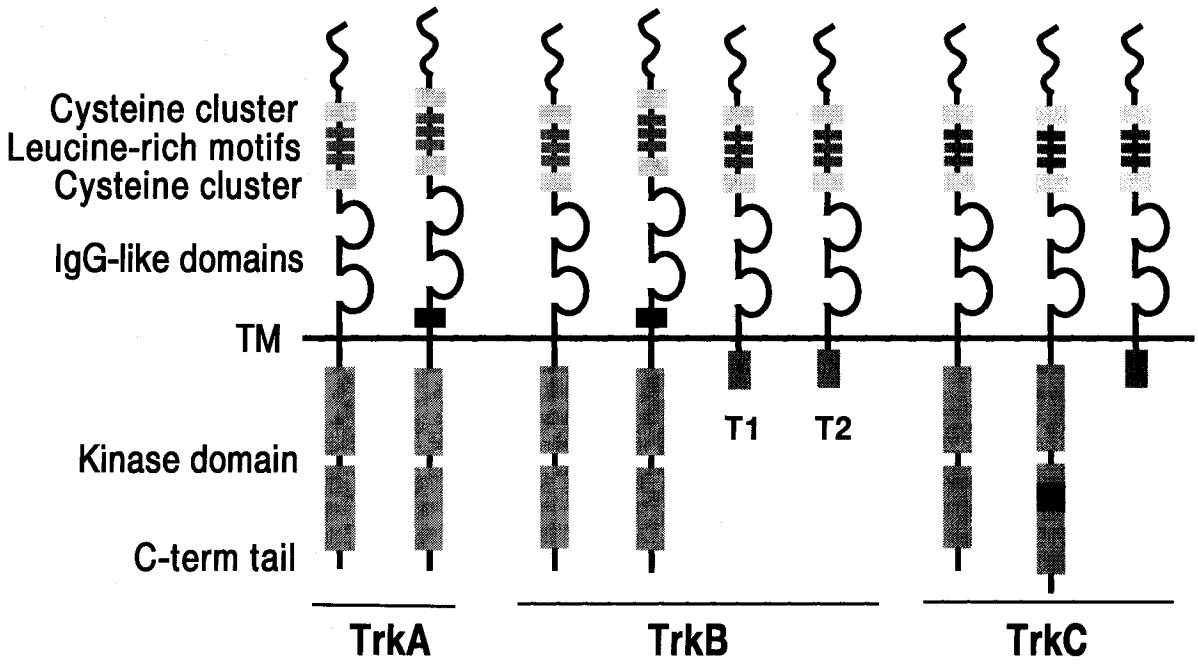

Figure 1.3. Schematic of the Trk tyrosine kinase isoforms. The three Trk genes (TrkA, B, and C) encode a full-length receptor, and multiple alternatively spliced isoforms. Splicing leads to TrkA and TrkB receptors that lack a short amino acid sequence in their extracellular domains, TrkB and TrkC receptors that lack the intracellular kinase domain, as well as TrkC receptor with an insert in the kinase domain.

Valenzuela et al., 1993). TrkB and TrkC variants which lack most of the intracellular domain are also produced (Klein et al., 1990; Middlemas et al., 1991; Okazawa et al., 1993; Tsouflas et al., 1993). Truncated TrkB receptors, designated T1 and T2, have cytoplasmic tails of 21 or 23 amino acids and are expressed at high levels in the adult brain (Fryer et al., 1996). The precise physiological function of these receptors is unclear, but they may act as dominant inhibitory modulators of Trk signaling, or either concentrate or prevent diffusion of neurotrophins (Eide et al., 1996; Baxter et al., 1997; Hapner et al., 1998).

1.2.2 Trk receptor signaling

Numerous gain and loss of function studies have shown that Trk signaling promotes survival and differentiation of most neuronal populations (Huang and Reichardt, 2001; Patapoutian and Reichardt, 2001). For example, ectopic expression of a Trk receptor is sufficient to confer a neurotrophin-dependent survival and differentiation response in many neurons (Allsopp et al., 1993; Barrett and Bartlett, 1994), and Trk receptor gene deletion results in extensive loss of specific neuronal populations (Snider, 1994). Several studies have indicated that Trk residues Y490 and Y785 are important ligand-induced phosphorylated docking sites for adaptor proteins that result in activation of Ras, PI3-K, and PLCγ (Loeb et al., 1994; Obermeier et al., 1994; Stephens et al., 1994), and attention has focused on the role of these pathways in neuronal survival, growth and differentiation.

1.2.2.1 Signaling through Ras

The Ras signaling pathway regulates neuronal survival and differentiation through activation of the mitogen-activated protein kinases (MAPK)/extracellular signal-regulated kinase (ERK) pathway (Grewal et al., 1999; Ballif and Blenis, 2001; Brunet et al., 2001). The adaptor protein Shc binds to phosphorylated Y490, and plays a crucial role in neuronal survival and outgrowth (Stephens et al., 1994; Meakin et al., 1999; Atwal et al., 2000). Trk-mediated phosphorylation of Shc promotes its interaction with Grb2 and the son of sevenless (SOS) Ras exchange factor which mediate Ras activation. Activated Ras induces a number of downstream signaling pathways that include p38MAPK and c-

Raf/B-Raf/ERK (Xing et al., 1996). Recent data indicates that ERK dependent activation of S6 kinases (RSK) and the subsequent phosphorylation of CREB by RSK and p38MAPK is an important survival pathway in neurons (Xing et al., 1998; Bonni et al., 1999; Riccio et al., 1999; Shimamura et al., 2000; Ballif and Blenis, 2001). The duration of ERK signaling is important for governing cellular responses to receptor tyrosine kinase activation (Marshall, 1995; Walton and Dragunow, 2000). Prolonged Trk-mediated ERK activation is necessary for neuronal differentiation of PC12 cells through a process involving fibroblast growth factor receptor substrate-2 (FRS2)(York et al., 1998; Meakin et al., 1999). FRS2 is a myristoylated protein that competes with Shc for Y490 binding, becomes tyrosine phosphorylated by Trk, and then associates with the Grb2/SOS complex to prolong Ras/ERK signaling (Meakin et al., 1999).

1.2.2.2 Signaling through PI3-K

PI3-K plays an important role in Trk-dependent survival in many neurons and Trk-dependent activation of PI3-K can occur through both Ras-dependent and Ras-independent pathways. The latter involve Shc-Grb2 complexes which bind insulin receptor substrate (IRS) -1, -2, and Grb-associated binder-1 (Gab-1), which induce association and activation of PI3-K (Holgado-Madruga et al., 1997; Nguyen et al., 1997; Yamada et al., 1997). Lipids generated by activated PI3-K induce membrane recruitment of the serine/threonine kinases 3-phosphoinositide-dependent kinase-1 (PDK1) (Vanhaesebroeck and Alessi, 2000) and Akt (protein kinase B)(Bellacosa et al., 1991; Coffer and Woodgett, 1991; Jones et al., 1991). Akt is activated by PDK1-dependent phosphorylation and in turn phosphorylates several substrates implicated in neuronal survival, including Bad (Datta et al., 1997; del Peso et al., 1997), Caspase 9 (Cardone et al., 1998; Rohn et al., 1998), Forkhead (FKHR) family members (Biggs et al., 1999; Brunet et al., 1999; Guo et al., 1999; Kops et al., 1999), IκB kinase (Kane et al., 1999), and glycogen synthase kinase-3 (GSK-3)(Cross et al., 1995; Shaw et al., 1997; Hajduch et al., 1998; Pap and Cooper, 1998; Ueki et al., 1998; van Weeren et al., 1998). Ras-dependent activation of PI3-K has also been observed in neurons. Ras can directly interact with PI3-K, and inhibition of Ras has been shown to suppress NGF-mediated

PI3-K activity (Rodriguez-Viciana et al., 1994; Klesse and Parada, 1998; Mazzoni et al., 1999).

1.2.2.3 Signaling through PLCγ

Phosphorylated Y785 lies near the carboxyl-terminus of the receptor, and serves as docking site for PLCγ (Obermeier et al., 1994). Trk phosphorylation of PLCγ generates an active enzyme capable of hydrolyzing phophatidyl inositides to generate inositol triphosphate (IP_3) and diacylglycerol (DAG) (Vetter et al., 1991). IP_3 increases the level of cytoplasmic Ca^{++}, whereas DAG activates protein kinase Cδ (PKCδ), which is involved in neurite outgrowth and activation of the ERK cascade (Corbit et al., 1999). However, mutation of Trk Y785 does not block neurite outgrowth, indicating that other Trk-mediated signals compensate by promoting neuritogenesis.

1.2.2.4 Other Trk-activated pathways

Yeast two-hybrid screens have resulted in the identification of two closely related Trk-interacting proteins termed rAPS (rat adaptor molecule containing PH and SH2 domains) and SH2-B (Qian et al., 1998). Both molecules function as adaptor proteins that bind to Grb2 and can induce activation of the Ras/ERK cascade and morphological differentiation of PC12 cells. Other potential interactors include CHK (Csk-homologous kinase), which promotes ERK activation (Yamashita et al., 1999a), and the c-Abl tyrosine kinase (Yano et al., 2000), which appears to interact with the TrkA juxtamembrane domain and play a role in differentiative responses (Meakin and MacDonald, 1998).

1.2.3 The K252a family of Trk receptor inhibitors

K252a, a glycosylated indolocarbazole alkaloid, was originally isolated from the culture broth of *Nocardiopsis* species, and was found to be a potent inhibitor of protein kinase C and calmodulin kinase (Kase et al., 1986, 1987). Later, several structurally-related compounds were isolated (K252b, c, and d), with varying kinase inhibitory specificities (reviewed in Knusel and Hefti, 1992). K252a and K252b potently inhibit TrkA, B and C, but exhibit relatively low activity against other tyrosine kinases, including the epidermal

growth factor receptor (EGFR), Src, and the insulin receptor (Berg et al., 1992; Tapley et al., 1992; Nye et al., 1992). K252a inhibits Trk kinase activity by antagonizing ATP binding to the kinase domain (Angeles et al., 1998).

K252a is widely used as a Trk kinase inhibitor, but paradoxically, K252a has neurotrophic effects on a variety of neuronal cells. K252a supports both neurite growth and survival of primary sensory neurons, neuroblastoma cells and PC12 cells (Hashimoto and Hagino, 1989; Borasio, 1990; Tischler et al., 1991), and induces ChAT activity in neurons derived from embryonic spinal cord, basal forebrain, and striatum (Glicksman et al., 1993, 1995). The mechanisms implicated in K252a-mediated "neurotrophic effects" are still unknown, but the survival-promoting activity of K252a has spurred the generation of novel neuroprotective compounds.

1.2.3.1 The K252a derivative CEP1347

CEP1347 (also known as KT7515) is a semisynthetic derivative of K252a that lacks Trk-inhibitory activity (Maroney et al., 1998). It is more effective than K252a at inhibiting cell death of motoneurons (Borasio et al., 1998; Glicksman et al., 1998; Maroney et al., 1998), cortical neurons (Namgung and Xia, 2000), and auditory hair cells (Pirvola et al., 2000), and is now in clinical trial for the treatment of Parkinson's and Alzheimer's diseases. The c-Jun amino-terminal kinase (JNK) pathway is thought to be an important regulator of neuronal apoptosis, and CEP1347 was found to inhibit JNK activation in several injury paradigms (Maroney et al., 1998; Friedman, 2000; Namgung and Xia, 2000; O'Ferrall et al., 2000; Wagner et al., 2000). In stressed cells, JNK becomes phosphorylated and activated by the dual specificity MAPK kinases (MKK) 4/7, which are regulated by several MAPKKKs, including the mixed lineage kinases (MLKs), dual leucine zipper kinase (DLK), leucine zipper-bearing kinase (ZLK), and MEKK1-4. The MLKs have been shown to mediate neuronal apoptosis after growth factor withdrawal in PC12 cells and sympathetic neurons (Mota et al., 2001; Xu et al., 2001), and CEP1347 was found to inhibit MLK-mediated JNK activation and apoptosis (Maroney et al., 2001; Xu et al., 2001). *In vitro* kinase assay experiments revealed that CEP1347 is a direct inhibitor of the MLKs, indicating that CEP1347 promotes survival by inhibiting MLK-

mediated JNK activation and apoptosis. However, these results did not rule out the implication of other targets in the neuroprotective effects of CEP1347.

1.3 THE P75 NEUROTROPHIN RECEPTOR

Binding studies on PC12 cells and primary peripheral neurons established that NGF responsive cells had high and low affinity binding sites, with dissociation constants (K_d) of $\sim 10^{-11}$ M and $\sim 10^{-9}$ M, respectively. With the cloning of p75NTR (Chao et al., 1986; Johnson et al., 1986), transfection studies revealed that p75NTR bound NGF at the lower of these affinities and the receptor was therefore termed the low affinity NGF receptor. With the expansion of the neurotrophin family, it became evident that p75NTR bound all of the neurotrophins with approximately equal affinity in most cells (Rodriguez-Tebar et al., 1990, 1992; Squinto et al., 1991). Intriguingly, NT-3 binds p75NTR with higher affinity ($K_d \sim 10^{-11}$ M) in embryonic chick sympathetic neurons (Dechant et al., 1997), but this high-affinity binding does not promote their survival (Dechant et al., 1993) and its biochemical basis remains uncertain.

The neurotrophins are the only mammalian ligands known to bind p75NTR, but the invertebrate ligand cysteine-rich neurotrophic factor (CRNF) has been shown to bind p75NTR with similar affinity than the neurotrophins. CRNF was isolated from the snail *Lymnaea stagnalis* (Fainzilber et al., 1996), and is not similar to any mammalian ligands identified to date. Nonetheless, this finding raises the possibility that CRNF is a prototype of an unidentified family of p75NTR ligands. Finally, p75NTR can serve as a receptor for rabies virus (Tuffereau et al., 1998), raising the possibility that p75NTR may provide a viral entry route into the cell, or that p75NTR-dependent signaling may play a role in rabies pathogenesis. One study has shown that rabies-induced neuropathology is not delayed or otherwise compromised in p75NTR-deficient mice (Jackson and Park, 1999), suggesting that p75NTR may not play an important role in disease progression. However, additional analysis of a novel p75NTR-deficient mouse may provide a definitive conclusion on this issue (see section 1.4.1 for descriptions of the p75NTR-deficient animals).

Sequence homologies in its extracellular and intracellular domains identified p75NTR as a member of the tumor necrosis factor (TNF) superfamily of receptors. p75NTR was the first identified member of this group of receptors that now contains about 25 receptors,

including TNFR1 and 2, Fas, RANK, and CD40 (Bazan, 1993). p75NTR is an unusual member of the TNFR family due to its propensity to dimerize rather than trimerize, because of its ability to act as a tyrosine kinase co-receptor, and because the neurotrophins are structurally unrelated to the TNF ligands which typically bind TNFR family members. With the exception of p75NTR, essentially all members of this TNFR family preferentially bind structurally related trimeric Type II transmembrane ligands that are members of the TNF ligand superfamily.

1.3.1 Structure of the p75NTR gene and promoter

The p75NTR gene contains six exons that spans approximately 23 kb at human chromosome region 17q12-17q22 (Huebner et al., 1986; Sehgal et al., 1988b). It encodes a 3.8 kb mRNA with a 5' untranslated region of about 300 nucleotides, and an unusually long (~2000 nucleotides) 3' untranslated region that contains a single consensus polyadenylation signal (Johnson et al., 1986). p75NTR is lacking in *C. elegans*. *D. melanogaster* and other invertebrates, but the *p75ntr* gene is present in jawless fish, which arose about 460 million years ago (Hallbook, 1999; Locksley et al., 2001). The high conservation of p75NTR between vertebrate species suggest that it may subserve higher functions specific to the vertebrates (Hutson and Bothwell, 2001; Jaaro et al., 2001).

The p75NTR promoter sequence is highly similar in rat, mouse, and human (Metsis, 2001), suggesting that its transcription is highly regulated, but the specific *cis*-acting elements controlling p75NTR expression are not well understood. The promoter lacks consensus TATA and CAAT sequences (Sehgal et al., 1988a), but contains several conserved GC rich sequences close to the initiator ATG which may represent Sp1 binding sites. Several E-box-like elements are present in the p75NTR promoter, with the most proximal capable of binding ME1, thereby repressing promoter activity (Chiaramello et al., 1995). The transcription factor NeuroD may compete with another bHLH factor for binding this same E-box, and activate p75NTR expression (Metsis, 2001). Moreover, specific regions of the p75NTR promoter have been shown to confer regulation by

retinoic acid, Vitamin D3, and testosterone (Metsis et al., 1992; Naveilhan et al., 1996), but the specific regulatory elements that function *in vivo* remain unknown.

1.3.2 Structure of the p75NTR protein

After cleavage of its 28 amino acid signal peptide, human p75NTR is a 399 amino acid Type I transmembrane protein that has a single N-linked carbohydrate at position 33 and several O-linked carbohydrates in the juxtamembrane stalk domain (Figure 1.4)(Large et al., 1989). The extracellular domain contains four repeated modules of six cysteines, a distinguishing feature of the TNFR superfamily members (Yan and Chao, 1991; Baldwin et al., 1992). These cysteine-rich domains (CRD1-4 from amino terminus) each form three intrachain disulfide bridges that create the receptor's elongated structure (Smith et al., 1994). Experimental and structural modeling studies have suggested that CRD3 may be primarily responsible for interaction with neurotrophins (Yan and Chao, 1991; Baldwin et al., 1992; Chapman and Kuntz, 1995; Shamovsky et al., 1999). In Fas, TNFR1 and CD40, CRD1 mediates ligand-independent receptor trimerization (Chan et al., 2000: Siegel et al., 2000), but it is unknown whether the CRD1 of p75NTR subserves a similar function.

The transmembrane domain and flanking juxtamembrane sequences are very well conserved between species (Heuer and Howitt, 1990; Large et al., 1989), and a peptide derived from the juxtamembrane region can induce apoptosis of chick sensory neurons (Coulson et al., 2000). The intracellular domain of p75NTR is palmitoylated at cysteine 279 (Barker et al., 1994), and is phosphorylated on serine and threonine residues (Grob et al., 1985; Taniuchi et al., 1986b). The function of these post-translational modifications are not known but could include roles in protein-protein interaction, proper intracellular folding of the receptor, or in directing the cellular localization of p75NTR. Intriguingly, p75NTR phosphorylation is enriched in monomeric p75NTR, suggesting that phosphorylation may regulate receptor multimerization (Grob et al., 1985).

TNFR superfamily members do not have intrinsic enzymatic activity and signaling occurs through association with cytoplasmic adaptor proteins. The p75NTR intracellular domain

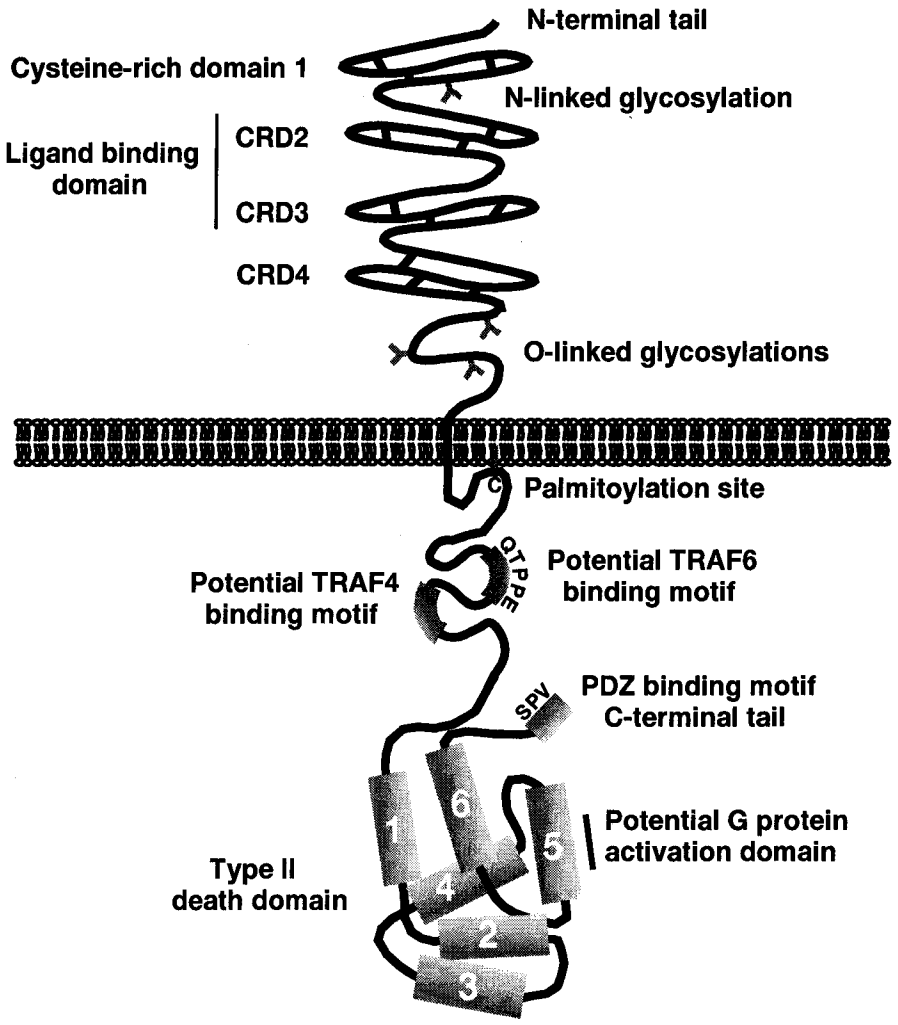

Fig. 1.4. Structure of the p75NTR protein. p75NTR is a Type I transmembrane receptor with an extracellular domain that contains four cysteine-rich domains (CRD), and multiple O- and N-linked glycosylation sites. The intracellular domain contains a palmitoylation site at cysteine 279, two potential TRAF-binding sites, a Type II death domain, a potential G protein activating domain, and a PDZ domain binding motif.

contains several regions likely to mediate interactions with downstream signaling elements. TNFR-associated factors (TRAFs) bind to many TNFR superfamily members and link receptor action to the JNK, nuclear factor-κB (NF-κB) and Src signaling pathways (reviewed in Bradley and Pober, 2001; Wajant et al., 2001). p75NTR binds TRAF4, TRAF6, and has been reported to strongly interact with one of two TRAF proteins present in *Drosophila*, but the precise binding domains responsible for these interactions are not known (Krajewska et al., 1998; Khursigara et al., 1999; Zapata et al., 2000). p75NTR does not contain any obvious TRAF binding consensus site, but the p75NTR sequence ^{293}QTPPPE^{298} does bear resemblance to QXPXEX, the motif defined as a TRAF6 binding site on CD40, RANK, and IRAK (Pullen et al., 1999). TRAF1, TRAF2, and TRAF3 bind the consensus sequence PXQX(T/S) on CD40, TNFR2, CD30, and ATAR, which is not present within p75NTR (Ye et al., 1999a).

The carboxyl-terminal tripeptide of p75NTR (*t*SPV) is conserved across all species, and is a consensus PDZ (present in PSD-95, Dlg, and ZO-1) domain binding site. PDZ-containing proteins are typically adaptor molecules that assemble supramolecular complexes necessary for localized signaling functions (Sheng and Sala, 2001). There is no evidence that p75NTR binds this class of PDZ proteins but instead, p75NTR binds FAP-1 (PTP1E), a PDZ-containing protein tyrosine phosphatase (PTPase)(Irie et al., 1999).

The most prominent intracellular feature of p75NTR is the death domain (DD), a ~80 amino acid association module initially identified in related pro-apoptotic TNFR superfamily members. In some proteins, the DD interacts with adaptor proteins that link receptors to Caspase activation, but DD interactions are not invariably associated with a pro-apoptotic function (Feinstein et al., 1995; Hofmann and Tschopp, 1995). DDs have been classified as Type I (e.g.. TNFR1 and Fas) and II (e.g. p75NTR and DAP kinase) on the basis of their overall similarity and the spacing between putative α-helices. The structure of the DDs from p75NTR and Fas have been solved by NMR, and both domains are compact globular structures consisting of ~80 amino acids arranged in two bundles which each contain three α-helices. Helices 2-6 have essentially identical orientations in

Fas and p75NTR, but the arrangement of helix 1 differs considerably between them, with its orientation differing almost 90 degrees between the two proteins (Huang et al., 1996; Liepinsh et al., 1997). This difference likely has important physiological consequences since the p75NTR DD does not show the self-aggregation property described for the Fas DD, and binding proteins which bind the Fas DD clearly do not bind p75NTR. It is therefore certain that DD-dependent signaling mechanisms employed by p75NTR differ considerably from those used by Fas and related receptors. p75NTR DD interactors described to date include NADE and RhoA. With regard to the latter, it is notable that the fifth helix of the p75NTR DD is similar to GTPase activation domains identified in several other proteins, including the peptide mastoparan (Feinstein and Larhammer, 1990; Myers et al., 1994; Liepinsh et al., 1997).

1.3.2.1 p75NTR isoforms

Truncated p75NTR isoforms are produced both by alternative splicing and proteolysis (Figure 1.5). The *p75ntr* gene is alternatively spliced to encode a p75NTR variant that lacks exon III, generating an isoform incapable of neurotrophin binding (Dechant and Barde, 1997). This p75NTR variant is predicted to be a Type I transmembrane protein with an intact intracellular and transmembrane domain, but its specific function remains uncertain. Mice lacking both p75NTR isoforms have recently been generated (von Schack et al., 2001), and are discussed in section 1.4.1.

Full-length p75NTR is cleaved by a constitutively active metalloproteinase to generate a soluble extracellular domain which is capable of binding neurotrophins, and a receptor fragment containing transmembrane and intracellular domains (Zupan et al., 1989; Barker et al., 1991; DiStefano et al., 1993). The soluble form of p75NTR (and presumably, the remaining transmembrane and intracellular domains) is produced at very high levels during development and following peripheral nerve injury (Zupan et al., 1989, DiStefano et al., 1991), but the biological role(s) of the proteolytic products remains completely unknown.

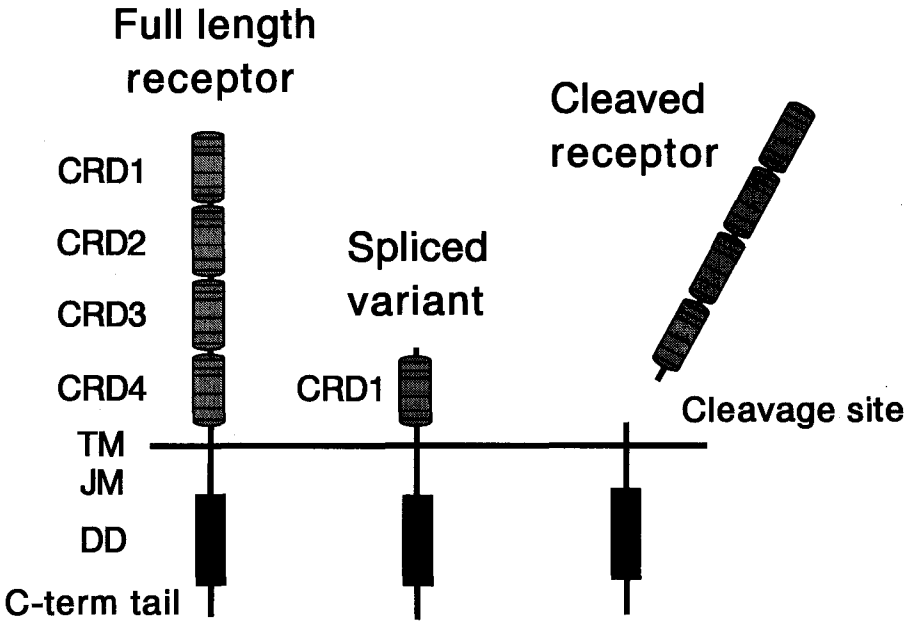

Figure 1.5. Schematic of the p75NTR isoforms. The p75NTR gene gives rise to a full length receptor and an alternatively spliced isoform lacking exon III, which comprises CRD2, 3 and 4. The full length receptor is also cleaved by a metalloproteinase that releases a free floating extracellular domain, and an intracellular domain linked to the membrane via the transmembrane region.

1.3.3 Endogenous Expression of p75NTR

1.3.3.1 Central nervous system

During early stages of avian and primate development, p75NTR mRNA is expressed in a wide variety of cell populations within the CNS (Buck et al., 1987; Ernfors et al., 1988; Schatteman et al., 1988; Escandon and Chao, 1989; Large et al., 1989; Heuer et al., 1990; Cotrina et al., 2000). p75NTR mRNA is present throughout the embryonic neural tube and its expression becomes restricted to postmitotic cells of the mantle layer, whereas proliferative neuroepithelial cells of the periventricular layer are essentially devoid of p75NTR mRNA and protein (Heuer et al., 1990; Cotrina et al., 2000; Salehi et al., 2000). In the retina, p75NTR mRNA is present in ganglion cells and in a subpopulation of cells of the developing inner nuclear layer starting at embryonic day 5 (E5).

p75NTR expression appears in several central neuronal populations as they differentiate, including spinal motor neurons and brain stem motor nuclei, lateral geniculate nucleus, medial terminal nucleus of the accessory optic tract, ventral and dorsal cochlear nucleus, thalamic nucleus, nucleus of the lateral lemniscus, amygdala, cortical subplate neurons, olivary pretectal nucleus, cuneate nucleus, gracile nucleus, as well as Purkinje cells, the external granule layer and deep nuclei of the cerebellum (Buck et al., 1988; Ernfors et al., 1988; Schatteman et al., 1988; Heuer et al., 1990; reviewed in Bothwell, 1991). Postnatally, p75NTR levels are reduced in most tissues and restricted to magnocellular neurons of the basal forebrain (Hefti et al., 1986; Springer et al., 1987; Yan and Johnson, 1988; Schatteman et al., 1988; Dreyfus, 1989), caudate/putamen neurons (Henry et al., 1994), motor neurons (Ernfors et al., 1989; Armstrong et al., 1991), cerebellar Purkinje cells (Shelton and Reichardt, 1986; Koh et al., 1989; Cohen-Cory et al., 1991), and to ventral premaxillary, mesencephalic trigeminal, hypoglossal, raphe, and suprachiasmatic nuclei (Schatteman et al., 1988; Koh et al., 1989; Sofroniew et al., 1989).

1.3.3.2 Peripheral nervous system

The p75NTR mRNA is expressed in the developing autonomic and sensory nervous system from the earliest stages of development (Heuer et al., 1990). Neural crest cells express p75NTR as they migrate and accumulate at sites of formation of sensory and

sympathetic ganglia, and levels increase substantially in cells of these ganglia as they aggregate and undergo neuronal differentiation. Parasympathetic and enteric neurons express p75NTR mRNA and all autonomic cranial ganglia show substantial levels of p75NTR expression. In the adult, p75NTR expression is retained in sympathetic and sensory neurons, and in subsets of enteric and parasympathetic neurons (Sutter et al., 1979; Yan and Johnson, 1988; Carroll et al., 1992; Schatteman et al., 1993).

1.3.3.3 Non-neuronal tissues

p75NTR is initially expressed very early in embryogenesis in cells derived from all three germinal layers (Thomson et al., 1988; Heuer et al., 1990) and becomes progressively restricted to specific cell populations as development proceeds. In the chick, p75NTR is expressed in the intermediate mesoderm and paraxial mesoderm at E1-E2 (Cotrina et al., 2000), and by E2, p75NTR is highly expressed in the rostral and caudal parts of the somites (Heuer et al., 1990; Cotrina et al., 2000). As somites subdivide into dermatome, myotome, and sclerotome, p75NTR levels decrease to undetectable levels in the myotome but remain high in the dermatome and sclerotome. p75NTR levels thereafter decrease to undetectable levels in the sclerotome and in the dermatome, and become restricted to dermal fibroblasts associated with hair follicles (Heuer et al., 1990).

p75NTR expression is enriched at a number mesenchymal/epithelial boundaries. In several developing organs (limb, kidney, maxillary pad, tooth, lung, muscle, testis, retina, and pituitary), mesenchymal cells located within the developing organs express p75NTR at very high levels, and p75NTR is abundant in mesenchyme surrounding developing epithelial structures (hair follicles, salivary glands, perivascular cells, meninges)(Byers et al., 1990; Sariola et al., 1991; von Bartheld et al., 1991; Wheeler and Bothwell, 1992; Alpers et al., 1993; Russo et al., 1994; Seidl et al., 1998; Wheeler et al., 1998).

1.3.3.4 Injury-induced expression

p75NTR expression is induced by injury in a wide variety of cell types. p75NTR mRNA and protein levels are increased in rat motor neurons and peripheral mechanoreceptors after sciatic nerve lesion (Ernfors et al., 1989; Armstrong et al., 1991; Stark et al., 2001),

in oligodendrocytes following spinal cord injury (Casha et al., 2001), in striatal and hippocampal neurons after experimentally induced focal cerebral ischemia (Baichwal and Baeuerle, 1997; Kokaia et al., 1998; Andsberg et al., 2001), in Schwann cells after nerve injury (Taniuchi et al., 1986a; Heumann et al., 1987), in rat Purkinje cells after axotomy and experimental allergic encephalomyelitis (Dusart et al., 1994; Martinez-Murillo et al., 1998; Nataf et al., 1998), in corticospinal neurons following axotomy (Giehl et al., 2001), and in cortical, hippocampal, and basal forebrain neurons after seizure (Roux et al., 1999; Oh et al., 2000).

1.3.4 Subcellular distribution and internalization of the neurotrophin receptors

Local effects of the neurotrophins, such as growth cone turning and modulation of synapses, may require a spatially restricted distribution of the neurotrophin receptors. Electron microscopic analysis of neurotrophin receptor mRNAs revealed that TrkA and TrkC mRNAs are restricted to the cell soma, but p75NTR and TrkB mRNAs have a somatodendritic distribution (Tongiorgi et al., 1997, 2000). In Madin-Darby canine kidney (MDCK) cells, p75NTR is delivered to the apical surface through a mechanism that requires the extracellular stalk region that links the CRDs to the transmembrane domain (Yeaman et al., 1997). TrkC and truncated TrkB receptors also appear on the apical surface of MDCK cells, but full-length TrkB receptors are sorted exclusively to the basolateral surface (Kryl et al., 1999). In dissociated neurons, there is no evidence of vectorial sorting of p75NTR, TrkB, or TrkC receptors, and all are observed within dendritic and axonal compartments (Kryl et al., 1999). However, p75NTR is enriched in axons and axon terminals *in vivo* (Dougherty and Milner, 1999), and TrkB is enriched in postsynaptic terminals (Aoki et al., 2000; Gan, 1999; Gonzalez et al., 1999). p75NTR bears a PDZ-binding motif which is present in many post-synaptic proteins, but there is no evidence that p75NTR accumulates in post-synaptic terminals.

Neurotrophic effects such as survival and differentiation, require internalization of the ligand-receptor complex, and its retrograde transport toward the cell body (Bartlett et al., 1998; Mufson et al., 1999; reviewed in Reynolds et al., 2000), but the processes

regulating internalization and transport of neurotrophins and their receptors remain elusive. In PC12 cells, internalization of NGF occurs through a clathrin-dependent mechanism (Grimes et al., 1996, 1997) that requires TrkA (Eveleth and Bradshaw, 1992; Loeb and Greene, 1993) and dynamin (Zhang et al., 2000). The mechanism by which retrograde transport is achieved is not yet resolved, but NGF and tyrosine phosphorylated TrkA are present in transport organelles (Grimes et al., 1996, 1997; Bhattacharyya et al., 1997). The kinase activity of TrkA appears crucial for this process (Reynolds et al., 1998) since inhibition of TrkA-mediated PI3-K activity blocked retrograde transport of NGF in sensory and sympathetic neurons (Reynolds et al., 1998, 1999).

p75NTR has also been implicated in the axonal transport of neurotrophic factors and the receptor has been shown to be transported both anterogradely and retrogradely in the sciatic nerve (Johnson et al., 1987). Analysis of p75NTR mutant mice has revealed that p75NTR is necessary for the retrograde transport of NT-4/5 and BDNF in sensory neurons, but is not required for the transport of NGF (Curtis et al., 1995). Consistent with this, antibodies to p75NTR or NGF treatment reduced the retrograde transport of BDNF and NT-3 from the retina to the isthmo-optic nucleus (von Bartheld et al., 1996). These results indicate that p75NTR can play a role in the internalization and retrograde transport of BDNF and NT-3, but does not seem to be required for the retrograde transport of NGF. However, in the absence of TrkA, p75NTR is capable of internalizing NGF (Kahle et al., 1994), and transporting NGF retrogradely from the retina to the isthmo-optic nucleus (von Bartheld et al., 1996). The precise mechanism for p75NTR endocytosis remains unclear.

Studies in PC12 cells have shown that both Trk and p75NTR localize to caveolae, a specialized cell membrane region where signaling molecules concentrate (Bilderback et al., 1997; Huang et al., 1999), and p75NTR and TrkA have been shown to directly bind caveolin-1, a caveolar component (Bilderback et al., 1999). The role of caveolae in neurotrophin receptor action remains uncertain, but examination of neurotrophin receptor trafficking in caveolin null mice will help resolve this issue.

1.4 FUNCTIONAL ASPECTS OF P75NTR

Assigning physiological functions to p75NTR and elucidating its signaling mechanisms have proven challenging. Nonetheless, present data suggest that p75NTR has two main physiological functions: modulating Trk receptor signaling and initiating autonomous signaling cascades that regulate survival and apoptosis.

1.4.1 Description of the p75NTR null mice

One of the most important tools in determining gene function is endogenous gene knockout achieved by homologous recombination. The p75NTR locus was initially targeted for deletion in 1992 and the mice generated have proven very useful for determining aspects of p75NTR function in a wide variety of physiological settings, many of which are described below (Lee et al., 1992). The strategy used to develop these mice was to delete exon III of the *p75ntr* gene which encodes the receptor's ligand binding domain, and these mice will therefore be referred to as p75NTR$^{exonIII-/-}$. A recent study has shown that the p75NTR gene generates an alternatively spliced form of p75NTR, and found that wild-type and p75NTR$^{exonIII-/-}$ mice produce this isoform in several tissues and therefore, p75NTR$^{exonIII-/-}$ mice still harbor p75NTR gene products (Dechant and Barde, 1997; von Schack et al., 2001). A complete gene knockout has now been achieved by deleting exon IV of the *p75ntr* gene, and both the p75NTR$^{exonIII-/-}$ and p75NTR$^{exonIV-/-}$ mice have been bred to near isogenecity in a C57Bl/6 background to directly compare phenotypes of these distinct gene ablations (von Schack et al., 2001). These studies have discovered significant phenotypic differences between p75NTR$^{exonIII-/-}$ and p75NTR$^{exonIV-/-}$ mice. Animals with the p75NTR$^{exonIV-/-}$ mutation are considerably smaller than their wild-type littermates and exhibit posterior limb ataxia. They show severe losses of peripheral sensory neurons which are more pronounced than in p75NTR$^{exonIII-/-}$ mice and show a dramatic loss of peripheral nerve volume, consistent with a severe loss of peripheral innervations. In addition, p75NTR$^{exonIV-/-}$ mice show large dilatations and ruptures of large blood vessels that results in partial perinatal lethality, a phenotype not observed in p75NTR$^{exonIII-/-}$ animals. These studies indicate that p75NTR$^{exonIII-/-}$ mice are functional hypomorphs and indicate that the alternatively spliced form of p75NTR plays a

significant functional role. Clearly, a detailed analysis of the p75NTR$^{exonIV-/-}$ mice will help provide novel insights into p75NTR function.

1.4.2 Interactions between p75NTR and the Trk receptors

Binding studies have indicated that p75NTR and Trk receptors interact independently with the neurotrophins with similar K_d of about 10^{-9} M (Rodriguez-Tebar et al., 1990, 1992; Kaplan et al., 1991a; Klein et al., 1991b; Squinto et al., 1991), which are lower than the high-affinity NGF binding sites (K_d~10^{-11} M) present on PC12 cells and sensory neurons (Greene and Tischler, 1976; Sutter et al., 1979; Green and Greene, 1986; Rodriguez-Tebar et al., 1990, 1992). Subsequent studies showed that, compared to cells that express each receptor individually, co-expression of p75NTR with TrkA receptors in transformed cells led to an increase in both high-affinity NGF binding sites (Hempstead et al., 1991; Rodriguez-Tebar et al., 1992; Mahadeo et al., 1994), and NGF-mediated TrkA activation (Barker and Shooter, 1994; Mahadeo et al., 1994; Verdi et al., 1994). The high-affinity binding site generated by p75NTR and TrkA has been well characterized kinetically (Hempstead et al., 1991; Mahadeo et al., 1994; Rodriguez-Tebar et al., 1992). In the absence of TrkA, p75NTR has a rapid rate of ligand association and dissociation whereas in the absence of p75NTR, Trk shows very slow association and dissociation kinetics. When the two receptors are co-expressed, the rate at which NGF can associate with TrkA increases by about 25 fold (Mahadeo et al., 1994), resulting in the generation of high-affinity binding sites. Consistent with this, TrkA activation that occurs in response to low concentrations is enhanced in the presence of p75NTR (Hantzopoulos et al., 1994; Verdi et al., 1994). The presence of p75NTR therefore appears to confer increased responsiveness to low neurotrophin concentrations, an important property for neurons which must bind neurotrophins that are present in target tissues in the subpicomolar range (reviewed in Barde, 1989). Indeed, p75NTR$^{exonIII-/-}$ mice have deficits in sensory and sympathetic innervation (Lee et al., 1992, 1994b) that correlates with reduced neurotrophin responsiveness in p75NTR$^{exonIII-/-}$-derived dissociated sensory and sympathetic neurons (Davies et al., 1993; Lee et al., 1994b, 1994c).

The precise molecular mechanisms that allow p75NTR to enhance NGF binding to TrkA and increase TrkA responsiveness to NGF remain uncertain, but two hypotheses have been put forward. First, p75NTR has been proposed to act as a co-receptor which binds NGF and either concentrates it locally or presents it to TrkA in a favorable binding conformation. Several studies have shown that disrupting NGF binding to p75NTR using monoclonal or polyclonal antibodies, or using BDNF to reduce NGF binding to p75NTR inhibits NGF-induced TrkA activation (Barker and Shooter, 1994; Lachance et al., 1997). Complementary studies have shown that a mutant form of NGF which binds TrkA but does not bind p75NTR is less effective than wild-type NGF in activating TrkA in cells where the two receptors are co-expressed (Barker and Shooter, 1994; Ryden et al., 1997). These findings are consistent with the results of Hantzopolous et al (1994) who showed that Trk activation was dramatically increased by wild-type p75NTR, but not by a mutant form of p75NTR deficient in neurotrophin binding. Together, these observations suggest that NGF binding to p75NTR is necessary for increasing the ability of TrkA to bind and respond to low levels of NGF.

An alternative view is that p75NTR may have an allosteric effect on Trk that confers high-affinity NGF binding to the TrkA receptor irrespective of NGF binding to p75NTR. In support of this model, a recent study has shown that high-affinity NGF binding sites can be generated when TrkA is co-expressed with either a p75NTR mutant deficient in neurotrophin binding, or at a lower frequency when using a chimeric receptor consisting of the EGF receptor extracellular domain linked to the transmembrane and cytoplasmic domains of p75NTR (Esposito et al., 2001). Additional experiments showed that both the transmembrane and intracellular domains of p75NTR were required for generation of high-affinity sites. Further work will be required to reconcile these two models. However, in future studies, it may be useful to distinguish between the generation of high-affinity binding sites and a functional effect on TrkA activation. The discordance between these approaches is shown by comparing the kinetic experiments of Esposito et al. (2001), who found that the transmembrane and intracellular domains of p75NTR were required for generation of high-affinity binding sites, versus the studies of Hantzopoulos et al. (1994), which showed that intracellular domain deficient p75NTR can facilitate TrkA, B and C

activation much more readily than full-length p75NTR. Indeed, most of the experiments that have established a role for neurotrophin binding to p75NTR in TrkA potentiation have examined measures of Trk activation or signaling, rather than generation of high-affinity binding sites (Barker and Shooter, 1994; Mahadeo et al., 1994; Verdi et al., 1994). Thus, while it seems certain that generation of kinetically distinguishable high-affinity sites contribute to the enhanced activation of TrkA observed in the presence of p75NTR, it appears unlikely that the profound effects of p75NTR on Trk activity can be explained through this mechanism alone. One possibility is that the membrane-anchored p75NTR binding domain collaborates with Trk to generate transient intermediate affinity sites that are difficult to resolve kinetically using standard binding protocols. Additional structure-function approaches that allow functional, physical and kinetic to be examined in parallel will be required to determine precisely how p75NTR facilitates Trk activation.

NGF is the preferred ligand for TrkA, but high concentrations of NT-3 and NT-4/5 can also activate the receptor. TrkB is readily activated by BDNF and NT-4/5, and can also be activated by high concentrations of NT-3. (Maisonpierre et al., 1990; Berkemeier et al., 1991; Squinto et al., 1991; Barker et al., 1993; Ip et al., 1993a; Benedetti et al., 1994; Lee et al., 1994a). Several studies have shown that the ligand specificity of Trk receptors is sharply limited by p75NTR. In the presence of p75NTR, NGF readily activates TrkA, but activation by NT-3 and NT-4/5 is greatly attenuated (Ip et al., 1993a; Lee et al., 1994a; Bibel et al., 1999; Mischel et al., 2001). Similarly, BDNF-mediated TrkB activation is not altered in the presence of p75NTR, but TrkB activation mediated by NT-3 and NT-4/5 is strongly reduced by p75NTR co-expression (Bibel et al., 1999). The physiological relevance of the attenuation of Trk signaling which is mediated by p75NTR has been illustrated by Brennan et al. (1999), who examined sympathetic neuron survival rates within superior cervical ganglia of mice with disrupted *p75ntr*^{exonIII}, *ngf* and *nt-3* alleles. In mice lacking one *ngf* allele (NGF$^{+/-}$), neuron number was reduced by 50%, but neuron numbers were normal in NGF$^{+/-}$, p75NTR$^{exonIII-/-}$ mice. This increase in neuronal survival was lost, however, in mice that were NGF$^{+/-}$ p75NTR$^{exonIII-/-}$, NT-3$^{+/-}$, indicating that in the absence of p75NTR, NT-3 can compensate for NGF by inducing TrkA activation. These findings suggest that p75NTR narrows neurotrophin specificity of TrkA *in vivo*.

The precise mechanism by which p75NTR attenuates TrkA activation by non-preferred ligands remains uncertain. Using a 293 cell transfection system, Bibel et al (1999) showed that p75NTR constructs lacking the intracellular domain were capable of attenuating TrkB activation induced by NT-3 and NT-4/5, and Mischel et al (2001), examining p75NTR-dependent attenuation of NT3-induced TrkA activation in *Xenopus* oocytes overexpressing mammalian receptors, also showed that the transmembrane and intracellular domain of p75NTR were dispensable for this effect. Intriguingly, this latter report also showed that binding of NT-3 to p75NTR was not required to inhibit TrkA signaling, and proposed that a physical association between p75NTR and TrkA regulates TrkA binding specificity. We have proposed an alternative, but not mutually exclusive, model for the regulation of Trk by p75NTR. It is well established that the activity of various receptor tyrosine kinases can be suppressed through receptor transmodulation, in which phosphorylation of specific serine and threonine residues within the cytoplasmic domain of tyrosine kinase receptors results in inhibition of ligand-mediated receptor activation (Countaway et al., 1989; Northwood and Davis, 1990; Olson and Pledger, 1990; Theroux et al., 1992). For example, activation of TNF signaling results in serine phosphorylation of the insulin receptor and its substrates IRS1 and IRS2, which strongly attenuates insulin signaling (Feinstein et al., 1993; Hotamisligil et al., 1994; Kanety et al., 1995). The physiological importance of this pathway has been underscored by recent studies which show that its dysregulation plays an important role in the development of Type II diabetes (Kim et al., 2001a; Yuan et al., 2001). Given the structural homology between p75NTR and TNF receptors, we hypothesized that the inhibition of Trk activity mediated by p75NTR could occur through a similar mechanism and, consistent with this, have shown that activation of p75NTR increases serine phosphorylation of the TrkA receptor (MacPhee and Barker, 1997). The serine residues within TrkA that are substrates of the p75NTR-induced activity are now being mapped, and it will be interesting to determine if their substitution inhibits the ability of p75NTR to reduce Trk activation.

The crosstalk between p75NTR and Trk receptors is not one sided, and several reports indicate that Trk activation modulates p75NTR signaling. In cells where the two receptors are co-expressed, TrkA-mediated survival appears to be a dominant signal that blocks

p75NTR-mediated apoptosis (Bamji et al., 1998; Yoon et al., 1998; Salehi et al., 2000). The mechanisms that allow Trk to suppress p75NTR-dependent apoptosis are not certain, but Dobrowsky's group has found that p75NTR-dependent ceramide production is blocked by a TrkA-derived pathway that appears to involve PI3-K activation (Dobrowsky et al., 1994, 1995; Bilderback et al., 2001).

Several recent studies suggest that the functional interactions between p75NTR and TrkA are supported by a physical interaction between the two receptors. Initial studies on this issue indicated that the two receptors co-localized within plasmalemmal patches through a mechanism involving their extracellular domains (Wolf et al., 1995; Ross et al., 1996), and more recent work has shown that when overexpressed in insect or 293 cells, p75NTR can be co-immunoprecipitated with each of the three mammalian Trk receptors and that intracellular and extracellular domains of both receptors are required for maximal interaction (Gargano et al., 1997; Bibel et al., 1999; Salehi et al., 2000). It is not yet certain whether this interaction is direct or if additional factors are required to facilitate the formation of a complex containing p75NTR and Trk. However, TrkA and p75NTR are both present in caveolae and both associate with caveolin, and it is possible that caveolin enhances p75NTR-TrkA complex formation (Bilderback et al., 1997, 1999; Huang et al., 1999). Another molecule that could play a role in this regard is ARMS (ankyrin-rich membrane spanning), a transmembrane protein that was recently identified in a two-hybrid screen using the cytoplasmic domain of p75NTR as a bait (Kong et al., 2001) and which is capable of physically interacting with both p75NTR and TrkA (Kong et al., 2001; Lee et al., 2001). NRAGE has also been found to interact with p75NTR and to disrupt a p75NTR/TrkA complex, suggesting that NRAGE and TrkA compete for p75NTR interaction (Salehi et al., 2000).

1.4.3 p75NTR regulates cell death

A growing body of *in vitro* and *in vivo* evidence suggests a role for p75NTR in apoptosis (Table 1.1), but the precise mechanisms involved in this process remain elusive. The discussion above shows that p75NTR is a Trk co-receptor which can inhibit Trk activity and, in some systems, it can be difficult to draw a distinction between pro-apoptotic

Table 1.1

In vivo and *in vitro* evidence of p75NTR-mediated cell death[a]

	Cell types	References
In vivo[b]	Oligodendrocytes	Casha et al., 2001
	Sensory neurons	Lee et al., 1992; Cheema et al., 1996; Majdan et al., 1997; Davey and Davies, 1998
	Sympathetic neurons	Taniuchi and Johnson, 1985; Majdan et al., 1997; Bamji et al., 1998
	Retinal ganglion cells	Frade et al., 1996; Frade and Barde, 1999
	Schwann cells	Ferri and Bisby, 1999; Syroid et al., 2000
	Motor neurons	Sekiya et al., 1986; Sendtner et al., 1992; Terrado et al., 2000; Frade and Barde, 1999; Ferri et al., 1997
	Isthmo-optic nucleus neurons	von Bartheld et al., 1994
	Somite cells	Cotrina et al., 2000
	Cortical neurons	Majdan et al., 1997; Roux et al., 1999
	Hippocampal neurons	Roux et al., 1999; Park et al., 2000
	Keratinocytes	Botchkarev et al., 2000
	Smooth muscle cells	Wang et al., 2000
	Cholinergic neurons	Yeo et al., 1997; Oh et al., 2000
In vitro[c]	Oligodendrocytes	Casaccia-Bonnefil et al., 1996; Yoon et al., 1998; Gu et al., 1999; Mukai et al., 2000; Kimura et al., 2001
	Sensory neurons	Barret and Bartlett, 1994; Coulson et al., 1999, 2000
	Sympathetic neurons	Bamji et al., 1998; Aloyz et al., 1998; Savitz et al., 2000
	Retinal ganglion cells	Frade, 2000
	Schwann cells	Soilu-Hänninen et al., 1999
	Cortical neurons	Park et al., 2000
	Hippocampal neurons	Friedman, 2000
	Neuronal cell lines	Rabizadeh et al.,1993; Barrett and Georgiou, 1996; Huang et al., 2000; Mukai et al., 2000; Roux et al., 2001a; Kimura et al., 2001
	Hepatic stellate cells	Trim et al., 2000
	Vascular smooth muscle cells	Wang et al., 2000
	Neuroblastoma cells	Bunone et al., 1997; Lièvremont et al., 1999
	Schwannoma cells	Gentry et al., 2000
	Sympathetic precursor cells	Salehi et al., 2000
	293T cells	Irie et al., 1999; Ye et al., 1999; Mukai et al., 2000; Wang et al., 2000; Kimura et al., 2001
	SH-SY5Y cells	Bono et al., 1999

[a] Reports demonstrating the implication of p75NTR in the induction of cell death, through ligand-independent or ligand-dependent mechanisms, using loss or gain of function experiments.

[b] Refers to work on cell populations *in situ*, which endogenously express p75NTR.

[c] Refers to work on cultured cells, looking at endogenous receptor or using an overexpression paradigm.

p75NTR signaling versus p75NTR-dependent inhibition of Trk activity. Indeed, p75NTR uses both mechanisms to facilitate cell death, with the relative dependence on one or another depending on the cellular context.

Direct evidence of p75NTR-mediated apoptosis was first described in 1993 by Bredesen and colleagues who reported that p75NTR overexpression facilitated apoptosis, an effect that was blocked by NGF (Rabizadeh et al., 1993). Since then, similar results have been reported in other cell culture paradigms (Bunone et al., 1997; Lievremont et al., 1999), but in many *in vitro* studies, NGF binding to p75NTR is required to induce apoptosis. For example, NGF binding to p75NTR induced apoptosis of differentiated rat oligodendrocytes (Casaccia-Bonnefil et al., 1996; Yoon et al., 1998; Gu et al., 1999), Schwann cells (Soilu-Hanninen et al., 1999), hepatic stellate cells (Trim et al., 2000), sympathetic neuron precursor (MAH) cells (Salehi et al., 2000), mesodermal cells (Cotrina et al., 2000), chick isthmo-optic nucleus neurons (von Bartheld et al., 1994), trigeminal mesencephalic sensory neurons (Davey and Davies, 1998), and retinal ganglion cells (Frade, 2000b). The complexity is further increased by other *in vitro* studies which show that ligand has no affect on p75NTR-induced apoptosis, and by several recent studies which indicate that p75NTR can promote survival (discussed in section 1.4.4 below).

This variance in findings presumably reflects the wide variance in experimental systems that have been examined and the cell specific complement of signaling molecules that can contribute to p75NTR's action. However, in many circumstances, the experimental paradigms examined have employed very high concentrations of NGF and/or highly overexpressed p75NTR, or have used primary cells whose p75NTR and neurotrophin expression levels bear little resemblance to the *in vivo* situation. It is probably fair to assume that the variance of results reported to date represent the extreme supraphysiological limits of p75NTR's action, and that the true physiological role of the receptor in regulating survival and death lies somewhere within this wide range of activities.

Several *in vivo* studies have addressed the role of p75NTR in developmental apoptosis. Developmental cell death in the avian retina can be reduced by the application of antibodies directed toward NGF or the extracellular domain of p75NTR (Frade et al., 1996), and mice lacking *ngf* alleles or bearing the $p75NTR^{exonIII}$ deletion have reduced apoptosis in developing retina and spinal cord (Frade and Barde, 1999). Transgenic mice which overexpress the intracellular domain of p75NTR within peripheral and central neurons display profound reductions in cortical, sympathetic, and sensory neurons (Majdan et al., 1997), and mice with the $p75NTR^{exonIII}$ deletion have supernumerary sympathetic neurons during the neonatal period (Bamji et al., 1998), indicating that p75NTR can activate the cell death machinery *in vivo*.

p75NTR expression levels are increased by nervous system injury in a wide variety of tissues, and in many cases have been tightly correlated with the induction of apoptosis (Ernfors et al., 1989; Armstrong et al., 1991; Dusart et al., 1994; Kokaia et al., 1998; Martinez-Murillo et al., 1998; Dowling et al., 1999; Roux et al., 1999; Oh et al., 2000; Syroid et al., 2000; Wang et al., 2000; Bagum et al., 2001; Casha et al., 2001). For example, in rats subjected to pilocarpine-induced seizure, neuronal expression of p75NTR becomes widespread in entorhinal, piriform, and hippocampal cortices, and is almost invariably associated with the presence of TUNEL positive nuclei (Roux et al., 1999).

Loss of function studies have indicated a causal role for p75NTR in injury-induced apoptosis. In axotomized sensory neurons, reduction of p75NTR levels using antisense oligonucleotides produces significant rescue (Cheema et al., 1996), and motor neuron loss occurring after transection of neonatal facial nerve is reduced in $p75NTR^{exonIII-/-}$ mice (Ferri et al., 1998; Ferri and Bisby, 1999). Intriguingly, NGF addition to transected neonatal facial nerve of wild-type animals is associated with increased cell loss (Sendtner et al., 1992). Although lacking in mechanistic details, taken together these *in vivo* studies strongly indicate a role for p75NTR in developmental and injury-induced apoptosis.

1.4.3.1 Mechanisms of p75NTR-induced apoptosis

A clear understanding of the physiological role of p75NTR in apoptosis will emerge from studies that define the precise signaling events that mediate receptor action *in vivo*. Although progress in this area is accelerating, the precise apoptotic signaling pathways activated by p75NTR remain elusive. For the purpose of this review, we will group cellular apoptotic mechanisms into two main pathways. In apoptosis induced by pro-apoptotic receptors, members of the TNF receptor superfamily assemble a death-inducing signaling complex (DISC) in which TRADD or FADD bind directly to the receptor's DD, thereby allowing aggregation and activation of Caspase 8 and subsequent Caspase cascade (reviewed in Aggarwal, 2000; Baud and Karin, 2001; Denecker et al., 2001). In the mitochondrial death pathway, active pro-apoptotic "BH3-domain only" members of the Bcl-2 family accumulate, bind and inactivate pro-survival Bcl-2 family members and thereby allow Bax and Bak to induce loss of mitochondrial integrity and cause Cytochrome c, IAF and Diablo/SMAC to accumulate in the cytoplasm (reviewed in Anderson and Tolkovsky, 1999; Gross et al., 1999; Harris and Johnson, 2001). The presence of Cytochrome c and Diablo/SMAC facilitate Caspase 9 activation and thereby induces a Caspase cascade that results in cell death (reviewed in Desagher and Martinou, 2000; Gottlieb, 2000; Kroemer and Reed, 2000; Matsuyama and Reed, 2000). These pathways are not mutually exclusive, and Caspase 8 activation can also result in cleavage and activation of Bid, a "BH3-domain only" member of the Bcl-2 family which results in activation of the mitochondrial death pathway.

Accumulating evidence indicates that the p75NTR apoptotic cascade is distinct from that induced by other pro-apoptotic TNF receptor superfamily members. p75NTR does not bind TRADD or FADD (P. Barker, unpublished results; Wang et al., 2001), consistent with NMR structural data showing that the Fas and the p75NTR DDs have significant structural differences (Liepinsh et al., 1997), and chimeric receptors that contain the extracellular portion of Fas and the intracellular portion of p75NTR do not induce apoptosis (Kong et al., 1999). Caspase 8 induction does not appear to be involved in p75NTR-mediated apoptosis, but Caspase 9 is activated during p75NTR-mediated killing (Gu et al., 1999; Wang et al., 2001; P. Barker, unpublished results) and a recent report

indicates that Bcl-X_L and a dominant negative form of Caspase 9 both attenuate p75NTR-induced cell death (Wang et al., 2001). Intriguingly, vFLIP E8, a viral inhibitor of apoptosis that binds effector death domains, appears to block p75NTR-induced cell death. Taken together, these results are consistent with the hypothesis that the signaling pathways responsible for p75NTR-dependent apoptosis involve DD effectors that result in preferential activation of the mitochondrial death pathway.

The mechanisms underlying neuronal cell death have been elegantly defined in primary culture systems in which cerebellar granule cells are subjected to potassium chloride (KCl) deprivation, and in peripheral neurons subjected to neurotrophin withdrawal. Sympathetic neurons deprived of NGF have been a particularly well studied model. In NGF-deprived sympathetic neurons, loss of Trk-mediated survival signals promotes the activation of a signaling cascade that ultimately results in loss of mitochondrial integrity, activation of Caspase 9 and cell death. Pioneering studies from Eugene Johnson's group performed in the late 1980's showed that apoptosis resulting from NGF withdrawal required both transcription and translation to occur (Martin et al., 1988), and more recent studies have shown that the JNK cascade and c-Jun-mediated transcription play a crucial role in mitochondrial Cytochrome c release, Caspase 9 activation and apoptosis of NGF-deprived sympathetic neurons (Deshmukh et al., 1996; Deshmukh and Johnson, 1998; Martinou et al., 1999; Putcha et al., 1999; Eilers et al., 2001; Bruckner et al., 2001; Harding et al., 2001).

The membrane proximal signaling events that lead to activation of the JNK pathway after neurotrophin withdrawal remain uncertain but downstream signaling events are falling into place. Following NGF withdrawal, the small GTPases Cdc42 and Rac1 become activated (Bazenet et al., 1998), and bind the Cdc42/Rac1 interactive binding (CRIB) motif of MAPKKKs that may include GCK, MEKK1-4, and MLK2-3 (Fan et al., 1996; Hirai et al., 1996; Rana et al., 1996; Tibbles et al., 1996; Sakuma et al., 1997). Activated MAPKKK(s) then phosphorylate and activate MKK4/7 (Xu et al., 2001), MAPKK family members leading to the phosphorylation and activation of specific JNK isoforms (Sakuma et al., 1997; Bruckner et al., 2001). Once phosphorylated, c-Jun binds promoters of genes

with appropriate AP-1 elements and induces transcription. The precise targets of c-Jun necessary for the induction of apoptosis has been the subject of intense interest and recently, Bim and Dp5, both "BH3-domain only" family members, have been identified as pro-apoptotic genes induced in a c-Jun-dependent manner in both sympathetic neurons subjected to NGF withdrawal and in cerebellar granule cells deprived of KCl (Harris and Johnson, 2001; Whitfield et al., 2001).

Several studies suggest that p75NTR can facilitate neuronal apoptosis resulting from neurotrophin withdrawal. Reduced expression of p75NTR in differentiated PC12 cells (Barrett and Georgiou, 1996) or sensory neurons (Barrett and Bartlett, 1994) correlates with reduced apoptosis following NGF-deprivation-mediated apoptosis, whereas increased p75NTR expression levels accelerates death following NGF withdrawal (Barrett, 2000). In sympathetic neurons derived from $p75NTR^{exonIII-/-}$ mice, apoptosis is significantly delayed following NGF withdrawal compared with neurons derived from wild-type littermates (Bamji et al., 1998). Ligand-mediated activation of p75NTR may play some role in this process since BDNF treatment of sympathetic neurons has been reported to increase apoptosis of NGF-deprived sympathetic neurons maintained in KCl (Bamji et al., 1998). p75NTR could contribute to neurotrophin-withdrawal mediated death either through inhibiting survival-promoting Trk activity, or through autonomous signaling that directly activates autonomous apoptotic pathways. This is a difficult issue to resolve, but recent results indicate that p75NTR facilitates apoptosis in neonatal sympathetic neurons derived from $TrkA^{-/-}$ mice, indicating that autonomous p75NTR signaling does play at least some role (Miller and Kaplan, 2001).

It is interesting to note the parallels between p75NTR-mediated and neurotrophin withdrawal-mediated apoptosis. First, Rac is induced in oligodendrocytes undergoing p75NTR-mediated death (Harrington et al., 2002). Second, p75NTR-mediated apoptosis correlates with activated JNK or c-Jun phosphorylation in oligodendrocytes (Casaccia-Bonnefil et al., 1996; Yoon et al., 1998), sympathetic neurons (Aloyz et al., 1998; Bamji et al., 1998), hippocampal neurons (Friedman, 2000), and PC12 cells (Roux et al., 2001a). Third, dominant-negative JNK or chemical inhibitors of the JNK pathway block

p75NTR-induced death (Friedman, 2000; Harrington et al., 2002). Fourth, p75NTR activation induces release of Cytochrome c from mitochondria and Caspase 9 is selectively activated during p75NTR-mediated killing (Gu et al., 1999; Wang et al., 2001; P. Barker, unpublished results). Although not yet proven, the available results suggest that p75NTR-induced apoptosis involves activation of small GTPases which communicate with MAPKKKs that lead to activation of the JNK pathway, and consequent mitochondrial release of Cytochrome c and Caspase activation. It should be noted that this potential pathway reflects results obtained from several different cell lines and has not yet been shown to mediate physiological p75NTR-dependent apoptosis. Nonetheless, it provides a useful framework from which to address issues in p75NTR signaling.

Another potential apoptotic pathway common to the NGF withdrawal and p75NTR-dependent cell death involves the activation of cell cycle regulatory molecules. The cyclin-dependent kinases (CDK) 4/5 phosphorylate the retinoblastoma (Rb) protein, and participate in NGF withdrawal-mediated sympathetic neuron death (Park et al., 1997, 1998, 2000a). Rb-mediated changes in E2F protein activity have not been shown to be involved in p75NTR-dependent apoptosis, however, NGF treatment of cultured retinal ganglion neurons induces the expression of cyclin B2, and results in cell cycle entry and apoptosis (Frade, 2000b). Apoptosis was blocked pharmacologically using the CDK inhibitor roscovitine, indicating that cell cycle regulatory proteins such as Rb and CDKs may be involved in p75NTR-mediated apoptosis. Two p75NTR interactors, SC-1 and NRAGE, can disrupt cell cycle functions, and in the case of NRAGE have also been shown to promote apoptosis (Frade, 2000a; Salehi et al., 2000).

Viewed from this perspective, some of the main outstanding signaling issues that need to be addressed include i) identifying the link between receptor and activation of small GTPases. As discussed below, none of the p75NTR-interacting proteins has motifs that suggest interactions with guanine nucleotide exchange factors or GTPase-activating proteins (GAP) that may affect Rac, Rho or Cdc42 activity. However, RhoA has been reported to bind p75NTR through an interaction with the mastoparan-like region of the p75NTR DD, and NGF binding to p75NTR appears to reduce RhoA activity (Yamashita

et al., 1999b), suggesting that the mastoparan-like domain may itself behave as a GAP under some circumstances. ii) identifying the MAPKKKs and MAPKKs that transduce the p75NTR apoptotic signal. To date, JNK has been shown to be activated by p75NTR and the MAPKKK inhibitor CEP1347 has been reported to block p75NTR-induced apoptosis (Friedman, 2000). However, the identity of specific p75NTR-regulated MAPKKKs and MAPKKs remains unknown. iii) determining if p53 or c-Jun-mediated transcription are required for p75NTR-induced apoptosis. p53 has been implicated in p75NTR-dependent death in sympathetic neurons (Aloyz et al., 1998) and may be activated through JNK-dependent phosphorylation (Milne et al., 1995; Hu et al., 1997) However, our recent studies indicate that p75NTR apoptosis is not hindered in cell lines lacking functional *p53* alleles (P. Roux and P. Barker, unpublished results). iv) examining whether "BH3-domain only" family members link p75NTR signaling to mitochondrial effects. Identification of particular "BH3-domain only" proteins involved in p75NTR-dependent apoptosis, either through post-translational modification (e.g. Bid, Bad) or through transcriptional regulation (e.g. Dp5, Bim), will provide important insights into the signaling pathways that mediate p75NTR-dependent apoptosis.

1.4.4 The p75NTR paradox - p75NTR also promotes cell survival

Much of the attention on autonomous p75NTR signaling has focused on p75NTR-induced cell death, but the receptor also appears to mediate several other effects. Indeed, the profound vascular phenotype recently reported in the $p75NTR^{exonIV-/-}$ mouse strongly suggests that the role of p75NTR may not be limited to functional collaborations with Trk receptors or apoptosis, and consistent with this, an accumulating body of evidence is beginning to reveal additional roles for the receptor.

Several studies have provided convincing data showing that, under some circumstances, p75NTR can facilitate cell survival rather than cell death. For example, NGF protects cultured cortical neurons, which express p75NTR but not TrkA, against glutamate-induced cytotoxicity (Shimohama et al., 1993; Kume et al., 2000), and NGF binding to p75NTR protects cortical and hippocampal neurons from calcium-mediated hypoglycemic damage (Cheng and Mattson, 1991; Cheng et al., 1993), suggesting a

protective role for the receptor. Recently, neurotrophin-binding to p75NTR has been shown to promote survival of neocortical subplate neurons during development (De Freitas et al., 2001) and NGF binding to p75NTR was reported to promote survival of a schwannoma cell line (Gentry et al., 2000), sensory neurons (Hamanoue et al., 1999), human breast cancer cells (Descamps et al., 2001), and cultured Schwann cells (Khursigara et al., 2001). Consistent with this, an NGF mutant that binds p75NTR but not TrkA inhibits serum deprivation-induced apoptosis of PC12 cells, and overexpression of the p75NTR intracellular domain enhances survival in a variety of cells, demonstrating that p75NTR can activate a survival pathway which is independent of Trk receptors (Hughes et al., 2001; Roux et al., 2001a).

1.4.4.1 Mechanisms of p75NTR-dependent survival

The NF-κB transcriptional complex is a family of transcription factors that regulate the expression of a number of genes involved in cell survival (reviewed in O'Neill and Kaltschmidt, 1997). Many members of the TNFR superfamily are bifunctional in that they activate apoptotic pathways through DD interactions, while promoting survival through activation of NF-κB (Pahl, 1999; Denk et al., 2000), thereby allowing fine regulatory control on life and death decisions.

Activation of the transcription factor NF-κB by p75NTR was initially reported in primary cultures of Schwann cells, where NGF binding to p75NTR increased NF-κB DNA binding activity, and p65 nuclear translocation (Carter et al., 1996). p75NTR-dependent NF-κB activation has since been shown in several other cell types (Ladiwala et al., 1998; Maggirwar et al., 1998; Yoon et al., 1998; Bhakar et al., 1999; Hamanoue et al., 1999; Gentry et al., 2000; Hughes et al., 2001), but the level of induction is typically modest compared to activities induced by other members of the TNFR superfamily and can sometimes be observed only after subjecting cells to temperature or oxygen stress (Carter et al., 1996; Bhakar et al., 1999; Hughes et al., 2001). In fibroblasts, p75NTR does not directly activate NF-κB, but indirectly enhances TNF-mediated NF-κB activation (Bhakar et al., 1999). Nonetheless, p75NTR-mediated NF-κB activation appears to promote survival of several other cell types (Maggirwar et al., 1998; Yoon et al., 1998;

Hamanoue et al., 1999; Gentry et al., 2000; Foehr et al., 2000; Descamps et al., 2001; Khursigara et al., 2001; Hughes et al., 2001).

The PI3-K/Akt pathway plays a major role in survival pathways regulated by Trk receptors (reviewed in Kaplan and Miller, 2000; Brunet et al., 2001) and surprisingly, recent data shows that p75NTR can independently activate survival pathways through a mechanism involving PI3-K and Akt (Roux et al., 2001a). The p75NTR-dependent activation of Akt is ligand-independent, blocked by inhibitors of PI3-K and associated with increased tyrosine phosphorylation of the p85 regulatory subunit of PI3-K, the Shc adaptor proteins, and at least one unidentified cell surface glycoprotein (Roux et al., 2001a). There are multiple targets of Akt that could play a role mediating p75NTR-dependent survival, but one of the more intriguing possibilities is that Akt-induced phosphorylation of IκB kinase 1 (IKK1) plays a role in the induction of NF-κB (Ozes et al., 1999; Romashkova and Makarov, 1999).

1.4.5 Additional functions of p75NTR

In addition to roles in regulating cell survival, several studies indicate that p75NTR may also promote cellular differentiation and growth. p75NTR$^{exonIII-/-}$ embryos show deficits in outgrowth of thoracic intercostal nerves (Yamashita et al., 1999b) and retarded axonal arborization in developing limb and ophthalmic branch (Yamashita et al., 1999b; Bentley and Lee, 2000), and adult p75NTR$^{exonIII-/-}$ animals have deficits in sensory and sympathetic target innervation (Lee et al., 1992, 1994b). It is likely that at least some of these effects are secondary to reduced Trk activation, but there are indications that p75NTR can directly affect cell growth and migration. For example, p75NTR enhances Schwann cell migration *in vitro* (Anton et al., 1994) and developmental Schwann cell migration from the DRG is greatly reduced in p75NTR$^{exonIII-/-}$ mice (Bentley and Lee, 2000). Since Schwann cells support and promote neurite outgrowth (Zimmermann and Sutter, 1983; Bixby et al., 1988), it is possible that some of the p75NTR effects on axon growth and arborization are indirect consequences of Schwann cell deficits. Paradoxically, some studies have observed accelerated outgrowth in the absence of p75NTR. The hippocampus of p75NTR$^{exonIII-/-}$ mice is hyperinnervated by cholinergic

afferents (Yeo et al., 1997), and cultured sympathetic neurons from these mice respond more robustly to NGF than those from wild-type littermates (Kohn et al., 1999). The absence of p75NTR in NGF overexpressing mice results in robust sympathetic axon growth on myelinated tracts in the mature CNS (Walsh et al., 1999), suggesting that neurotrophins may also act as growth inhibitors through p75NTR. Recent studies showing that p75NTR can interact and modulate the growth regulatory protein RhoA raises the possibility that p75NTR may regulate positively or negatively axonal growth by modulating small GTPases, such as Rho proteins (Yamashita et al., 1999b).

Hair follicles (HF) begin to form in the mouse at around E16-E18 with the development of a mesenchymal condensation that forms the dermal papillae (Holbrook and Minami, 1991; Holbrook et al., 1993). Dermal papillar (DP) fibroblasts express high levels of p75NTR and recent findings show that $p75NTR^{exonIII-/-}$ animals show significant acceleration of HF morphogenesis. Intriguingly, DP fibroblasts within $p75NTR^{exonIII-/-}$ animals show reduced proliferative activity, suggesting that their transition from a proliferative to differentiated state is accelerated and that p75NTR may normally negatively control HF development (Botchkareva et al., 1999). p75NTR also plays a role in mature HF. Catagen, the natural process of HF regression, is significantly retarded in $p75NTR^{exonIII-/-}$ mice. Whereas NGF, BDNF and NT-3 all promote catagen in organotypic cultures of wild-type skin, they have no effect on skin derived from $p75NTR^{exonIII-/-}$ mice. Consistent with this, there is a significant reduction of HF regression in skin of transgenic mice overexpressing NGF from a keratin promoter (Botchkarev et al., 2000). Together, these studies suggest that ligand-dependent activation of p75NTR plays a significant role in hair catagen.

1.4.6 p75NTR interactors

Key requirements for understanding p75NTR function include the identification of cytosolic proteins that physically interact with the receptor's intracellular domain and allow the receptor to modulate intracellular signaling pathways. Many groups have taken up this challenge and in the last several years, numerous p75NTR interacting proteins have been identified (Figure 1.6). Surprisingly, in many cases these proteins are novel and

their identification has tended to increase the complexity surrounding p75NTR rather than placing it into established signaling mechanisms. Indeed, a major challenge facing the field is to link the various interactors to specific p75NTR-dependent functions and signaling events. Another is to develop an accurate structure-function map of p75NTR that links domains within the p75NTR intracellular domain to specific interactors and specific signaling events (Table 1.2). It is notable that it is still unclear whether p75NTR-mediated cell death requires the receptor's juxtamembrane region (Coulson et al., 1999, 2000) or the death domain (Mukai et al., 2000; Wang et al., 2001), since evidence favoring the involvement of both domains has been presented. It is possible that each domain signals independently, perhaps in a tissue specific manner, but this will only be clarified with an accurate p75NTR structure-function map.

NRIF is a 94 kDa zinc finger protein of the C2H2 type (Klug and Schwabe, 1995) that appears to interact with both the juxtamembrane and death domains of p75NTR (Casademunt et al., 1999). It contains two Krüppel boxes (KRAB) at the amino-terminus, five zinc finger motifs followed by a potential nuclear localization signal (NLS) at the carboxyl-terminal. NRIF mRNA is ubiquitously expressed during mouse development, but decreases by adulthood. Interestingly, NRIF is structurally similar to Zac1, a putative tumor suppressor gene that regulates apoptosis and induces cell cycle arrest (Spengler et al., 1997), and overexpression of NRIF induces death of 293 cells (Casademunt et al., 1999). Normally, NRIF is present in the nucleus, but in the presence of p75NTR, it also accumulates in the cytosol. $NRIF^{-/-}$ mice show strain specific effects. In a mixed 129sv/BL6 background, $NRIF^{-/-}$ mice are viable and fertile but in a congenic BL6 strain, $NRIF^{-/-}$ embryos die by E12. Intriguingly, developmental cell death is reduced in the retina of $NRIF^{-/-}$ embryos from 129sv/BL6, similar to the reductions observed in mice lacking p75NTR (Frade and Barde, 1999).

NRAGE is a 86 kDa protein that interacts *in vivo* and *in vitro* with the juxtamembrane region of the p75NTR cytoplasmic domain (Salehi et al., 2000). NRAGE is a member of the MAGE family of proteins, all of which contain a ~200 amino acid MAGE homology domain (MHD). The MAGE domain in NRAGE is responsible for binding to the

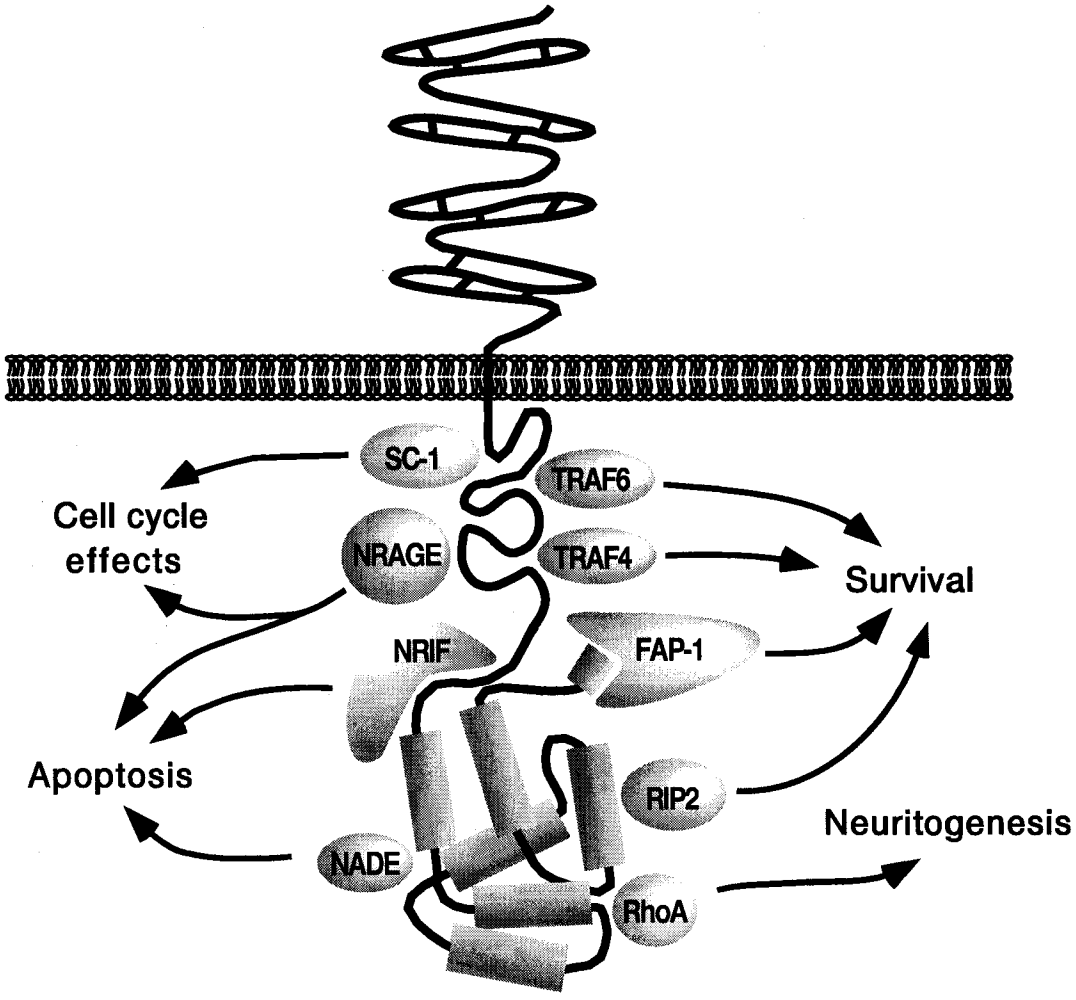

Figure 1.6. Adapter proteins involved in p75NTR-mediated signaling. Schematic representation of the different p75NTR interactors identified to date, in contact with the reported intracellular region of p75NTR. NRAGE, SC-1, TRAF4, and TRAF6 bind to the p75NTR juxtamembrane domain, NRIF interacts with both the juxtamembrane and death domains, and NADE, RIP2, and RhoA bind to the death domain.

Table 1.2

Adaptor proteins that interact with p75NTR

Interactors[a]	Particularities	p75NTR region[b]	References
NRIF (Neurotrophin receptor-interacting factor)	Five zinc finger domains Two Krüppel boxes (KRAB) Nuclear localization signal (NLS)	JM and DD a.a. 244-396	Casademunt et al., 1999
NRAGE (Neurotrophin receptor-interacting MAGE homolog)	MAGE homology domain (MHD) Interspersed WQXPXX repeats	JM a.a. 250-330	Salehi et al., 2000
NADE (p75NTR-associated death executor)	Nuclear export signal (NES) Two boxes for ubiquitination	DD a.a. 338-396	Mukai et al., 2000
SC-1 (Schwann cell factor-1)	Six zinc finger domains Positive regulatory (PR) domain N-terminal PEST sequence	JM a.a. 250-322	Chittka and Chao, 1999
RhoA	Small GTPase	DD a.a. 331-416	Yamashita et al., 1999
FAP-1 (Fas-associated phosphatase-1)	Protein tyrosine phosphatase Six PDZ domains (GLGF repeats)	*t*SPV a.a. 396-396	Irie et al., 1999
ARMS (Ankyrin-rich membrane spanning)	Eleven ankyrin repeats Polyproline stretch (SAM domain) PDZ binding motif	ND	Kong et al., 2001
TRAF4 (Tumor necrosis factor receptor-associated factor 4)	TRAF domain Seven zinc finger domains RING finger	JM a.a. 245-313	Krajewska et al., 1998; Ye et al., 1999
TRAF6	TRAF domain Five zinc finger domains RING finger	JM a.a. 268-283	Khursigara et al., 1999; Ye et al., 1999
DTRAF-1 (*Drosophila* TRAF-1)	TRAF domain Seven zinc finger domains	ICD	Zapata et al., 2000
RIP2 (Receptor interacting protein-2)	Serine/threonine kinase domain Caspase recruitment domain (CARD)	DD	Khursigara et al., 2001
Caveolin-1	Caveolin-scaffolding domain (CSD)	ICD	Bilderback et al., 1998
TrkA, B, and C	tyrosine kinase domain Two immunoglobulin-like domains Two cysteine-rich clusters Three leucine-rich repeats	ECD ICD	Gargano et al., 1996; Bibel et al., 1999

[a] p75NTR interacting proteins detemined by two-hybrid assays or in vitro and *in vivo* pull-outs.

[b] Region within p75NTR shown to be required for interaction with respective adaptor protein. JM: juxtamembrane domain; DD: death domain; ICD: intracellular domain; ECD: extracellular domain; ND: not determined; a.a.: amino acid.

juxtamembrane domain of p75NTR. NRAGE expression is enriched in the developing brain and spinal cord, and decreases markedly by adulthood. During development, NRAGE colocalizes with p75NTR in the trigeminal and dorsal root sensory ganglia, within the facial motor nucleus, and in the mantle layer of the medulla oblongata. The mantle layer was found to be a site of p75NTR-dependent apoptosis (Frade and Barde, 1999), which suggests that NRAGE may participate in p75NTR-mediated apoptotic signaling. Consistent with this, NRAGE was found to be necessary for NGF-mediated p75NTR-dependent MAH cell apoptosis. Conversely, expression of TrkA abrogated this effect, demonstrating that TrkA can inhibit NRAGE-dependent p75NTR-mediated cell death. In fact, TrkA and NRAGE appear to compete for the same site on p75NTR, suggesting a new mechanism of Trk and p75NTR transmodulation. The functional roles of most MAGE family members are unknown, but Necdin induces growth arrest and has been suggested to play a role in neuroblast cell cycle exit (Taniura et al., 1998, 1999). Interestingly, NRAGE also facilitates cell cycle exit of 293 cells, suggesting that it may interact with a common set of cell cycle regulators.

NADE was identified in a yeast two-hybrid screen using the p75NTR DD as a bait (Mukai et al., 2000). NADE has two consensus motifs, a leucine-rich nuclear export signal (NES), and two ubiquitination boxes. Both appear functional since NADE expression in PC12 and PCNA cells is only observed in the presence of proteasome inhibitors, and NADE normally localizes to the cytoplasm, but NADE with a mutated NES accumulates in the nucleus. NADE associates with the p75NTR death domain in a NGF-dependent fashion in transfected cells, and co-transfection of p75NTR and NADE lead to NGF-dependent Caspase activation and apoptosis in 293T cells. Zinc treatment of cortical neurons induces both p75NTR and NADE expression, and a function-blocking antibody to p75NTR inhibits both NADE association with p75NTR and zinc-induced toxicity in cortical neurons (Park et al., 2000b). Intriguingly, *in vivo* experiments show that ischemia induces both p75NTR and NADE expression in degenerating CA1 neurons, and that zinc chelators completely block the induction of p75NTR, NADE, and cell death.

TRAF proteins are adaptor molecules that link TNF receptor and interleukin receptor superfamily members to the JNK and NF-κB signaling pathways (reviewed in Wajant et al., 2001). Six TRAF proteins have been identified in mammals and these have been examined for their ability to interact with p75NTR. Initial reports showed that TRAF4 (Krajewska et al., 1998) and TRAF6 (Khursigara et al., 1999) specifically bound p75NTR, but a subsequent paper indicated that all six TRAF proteins were capable of interacting with the receptor (Ye et al., 1999b). We have recently compared TRAF binding specificity and found that, under our conditions, p75NTR binds only TRAF4 and TRAF6 (M. Grapes, unpublished results). TRAF4 and TRAF6 represent the most phylogenetically ancient members of the TRAF family (Wajant et al., 2001), and recent studies have shown that *Drosophila* TRAF1, which is highly related to mammalian TRAF4, readily interacts with mammalian p75NTR intracellular domain (Zapata et al., 2000). Although p75NTR is not present in *Drosphila*, these findings suggest that signaling cascades involving p75NTR, TRAF4 and TRAF6 have been well conserved. TRAF6 activation has been linked to activation of the JNK and NF-κB pathways in other systems, and consistent with this, TRAF6 appears to play a role in p75NTR-mediated NF-κB activation (Khursigara et al., 1999). In contrast, the role of TRAF4 is not well characterized. TRAF4 does not seem to activate NF-κB or JNK, but recent data from our group suggests that it may play a role in p75NTR-mediated Akt activation (M. Grapes, unpublished results).

RIP-2 is an adaptor protein that contains a serine/threonine kinase domain and a Caspase recruitment domain (CARD), and binds to the DD of p75NTR in a NGF-dependent manner through its CARD domain (Khursigara et al., 2001). RIP-2 seems to play a role in p75NTR-mediated NF-κB activation, since a dominant inhibitory isoform of RIP-2 blocks NGF-dependent NF-κB activation and facilitate cell death of Schwann cells.

FAP-1 is a large PTPase that contains six PDZ domains. FAP-1 was identified as a Fas receptor interactor in 1995, and more recent studies have shown that FAP-1 also binds to

the terminal SPV motif present at the carboxyl end of p75NTR (Sato et al., 1995; Irie et al., 1999). The precise role of FAP-1 in p75NTR function is not known, but FAP-1 binding correlates with modestly enhanced p75NTR-mediated NF-κB activation in transfected 293T cells, and mutant isoforms of p75NTR that lack the FAP-1 binding site increased the susceptibility of 293T cells to tamoxifen-induced death. p75NTR-mediated survival was found to correlate with decrease PTPase activity (Roux et al., 2001a), suggesting that p75NTR might bind and inactivate FAP-1 to mediate this effect.

ARMS is a large protein containing four putative transmembrane domains, several ankyrin repeats, a sterile alpha motif domain and a potential PDZ-binding motif, and was originally identified as a p75NTR-interacting protein (Kong et al., 2001). ARMS can be identified in association with both p75NTR and TrkA, and becomes phosphorylated on tyrosine residues in response to activation of TrkA, TrkB or Ephrin B2 receptors. Therefore, ARMS appears be an integral component of a Trk-p75NTR complex and may behave as an adaptor protein capable of linking these receptors physically and functionally.

SC-1 is a p75NTR interacting protein that is composed of a zinc finger domain, a positive regulatory (PR) domain, and a potential amino-terminal PEST sequence (Chittka and Chao, 1999). The PR domain of SC-1 is a motif common to several transcription factors, such as PRD1-BF1 and RIZ (Buyse et al., 1995; Ren et al., 1999), which act as transcriptional repressors, suggesting that SC-1 might subserve similar functions. SC-1 is normally present in the cytoplasm, but NGF binding to p75NTR and not TrkA results in nuclear translocation of SC-1. As with NRAGE, SC-1 induces cell cycle arrest when overexpressed, suggesting a common mechanism linking these disparate proteins to p75NTR's action.

RhoA is part of a family of small GTPases involved in several aspects of neuronal morphogenesis, including migration, polarity, axon growth and guidance, dendrite plasticity and synapse formation (reviewed in Luo, 2000), and has been recently found to interact with the intracellular domain of p75NTR (Yamashita et al., 1999b). Transfection

of p75NTR, constitutively activated RhoA and RhoB in 293 cells, and neurotrophin binding to p75NTR caused loss of RhoA activation, suggesting that this functional interaction might play some role in p75NTR-dependent regulation of neurite outgrowth.

Ceramide. Neurotrophin binding to p75NTR induces activation of sphingomyelinase and hydrolysis of sphingomyelin leads to the accumulation of ceramide (Dobrowsky et al., 1994, 1995). To date, the link between p75NTR activation and ceramide generation remains uncertain, but likely involves acidic and/or neutral sphingomyelinases, enzymes capable of mediating sphingomyelin hydrolysis. Caveolin is a key structural protein in the formation of caveolae (Parton, 1996), which are invaginations of the plasma membrane and the major site of tyrosine kinase and sphingolipid signaling (Okamoto, 1998). The p75NTR and TrkA receptor localize to caveolae and can associate with the caveolin protein, generating an arrangement that can facilitate p75NTR-mediated sphingomyelin hydrolysis, as well as their interactive effects (Bilderback et al., 1997, 1999; Huang et al., 1999). p75NTR-dependent ceramide production did not induce death of T9, NIH-3T3, and PC12 cells (Dobrowsky et al., 1994, 1995), but NGF-mediated ceramide production correlates with JNK activation and apoptosis in oligodendrocytes (Casaccia-Bonnefil et al., 1996).

RATIONALE AND OBJECTIVES

Rationale

Present data suggest that p75NTR can modulate Trk receptor signaling and initiate Trk-independent signal transduction cascades, which in some cellular contexts, lead to apoptosis. Specifically, p75NTR stimulates the generation of ceramide through activation of sphingomyelinase, activates JNK and NF-κB. The elements linking p75NTR to proximal pathways are unknown, but several proteins have been shown to directly interact with p75NTR, including TRAF proteins, SC-1, NRIF, RhoA, NADE and NRAGE. The requirement for these different molecules in p75NTR-mediated signaling are not well understood, and thus, there is a critical need for *in vivo* and *in vitro* models of p75NTR signaling. Moreover, the contribution of different signaling pathways involved in the regulation of neuronal death and survival remains elusive, and the careful analysis of these pathways will be required to understand this finely regulated process.

Objectives

There are three main objectives to this thesis:

1. Given that the function of p75NTR after injury is still unknown, we attempted to develop an *in vivo* model to determine the post-traumatic role of p75NTR in the central nervous system of the rat.

2. Little is known about the exact signaling pathways involved in p75NTR-mediated apoptosis. Consequently, we developed an *in vitro* model to identify the mechanisms and signaling pathways used by p75NTR to mediate life and death decisions.

3. Finally, given that the survival promoting mechanisms induced by the related compounds, K252a and CEP1347, are unknown, we decided to further characterize the signaling pathways involved in the neuroprotective effects induced by these compounds.

PREFACE TO CHAPTER 2

The exact function of p75NTR is still unclear, but growing evidence suggest a death-inducing role for the receptor. Several signaling pathways have been shown to be activated by p75NTR, but their contributions to p75NTR-mediated apoptosis remain elusive. Most of the current knowledge on p75NTR comes from *in vitro* studies, and *in vivo* models are required to study p75NTR function. Interestingly, p75NTR expression has been shown to increase drastically following nervous system injuries, such as ischemia and nerve transection. Whilst the role of p75NTR after injury is still unknown, the induction of p75NTR may represent an interesting means to study its function. Using the pilocarpine-induced seizure model of traumatic injury in the rat, we asked (a) whether apoptosis was an important form of cell death contributing to seizure-induced damage, (b) whether p75NTR expression was induced in this model of nervous system injury, and (c) if injury-induced p75NTR expression correlated with apoptotic cell death in the central nervous system.

CHAPTER 2

P75 NEUROTROPHIN RECEPTOR EXPRESSION IS INDUCED IN APOPTOTIC NEURONS AFTER SEIZURE

p75 Neurotrophin Receptor Expression is Induced in Apoptotic Neurons Following Seizure

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ABSTRACT

Seizure causes neuronal cell loss in both animal models and human epilepsy. To determine the contribution of apoptotic mechanisms to seizure-induced neuronal cell death, rat brains were examined for the occurrence of TUNEL-positive nuclei following pilocarpine-induced seizure. Numerous TUNEL-positive cells were observed throughout the post-seizure hippocampus, piriform cortex, and entorhinal cortex. Combined TUNEL/NeuN immunocytochemistry demonstrated that the vast majority of TUNEL-positive cells were neurons. To identify components of the signal transduction cascade promoting post-seizure apoptosis, the expression of the p75 neurotrophin receptor (p75NTR) was examined. Seizure-induced increases in p75NTR protein and mRNA were detected in hippocampus, piriform cortex, and entorhinal cortex. Immunohistochemical double labeling revealed almost complete correspondence between TUNEL-positive and p75NTR expressing cells, suggesting that seizure-induced neuronal loss within the central nervous system occurs through apoptotic signaling cascades involving p75NTR.

INTRODUCTION

The neurotrophins are a conserved family of proteins that play a critical role in the development and maintenance of the nervous system (reviewed in Barde, 1989). Their cellular effects are mediated by two distinct classes of cell surface receptors. The Trk receptors are a family of transmembrane receptor tyrosine kinases that selectively bind different members of the neurotrophin family, with TrkA preferentially binding NGF, TrkB preferring BDNF and NT-4/5, and TrkC interacting with NT-3 (reviewed in Kaplan and Miller, 1997). The second class of neurotrophin receptor contains a single family member, the p75 neurotrophin receptor (p75NTR), that binds all the neurotrophins (reviewed in Barker, 1998; Casaccia-Bonnefil et al., 1998). p75NTR is a member of the TNF receptor superfamily that includes CD27, CD30, CD40, 4-1BB, OX40, the fas antigen and the tumor necrosis factor receptors TNFR1 and TNFR2 (Bazan, 1993). Two opposing functions have been proposed for p75NTR. When coexpressed with TrkA, p75NTR enhances NGF-mediated survival by increasing the amount of NGF that binds the TrkA receptor (Barker and Shooter, 1994; Mahadeo et al., 1994; Verdi et al., 1994). Conversely, in some systems, p75NTR appears to behave as a ligand-regulated pro-apoptotic receptor (Frade et al., 1996; Casaccia-Bonnefil et al., 1996; Majdan et al., 1997; Bamji et al., 1998; Frade and Barde, 1998). The signaling cascades that allow p75NTR to promote apoptosis remain unknown but may involve ceramide production through activation of sphingomyelinase (Dobrowsky et al., 1994, 1995), activation of c-Jun N-terminal kinase (JNK; Casaccia-Bonnefil et al., 1996; Yoon et al., 1998) and accumulation of p53 (Aloyz et al., 1998).

Neuronal cell death has been well documented in both human epilepsy and experimental seizure models (reviewed in Represa et al., 1995; Sloviter, 1996; Treiman, 1996, Morrison et al., 1996). Although the specific contribution of cell death to the pathophysiology of epilepsy remains unclear, multiple studies suggest that damage produced by status epilepticus (SE) promotes the development of the recurrent spontaneous seizures characteristic of epilepsy (Aicardi and Chevrie, 1983; Cavalheiro et al., 1991; Priel et al., 1996). Pilocarpine-induced SE in the rat results in damage in multiple brain regions (Turski et al., 1983, 1987; Olney et al., 1986). Dystrophic neurons

can be detected as early as twenty minutes after induction of SE, and much of this cell damage is likely due to necrosis (Fujikawa, 1996). Apoptotic cell death has been reported in some seizure models (Pollard et al., 1994; Bengzon et al., 1997, Morrison et al., 1996), but the specific contribution of apoptotic or necrotic death to seizure-induced neuronal loss is not clear and the cellular mechanisms leading to the induction of apoptosis following seizure are unknown.

In addition to necrotic and apoptotic cell death, seizure also induces changes in gene expression, including marked alterations in neurotrophin and trk receptor expression (Gall et al., 1991a; reviewed in Gall, 1993). Neurotrophins have been suggested to play a trophic role post-seizure; however, the recent demonstration of a pro-apoptotic role for the p75 neurotrophin receptor suggests that increased neurotrophin expression after seizure could potentially promote cell death via p75NTR-dependent apoptotic mechanisms.

In this study, we demonstrate that pilocarpine-induced seizure results in marked and persistent apoptosis within hippocampal, piriform, and entorhinal cortical neurons. We show that this region-specific increase in neuronal apoptosis is accompanied by expression of p75NTR mRNA and protein. Furthermore, we demonstrate that neurons undergoing seizure-induced apoptosis invariably show strong induction of p75NTR, suggesting that upregulation of p75NTR expression and activation of p75NTR signaling cascades may facilitate neuronal apoptosis following seizure.

EXPERIMENTAL PROCEDURES

Seizure induction. Adult male Sprague-Dawley rats (200-300 g; Charles River Canada, PQ) were used for all experiments and housed under environmentally controlled conditions. Status epilepticus (SE) was induced by the administration of pilocarpine (380 mg/kg, i.p.; ICN, PQ). Thirty minutes before administering pilocarpine, animals received scopolamine methyl-bromide (1 mg/kg, i.p.; Sigma, ON) to reduce the peripheral cholinergic effects of pilocarpine. During SE, the animals exhibited 2 to 5 stage 5 seizures, behaviorally similar to kindled stage 5 seizures (Racine, 1972). To reduce mortality due to seizure, diazepam (10 mg/kg, i.p.; Hoffmann-LaRoche, ON) was injected one hour after the onset of SE. Control animals were treated identically to the experimental group except that they received saline instead of pilocarpine. Animals were euthanized and tissue removed 1, 3, 7 or 14 days after pilocarpine injection.

Tissue preparation. For immunocytochemical and TUNEL assays, animals were anaesthetized by injection of sodium pentobarbital (50 mg/kg, i.p.; MTC Pharmaceuticals, ON), and perfused intracardially with phosphate-buffered saline (PBS) plus heparin (5 mg/ml) followed by 4% paraformaldehyde, 15% picric acid, in PBS at pH 8 and 37 °C. Following perfusion, brains were removed, postfixed for 3 days at room temperature (RT) and cryoprotected in 30% sucrose-containing fixative at RT for 48 hours before sectioning. Frozen 40 μm cryostat serial sections were stored in cryoprotectant at -20 °C (30% sucrose and 30% ethylene glycol in PBS) and assayed within three months of sectioning. For immunoblot analysis, animals were euthanized with pentobarbital as above, the brain removed, cortex (combined neo-, and paleo-cortical tissue excluding hippocampus) and hippocampus rapidly dissected, and total protein extracted using Trizol (Life Technologies, MD) as suggested by the manufacturer.

Immunoblotting. p75NTR immunoreactivity was detected using anti-p75NTR-B1, a rabbit polyclonal antibody directed against a GST-fusion protein containing amino acids 276-425 of the intracellular domain of rat p75NTR (Majdan et al., 1997). MC192 ascites fluid was produced as described previously (Barker and Shooter, 1994) and purified using an Immunopure column (Pierce, IL). Protein content of brain tissue extracts was

normalized using the BCA assay (Pierce). Twenty five μg of protein was then solubilized in sample buffer (Laemmli, 1970), separated on 10% SDS-polyacrylamide gels (SDS-PAGE) and electroblotted to nitrocellulose. Blocking, primary antibody, and secondary antibody incubations for p75NTR immunoblots were all performed in 10 mM Tris (pH 7.4), 150 mM NaCl, and 0.2% Tween 20 with 5% (w/v) dry skim milk powder using anti-p75NTR-B1 (1:2000). HRP-conjugated donkey anti-rabbit IgG (Jackson Laboratories, PA) was used at a dilution of 1:10000. Immunoreactive bands were detected using enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Dupont, ON). The immunoreactive band detected in brain homogenates comigrated with an immunoreactive band of the appropriate molecular weight present in cell homogenates derived from p75NTR-transfected 293 cells and immunoreactivity could be blocked by a 6XHis-fusion protein corresponding to the intracellular domain of p75NTR (data not shown). Densitometry and quantification of the relative level of p75NTR protein was performed on scanned images of immunoblots (Epson ES 1200C) using NIH Image (US National Institutes of Health). The mean densitometric value corresponding to p75NTR expression was calculated for each time point (n=3 per time point with the exception of the one day post-seizure time point where n=2), and the percent increase from controls determined by direct comparison with samples on the same immunoblot.

Immunocytochemistry. Following cryostat sectioning, brain sections were washed briefly in PBS, and endogenous peroxidase activity was reduced by incubation in 75% methanol and 3% H2O2 for 30 minutes at RT. Blocking, primary, and secondary antibody incubations were performed in blocking solution (2% bovine serum albumin, 2% heat inactivated normal goat serum and 0.2% Triton X-100). Anti-p75NTR-B1 was used at a dilution of 1:500 and HRP-conjugated goat anti-rabbit IgG at a dilution of 1:1000 (Jackson Labs). Immunocytochemistry for c-Jun expression was performed as described for p75NTR, using a monoclonal antibody against c-Jun at 1:2000 (Transduction Laboratories, KY), and an HRP-conjugated goat anti-mouse IgG at 1:1000 (Jackson Labs). Antibody complexes were detected with diaminobenzidine (DAB) and H2O2 as described (Vector Laboratories, CA). For p75NTR and TUNEL costaining, sections were directly blocked for two hours following the TUNEL reaction, followed by an overnight

incubation with purified MC192 (3 μg/ml at 4 °C), a monoclonal antibody which recognizes rat p75NTR (Chandler et al., 1984). Secondary antibody incubation was performed at RT for 2 hours using Cy3-conjugated goat anti-mouse IgG (Jackson Labs) at a dilution of 1:1000. NeuN/TUNEL double labeling was performed as for p75NTR, using the NeuN mouse monoclonal antibody at a dilution of 1:25 (gift of Richard Mullen). During the washes, nuclei were stained using Hoechst 33258 as described (Molecular Probes, OR). Bright field images of p75NTR and c-Jun immunoreactivity, and TUNEL were photographed using a Zeiss Axiophot microscope. Fluorescence was visualized using a Zeiss Axioscop microscope and photographed using a CCD camera and Northern Eclipse software (Empix Imaging, ON).

In situ hybridization. Following intracardial perfusion with 100 ml of 37 °C saline with 5 μg/ml heparin, brains were immediately dissected, placed in ice cold PBS, and frozen in isopentane (2-methyl butane) chilled in liquid nitrogen. Five micron cryostat sections were cut and fixed to slides (Superfrost/Plus; Fisher, ON) with 4% paraformaldehyde, 15% picric acid in PBS. In situ hybridization was performed as described (Braissant and Wahli, 1998) using digoxigenin labeled RNA probes, signal amplified using the Tyramide Signal Amplification kit (NEN, MA), and peroxidase/DAB detection. Probes were sense and antisense transcripts of a 300 base pair fragment corresponding to nucleotides 400 to 700 of rat p75NTR cDNA (Radeke et al., 1987).

In situ detection of DNA cleavage. End-labeling of nicked DNA with fluorescein-dUTP and terminal transferase (TUNEL) was performed using an in situ cell death detection kit as per the manufacturer's instructions (Boehringer Mannheim, PQ). TUNEL-positive nuclei were detected using DAB and H_2O_2 with an HRP-conjugated anti-fluorescein antibody. For the colabeling studies described above, TUNEL-positive cells were identified directly using the FITC fluorescence of the incorporated dUTP. Positive cells in 12 fields sampled from layers II and III of the entorhinal cortex derived from 2 different rats (6 fields each) at three days post-seizure were scored for p75NTR immunoreactivity, TUNEL reactivity, and their co-localization. In separate experiments, coincidence of NeuN and TUNEL reactivity was similarly determined.

DNA extraction and agarose gel electrophoresis. Samples of hippocampi as well as frontal and temporal lobe were dissected from brains of control rats or from rats seized one or three days earlier and then immediately frozen in dry ice, and stored at -70 °C. DNA was purified as described in Sankar et al. (1998) with some modifications. Tissues were homogenized using a dounce homogenizer with a loose pestle in 5 volumes of a buffer containing 15 mM HEPES (pH 7.2), 0.25 M sucrose, 60 mM KCl, 10mM NaCl, 1 mM EGTA, 5 mM EDTA and 1 mM PMSF. Cells were then centrifuged at 2000 g for 10 minutes and incubated overnight in a buffer containing 50 mM NaCl, 10 mM Tris pH 8.0, 25 mM EDTA, 0.5% SDS, 0.5 mg/ml proteinase K and 0.1 mg/ml DNase-free RNase A at 55 °C. The lysate was extracted twice using equal proportions of phenol:chloroform (1:1) and then the aqueous layer was incubated at 37 °C with 0.1 mg/ml RNase A for 90 minutes. The phenol:chloroform extraction was repeated and DNA was precipitated overnight with 2.5 volume of ethanol and 1/10 volume of sodium acetate at -20 °C. Precipitated DNA was spun at 15,000 g for 30 minutes and washed three times with 70% ethanol. The DNA pellet was then air-dried and resuspended in 0.5-1 ml of 10 mM Tris and 1 mM EDTA. Spectrophotometry revealed A260/280 ratios of 1.6-1.9, indicating relatively pure DNA in concentrations of 0.8-1.3 mg/ml. Thirty mg of DNA was run on each lane of a 1% agarose gel containing 0.5% ethidium bromide at 5 V/cm gel length. The gel was viewed under UV transillumination and photographed using a Kohu CCD camera.

RESULTS

Pilocarpine-induced seizure leads to apoptosis in the adult rat brain. Brains of rats subjected to pilocarpine-induced seizure were examined for evidence of cell death using TUNEL, a method that detects apoptotic cells in situ (Gavrieli et al., 1992; Sgonc et al., 1994). Induction of SE by injection of pilocarpine caused severe generalized seizures (multiple class 5) that resulted in numerous TUNEL-positive nuclei in multiple brain regions. TUNEL-positive cells were clearly detected at both one and three days following seizure in piriform cortex, entorhinal cortex, and hippocampus, but TUNEL-positive cells were not observed in brain sections of control animals (Figure 2.1). The incidence of TUNEL-positive cells was greater one day after seizure in both the piriform and entorhinal cortices compared to three days. However, in the hippocampus, TUNEL staining was maximal at three days post-seizure ($n > 4$ for each timepoint). Although the incidence of TUNEL-positive nuclei peaked at one day in entorhinal cortex after seizure, they were still detected in layers II and III of the entorhinal cortex fourteen days after seizure (not shown). In the hippocampus, most TUNEL-positive nuclei were concentrated in the CA1 region, with lower numbers in the granule cell layer of the dentate gyrus (Figure 2.1I). Consistent with previous histochemical descriptions of the distribution of dystrophic cells produced by pilocarpine-induced seizure (Mello et al., 1993; Fujikawa, 1996), the amygdala, the perirhinal cortex and the lateral posterior thalamic nucleus showed fewer but detectable TUNEL-positive nuclei following seizure (not shown). Incidence of TUNEL-positive cells varied somewhat between animals but the regional distribution of TUNEL-positive cells was consistent.

To confirm that TUNEL staining within seized brain reflects seizure-induced apoptosis, genomic DNA was extracted from brains of control and seized rats and analyzed for oligosomal fragmentation, a biochemical hallmark of apoptosis. Figure 2.2 shows that DNA fragmentation was undetectable in brains from control animals; however, DNA fragments with a periodicity of approximately 180 base pair were present in extracts of temporal cortex from seized animals at both one and three days after SE. DNA fragmentation was most prevalent in temporal cortex, consistent with the high level of TUNEL reactivity observed in the piriform and entorhinal cortices, but was also clearly

detected in extracts of hippocampus and the frontal cortex. Sections costained with TUNEL and Hoechst 33258 demonstrate that a subset of the TUNEL positive nuclei are condensed in a manner characteristic of apoptotic cell death (Figure 2.7). Together, these data demonstrate the presence of apoptotic cells within multiple CNS structures in the rat following pilocarpine-induced seizure.

Signaling pathways that result in c-Jun expression and phosphorylation are involved in neuronal apoptosis (reviewed in Dragunow and Preston, 1995; Herdegen et al., 1997) and we therefore examined whether c-Jun protein expression was induced following pilocarpine-induced seizure. Consistent with previous results which showed induction of immediate-early response genes in other seizure models (Dragunow et al., 1993; Herdegen et al., 1997), prominent c-Jun expression was detected throughout the brain one day after pilocarpine-induced SE (Figure 2.1L).

p75NTR protein expression increases following seizure. Neurotrophins promote neuronal survival by activating trk receptors but recent studies also suggest that p75NTR may facilitate neuronal apoptosis. As an initial step toward identifying molecules that may promote neuronal apoptosis following seizure, levels of p75NTR protein were assayed following pilocarpine-induced seizure. Immunoblot analysis of tissue isolated from non-seized controls show that the level of p75NTR protein is low but detectable in protein extracted from hippocampal and cortical tissue. Following seizure, p75NTR protein expression increased in a time-dependent manner (Figure 2.3). In the hippocampus (Figure 2.3B), p75NTR protein levels increased one day after seizure and then persisted as a 4-6 fold increase for at least 7 days. Two weeks after seizure, the level of p75NTR protein had returned to that of sham-treated controls. In cortical lysates (Figure 2.3A) the increase was delayed and more transient, with a 5-fold increase in p75NTR expression three days after seizure but sharply reduced p75NTR levels by seven days.

To identify the cellular distribution of p75NTR protein, brain sections from seized and control rats were analyzed for p75NTR immunoreactivity one and three days after pilocarpine treatment. In control animals, p75NTR immunoreactivity was restricted to

basal forebrain, as previously described (Kiss et al., 1988; Lee et al., 1998; data not shown). Following pilocarpine-induced seizure, p75NTR immunoreactivity was detected in the piriform cortex, entorhinal cortex, perirhinal cortex, and hippocampus (Figure 2.4). Of these regions, entorhinal cortex showed the strongest p75NTR immunoreactivity, particularly three days after seizure. At one day post-seizure, a diffuse increase in immunoreactivity was detected in entorhinal, piriform, and perirhinal cortices. By three days post-seizure, strong p75NTR immunoreactivity was concentrated in cortical layers II and III and was clearly associated with cell bodies and processes. Immunoreactivity decreased gradually dorsal to perirhinal cortex. The immunoblotting studies analyses did not reveal an increase in p75NTR protein in the cortex one day after seizure (Figure 2.3) which is consistent with the restricted cortical expression observed by immunocytochemistry at this time point. In the hippocampus, increased p75NTR expression was observed in the dentate granule cell layer, dentate hilus, and CA1 pyramidal cell layer, and was most prominent 3 days after seizure.

p75NTR mRNA expression is increased in seized brain. To identify the cellular source of p75NTR expression following pilocarpine seizure, p75NTR mRNA distribution was determined by *in situ* hybridization in sections taken from pilocarpine-treated animals one and three days after seizure. Figure 2.5A shows that p75NTR mRNA was readily detected in layers II and III of the entorhinal cortex using an antisense p75NTR cRNA. Within the hippocampus, p75NTR mRNA was detected primarily in the CA1 pyramidal cell layer and dentate granule cell layer, but lower levels were present in the hilus (Figure 2.5D). No specific hybridization was observed when a control sense probe was used in the entorhinal cortex or the hippocampus (Figure 2.5B, E), and sense and antisense p75NTR probes produced no detectable signal in sections of entorhinal cortex and hippocampus from control, non-seized animals (not shown).

p75NTR expression is induced following seizure by neurons undergoing apoptosis. To determine if the p75NTR immunoreactive and TUNEL-positive cells were neurons, we first examined the coincidence of TUNEL and NeuN, a neuron specific epitope (Mullen et al., 1992). Double label immunofluorescence showed that greater than 90% of the

TUNEL-positive cells in layers II and III of the entorhinal cortex three days after seizure were NeuN-positive, identifying them as neurons (Figure 2.6).

To determine if p75NTR expression correlated with TUNEL labeling of individual cells, co-labeling was performed on sections of entorhinal cortex derived from animals three days after pilocarpine-induced SE. The mouse monoclonal antibody MC192 was used to detect p75NTR in these experiments because p75NTR-B1 antigenicity was incompatible with the TUNEL reaction. Control studies on serial sections demonstrated that MC192 and p75NTR-B1 produce identical patterns of immunoreactivity (not shown). Co-labeling within the entorhinal cortex demonstrated that most of the TUNEL-positive cells present in layers II and III three days after seizure were also p75NTR immunoreactive (Figure 2.7A-G). An almost complete coincidence of TUNEL staining with p75NTR immunoreactivity was also observed in the CA1 region of the hippocampus (Figure 2.7H-I). Cell counts of layers II and III in the entorhinal cortex revealed a strong correlation between p75NTR immunoreactivity and TUNEL ($n>600$, Table 2.1): greater than 83% of the cells which were positive for p75NTR were also TUNEL-positive and more than 85% of TUNEL-positive cells were immunoreactive for p75NTR (Table 2.1). Therefore, there was an almost complete overlap between p75NTR expression and the presence of TUNEL-positive nuclei.

DISCUSSION

In this study we demonstrate that pilocarpine-induced seizure produces a large increase in TUNEL-positive neurons in the hippocampal, entorhinal, and piriform cortices and a dramatic rise in cellular DNA cleavage, a hallmark of apoptosis. This regional damage is accompanied by a large increase in levels of p75NTR mRNA and protein in neurons within these same structures. The incidence of TUNEL within individual neurons correlates tightly with p75NTR expression, with over 85% of the cells with TUNEL-positive nuclei showing induced p75NTR expression. TUNEL-positive cells are still observed many days after pilocarpine administration, indicating that apoptotic mechanisms, potentially mediated by p75NTR, may contribute to long-term cell loss after status epilepticus.

The relative proportions of necrotic and apoptotic cell death are not known in any seizure model but both necrosis and apoptotic cell death occur following kainic acid-induced seizure and during kindling (Pollard et al., 1994; Morrison et al., 1996; Bengzon et al., 1997). Histochemical assays have shown that pilocarpine-induced seizure induces cell damage in numerous sites throughout the brain that include the hippocampal gyrus (CA1 and CA3 cell layers), the dentate gyrus (both granule cell and hilar layers), piriform cortex, and entorhinal cortex (Fujikawa, 1996). This pilocarpine-induced cell loss can be inhibited by NMDA antagonists (Rice and DeLorenzo, 1998), suggesting that much of it is triggered by excitotoxic mechanisms. We have found that pilocarpine-induced seizure results in a profound increase in TUNEL-reactivity in neurons, particularly in entorhinal and piriform cortices but also within the hippocampus.

Necrosis and apoptosis are defined on the basis of morphological criteria but in mechanistic terms, apoptosis refers to active intracellular signaling that results in cellular suicide. In some systems, dying cells can show morphological features of both necrosis and apoptosis; for example, cells showing morphological hallmarks of necrotic death can also be TUNEL positive (Charriaut-Marlangue and Ben-Ari, 1995). Pyknotic nuclei, which are characteristic of apoptosis, become numerous following pilocarpine-induced seizure paradigm (Figure 2.7) but to confirm that intracellular apoptotic signaling

cascades contribute to the cell death which occurs following pilocarpine-induced SE, DNA extracted from various areas of seized brains was examined for the DNA cleavage pattern characteristic of intracellular apoptotic mechanisms. Our demonstration that seizure induced by pilocarpine results in DNA fragmentation which correlates with the region specific increase in TUNEL staining and pyknotic nuclei indicates that this seizure model results in widespread activation of intracellular apoptotic cascades. Kainate, which is widely used to induce seizure, results in damage primarily to the hippocampus, particularly within CA1, CA3, the hilus and the subiculum (Morrison et al., 1996); our data suggest that pilocarpine-induced seizure results in a much more profound apoptotic response within the central nervous system, ultimately resulting in more widespread neuronal damage.

The expression of neurotrophins and trk receptors is regulated by kindling and following chemically-induced seizure. A transient increase in mRNA for NGF, BDNF, TrkB and TrkC in the hippocampus and neocortex has been demonstrated during kindling (Ernfors et al., 1991; Bengzon et al., 1993; Merlio et al., 1993) and kainic acid or bicuculline-induced seizure results in increased expression of NGF, BDNF, and TrkB mRNA levels (Zafra et al., 1990; Ballarin et al., 1991; Gall et al., 1991b; Isackson et al., 1991; Dugich-Djordjevic et al., 1992, 1995; Humpel et al., 1993; Wetmore et al., 1994). Studies examining NT-3 expression have suggested either no change (Ballarin et al., 1991; Ernfors et al., 1991; Merlio et al., 1993) or a decrease in expression by hippocampal neurons following seizure (Bengzon et al., 1992; Gall, 1992; Rocamora et al., 1992), suggesting that increases in neurotrophin expression are restricted to specific family members. In addition, TrkA mRNA is unchanged by kindling or pilocarpine-induced seizure (reviewed in Gall, 1993; Persson and Ibanez, 1993; Mudo et al., 1996). Traditionally, the injury-induced increase in neurotrophin expression has been thought to mediate cell survival or synaptic plasticity; however, the recently discovered apoptotic function of p75NTR may require reevaluation of this hypothesis.

The p75NTR is widely expressed in the nervous system during development, but in the adult CNS, p75NTR expression is limited mainly to magnocellular neurons of the basal

forebrain, cells within the caudate putamen and cerebellar Purkinje cells (reviewed in Barker, 1998). The expression profile of p75NTR following chemically-induced seizure has not been previously addressed but p75NTR mRNA levels are unchanged by kindling (Merlio et al., 1993). p75NTR mRNA levels are increased in some forms of neuronal injury such as in motoneurons following sciatic nerve crush (Ernfors et al., 1989), in adult striatal cholinergic neurons following experimentally induced focal cerebral ischemia (Kokaia et al., 1998), and in Purkinje cells following axotomy (Armstrong et al., 1991; Dusart et al., 1994; Martinez-Murillo et al., 1998). The functional consequences of these changes in p75NTR expression following neuronal trauma are uncertain since p75NTR can, on the one hand, facilitate TrkA activation and increase survival effects of the neurotrophins (Barker and Shooter, 1994; Verdi et al., 1994; Ryden et al., 1997) but also play a pro-apoptotic role (Rabizadeh et al., 1993; Barrett and Bartlett, 1994; Frade et al., 1996; Majdan et al., 1997; Bamji et al., 1998). This contrast in p75NTR function is illustrated by comparing wild-type and $p75NTR^{exonIII-/-}$ sympathetic neurons under different experimental conditions. Sympathetic neurons derived from $p75NTR^{exonIII-/-}$ mice require increased amounts of NGF to maintain survival (Lee et al., 1994; Ryden et al., 1997), indicating that p75NTR normally facilitates trkA activity, yet $p75NTR^{exonIII-/-}$ neurons undergo apoptosis in response to neurotrophin withdrawal considerably more slowly than their wild-type counterparts (Bamji et al., 1998), indicating that p75NTR also normally facilitates apoptosis. It now seems very likely that there is considerable cell and developmental specificity to p75NTR function *in vivo*, with p75NTR enhancing survival in some circumstances and facilitating apoptosis in others.

We favor the hypothesis that p75NTR is induced following seizure through an activity-dependent mechanism and is then capable of activating apoptotic signaling cascades in response to bound neurotrophin. This model is consistent with findings which show that p75NTR expression is increased by potassium chloride treatment of cultured Purkinje cells (Cohen-Cory et al. 1993) with the action of other related apoptotic receptors, where regulated receptor expression is necessary and sufficient for the initiation of an apoptotic cascade (Muller et al., 1998; Chan et al., 1999) and with findings which show that p75NTR activates apoptotic pathways in a ligand-dependent manner (Frade et al., 1996;

Casaccia-Bonnefil et al., 1996; Majdan et al., 1997; Bamji et al., 1998; Frade and Barde, 1998, 1999).

Signaling pathways activated by p75NTR remain poorly characterized but likely candidates that may be involved in this cascade include JNK and the p53 tumor suppressor. Mice lacking the gene for JNK3, an isoform of JNK enriched in the CNS, show reduced apoptosis in response to excitotoxic injury (Yang et al., 1997) and although p75NTR-mediated activation of JNK has not been reported in central neurons, JNK is induced following p75NTR activation in oligodendrocytes and sympathetic neurons (Casaccia-Bonnefil et al., 1996; Yoon et al., 1998; Bamji et al., 1998). Our results show that seizure resulted in increased c-Jun immunoreactivity yet the distribution of both p75NTR and TUNEL staining was considerably more restricted than the increase in c-Jun expression. It is possible that production of the c-Jun protein may be a necessary prerequisite for seizure-induced neuronal apoptosis but is insufficient to mediate apoptosis on its own. Indeed, c-Jun may become phosphorylated by JNK (and therefore active) only in those cells expressing p75NTR. It is noteworthy that following brain ischemia, c-Jun is widely expressed yet c-jun phosphorylation occurs only in a proportion of piriform cortical cells undergoing apoptosis (Herdegen et al., 1998). Several findings also indicate that p53 may be involved in neuronal apoptosis following seizure. Morrison and colleagues (1996) have shown that hippocampal neurons which are normally lost following kainic acid-induced seizure are protected in mice lacking functional alleles for p53. Furthermore, p53 may be implicated in a p75NTR-dependent apoptotic pathway induced in sympathetic neurons withdrawn from NGF (Aloyz et al., 1998). Given the links between p75NTR, JNK activation and p53, it will be interesting to test if p75NTR acts as an apoptotic receptor which mediates JNK and/or p53 activation in adult neurons and to determine if p75NTR expression constitutes the first step of a death process triggered by seizure.

The roles of p75NTR in enhancing trk activity and mediating apoptosis are complex and signaling events evoked by p75NTR are not fully understood. There is a critical need for *in vitro* and *in vivo* models that will allow the elucidation of p75NTR function. Here, the

concurrence of apoptosis and p75NTR expression observed in the CNS following pilocarpine-induced seizure indicates that analysis of the role of p75NTR in seizure-induced apoptosis will prove useful for identifying both p75NTR signaling mechanisms and p75NTR's potential contribution to neuronal cell death *in vivo*.

Table 2.1

Proportion of TUNEL and p75NTR co-labeled cells in the entorhinal cortex three days post-seizure.

TUNEL positive	**p75NTR positive**
530 (100%)	456 (85.8 ± 7.6%)
	p75NTR negative
	74 (14.2 ± 2.4%)
p75NTR positive	**TUNEL positive**
545 (100%)	456 (83.6 ± 7.4%)
	TUNEL negative
	89 (16.4 ± 1.2%)

Three days post-seizure, brain sections were assayed for nicked DNA using TUNEL followed by p75NTR immuno-cytochemistry as shown in figure 2.5. TUNEL positive (FITC) and p75NTR positive (Cy3) cells were counted and the overlap between the two groups determined. Values represent the percentage of each group compared to either the total p75NTR or total TUNEL positive cells ± SE of 12 separate fields derived from 2 different animals.

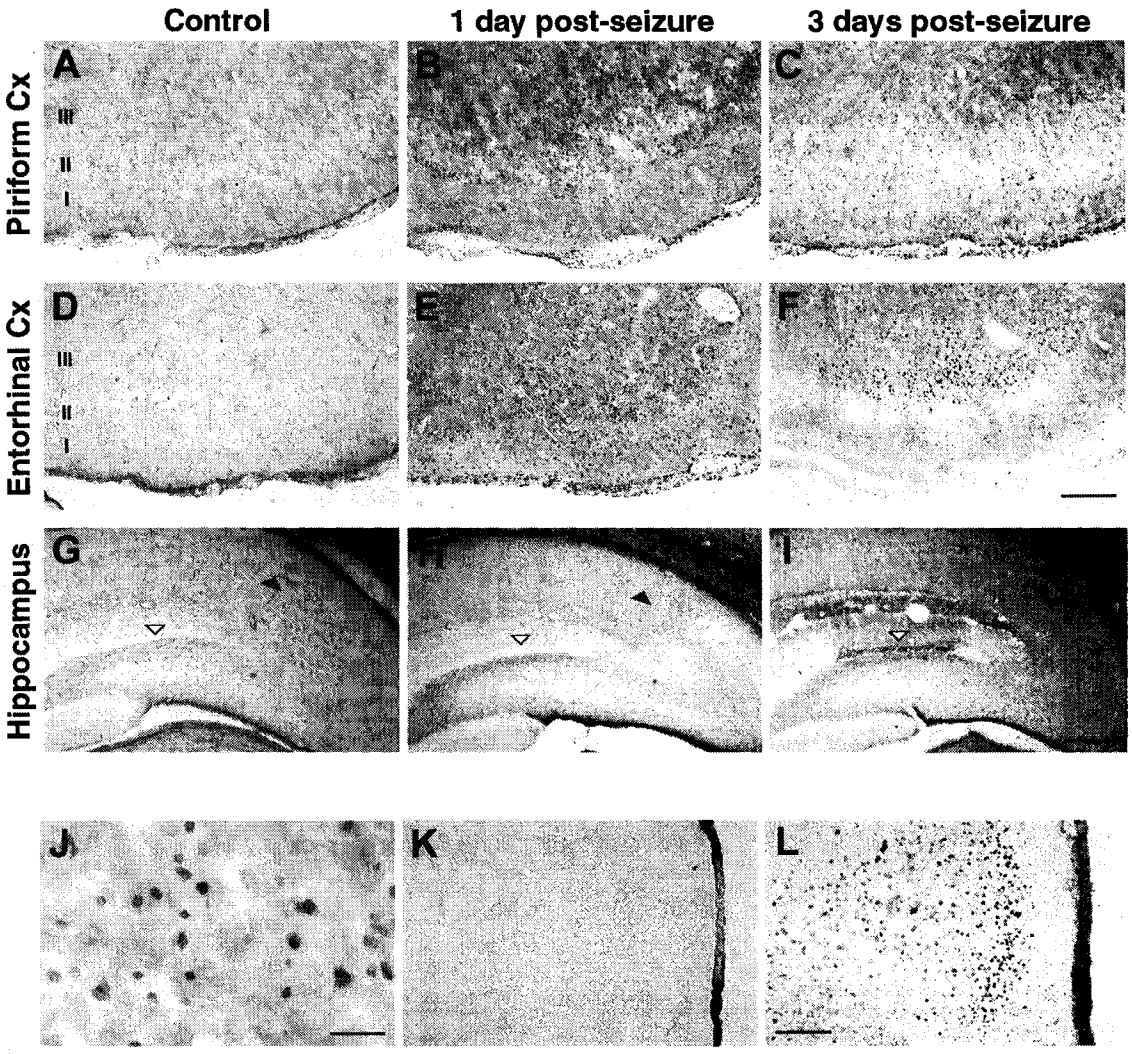

Figure 2.1. TUNEL staining and expression of c-Jun following pilocarpine-induced seizure in the rat brain. Cells positive for TUNEL reactivity (A-J) and for c-Jun expression (K, L) were visualized using peroxidase/DAB in brain sections from control rats (A, D, G, K), one day (B, E, H, L), or three days after seizure (C, F, I) in the piriform cortex (A-C), entorhinal cortex (D-F, K, L), and hippocampus (G-I). Panel J is a higher magnification of the tissue in panel E showing TUNEL-positive nuclei. Filled arrowheads identify the hippocampal CA1 pyramidal cell layer. Open arrowheads identify the upper blade of the dentate granule cell layer. Cortical layers are indicated. Cx: cortex. Scale bars=200 μm (F), 400 μm (I), 20 μm (J), and 160 μm (L).

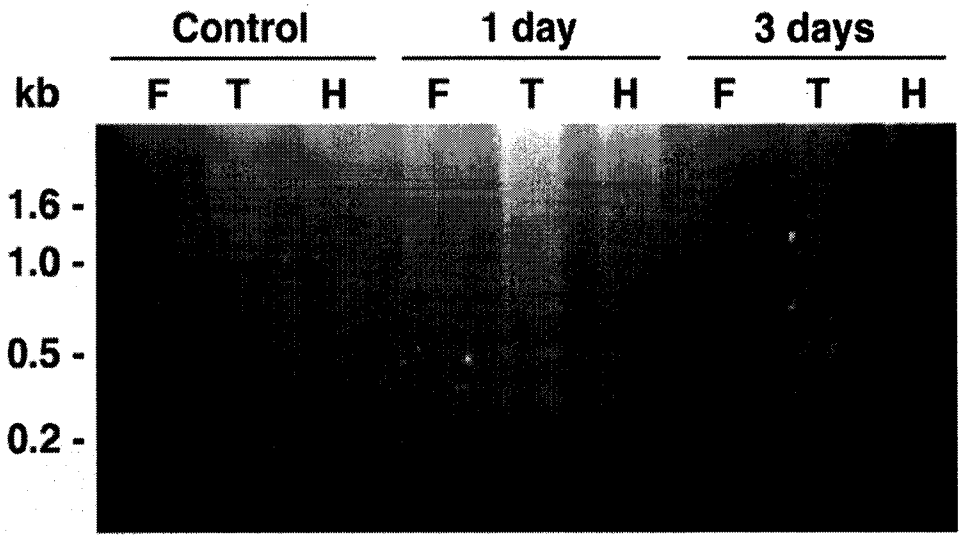

Figure 2.2. DNA fragmentation following pilocarpine-induced seizure. DNA extracted from control, one day and three days post-seizure tissue was analyzed by agarose gel electrophoresis. DNA laddering is visible in extracts of rat brain one and three days following pilocarpine-induced seizure. DNA was extracted from dissected samples of frontal cortex (F), temporal cortex (T), and hippocampus (H).

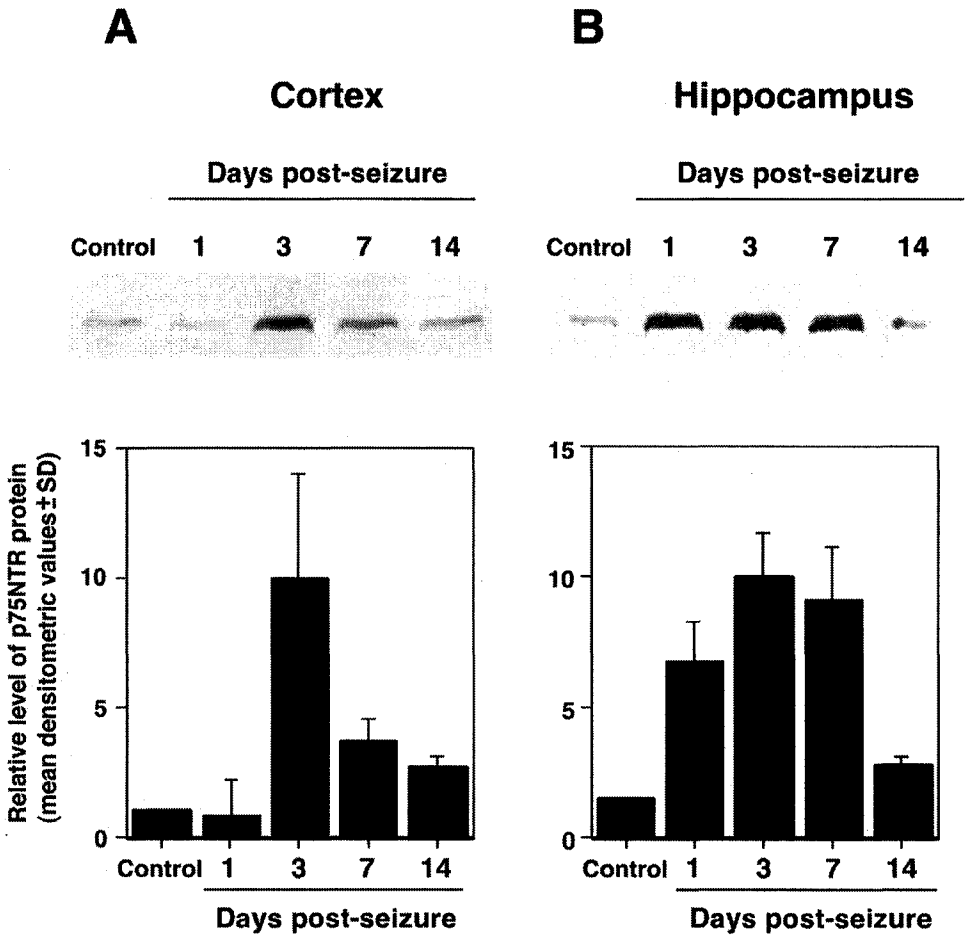

Figure 2.3. Increased p75NTR expression following seizure. Protein homogenates from the hippocampus and cortex (neo- and paleo-cortical tissue) of control, one day, three days, seven days, and fourteen days post-seizure. Representative immunoblots show the relative amount of p75NTR protein in cortex (A) and hippocampus (B). Each graph represents the normalized densitometric index of immunoblots from three animals per timepoint (± SD) except for the one day timepoint, where two animals were analysed.

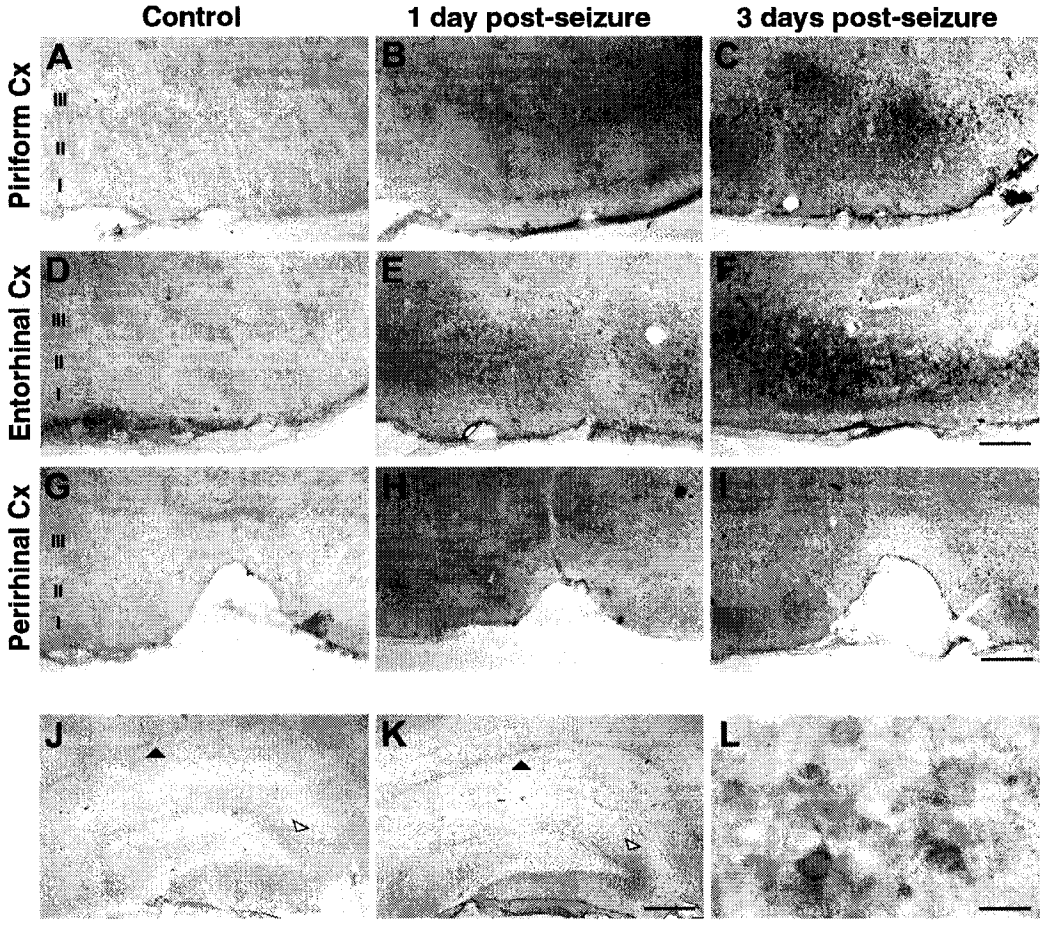

Figure 2.4. Cellular localization of p75NTR protein following seizure. p75NTR expression visualized with peroxidase/DAB in brain sections from control (A, D, G, J), one day (B, E, H), or three days after seizure (C, F, I, K). Panel A-C show the piriform cortex, D-F the entorhinal cortex, G-I the perirhinal cortex, and J-K the hippocampus. Panel L is a higher magnification of the tissue in panel F showing specifically stained cell bodies and processes. Filled arrowheads identify the hippocampal CA1 pyramidal cell layer. Open arrowheads identify the upper blade of the dentate granule cell layer. Cortical layers are identified as indicated. Cx: cortex. Scale bars=160 μm (F), 250 μm (I), 400 μm (K), and 20 μm (L).

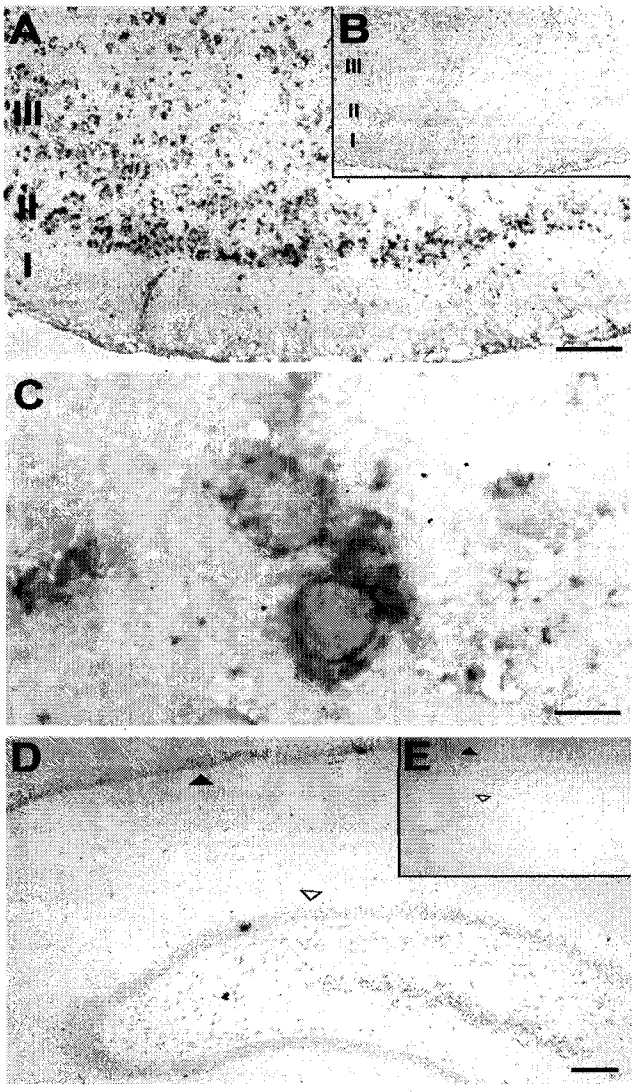

Figure 2.5. p75NTR mRNA expression post-seizure. p75NTR mRNA was detected by in situ hybridization one day after seizure in the entorhinal cortex. (A) p75NTR mRNA detected with an antisense probe in entorhinal cortex one day post-seizure. (B) No signal was detected using the corresponding sense probe on a adjacent section from the same brain. (C) Higher magnification of the tissue in panel A illustrating the cytoplasmic localization of the specific hybridization signal. (D) p75NTR mRNA detected in the hippocampus three days after seizure using the antisense probe, and (E) no signal was detected using the sense probe on a adjacent section from the same brain. Filled arrowheads identify the hippocampal CA1 pyramidal cell layer. Open arrowheads identify the upper blade of the dentate granule cell layer. Cortical layers are indicated. Scale bar=120 μm (A), 12 μm (C), and 200 μm (D).

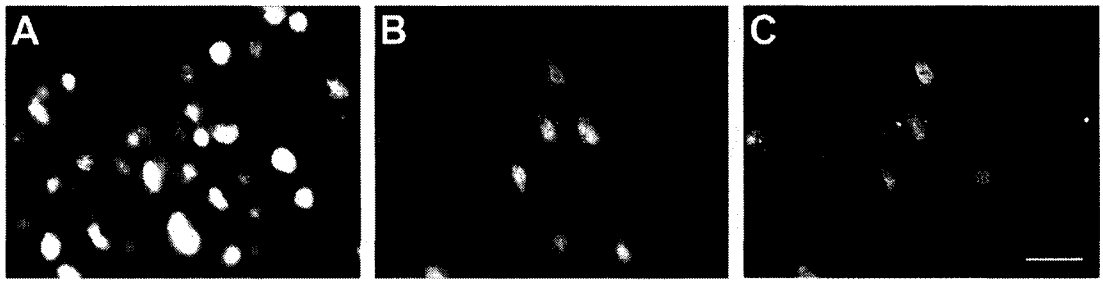

Figure 2.6. Cells undergoing apoptosis are neurons. Triple-immunofluorescence demonstrating colocalization of Hoechst 33258 (A), NeuN (B), and TUNEL (C) in entorhinal cortex three days after pilocarpine-induced seizure. NeuN is neuron-specific, but not a pan-neuronal marker (Mullen et al., 1992), suggesting that some proportion of NeuN negative cells may also be neuronal. Scale bar=20 μm (C).

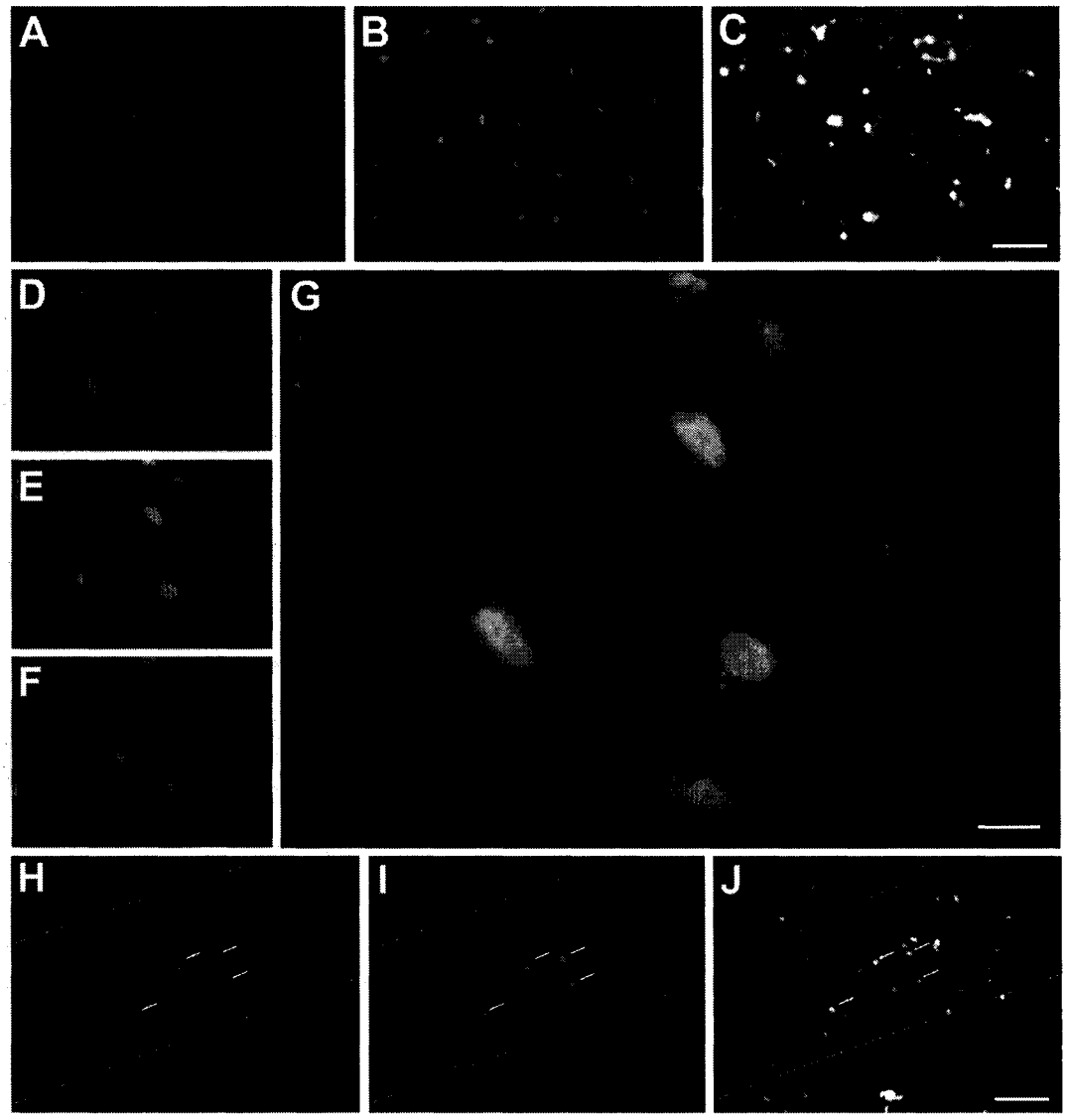

Figure 2.7. p75NTR expression is induced following seizure in neurons undergoing apoptosis. Triple-immunofluorescence of p75NTR (A, D, H), TUNEL (B, E, I) and Hoechst 33258 (C, F, J) in layer III of the entorhinal cortex (between bregma -7.04 and -7.30 – Panels A-F) and within hippocampus (H-J), three days after pilocarpine-induced seizure. Panel G is a composite of panels D-F, with p75NTR immunoreactivity visualized in red (Cy3 conjugated secondary ab), TUNEL reaction in green (FITC), and Hoechst-stained nuclei in blue. In Panels H-J, the dashed line indicates the boundary of the hippocampal CA1 layer and the white bars indicate cells containing pyknotic nuclei. Scale bar=50 μm (C, J), 10 μm (G).

PREFACE TO CHAPTER 3

The study presented in Chapter Two suggests that p75NTR plays a pro-apoptotic role after seizure. To determine if p75NTR directly contributes to neuronal death in this model, we analyzed the response of the p75NTR mutant mouse to seizure-induced damage. To our surprise, we found that the genetic background of this mouse was incompatible with the pilocarpine model, and since then, have been breeding the p75NTR mutant mouse in a compatible strain. This part of the study is currently ongoing.

The second objective of this thesis was to identify the signaling mechanisms mediated by p75NTR. Because the ligand dependency of p75NTR is still unresolved, we have used recombinant adenoviruses encoding various isoforms of p75NTR to constitutively activate p75NTR signaling cascades in a variety of cellular circumstances *in vitro*. We observed that overexpression of p75NTR induced cell death, suggesting that these results reflect our *in vivo* findings. However, when we analyzed the effects of p75NTR on survival pathways, we also have found that under certain circumstances, p75NTR can promote survival through the activation of the PI3-K/Akt pathway.

CHAPTER 3

THE P75 NEUROTROPHIN RECEPTOR (P75NTR) ACTIVATES AKT (PROTEIN KINASE B) THROUGH A PHOSPHATIDYLINOSITOL 3-KINASE-DEPENDENT PATHWAY

The p75 neurotrophin receptor (p75NTR) activates Akt (protein kinase B) through a phosphatidylinositol 3-kinase-dependent pathway.

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ABSTRACT

The Akt kinase plays a crucial role in supporting Trk-dependent cell survival whereas the p75 neurotrophin receptor (p75NTR) facilitates cellular apoptosis. The precise mechanism that p75NTR uses to promote cell death is not certain but one possibility is that p75NTR-dependent ceramide accumulation inhibits PI3-kinase-mediated Akt activation. To test this hypothesis, we developed a system for examining p75NTR-dependent apoptosis and determined the effect of p75NTR on Akt activation. Surprisingly, p75NTR increases, rather than decreases Akt phosphorylation in a variety of cell types, including human Niemman-Pick fibroblasts which lack acidic sphingomyelinase activity. The p75NTR expression level required to elicit Akt phosphorylation was much lower than that required to activate the JNK pathway or mediate apoptosis. We show that p75NTR-dependent Akt phosphorylation was independent of TrkA signaling, required active PI3-K and was associated with increased tyrosine phosphorylation of p85 and Shc and with reduced cytosolic tyrosine phosphatase activity. Finally, we show that p75NTR expression increased survival in cells exposed to staurosporine or subjected to serum withdrawal. These findings indicate that p75NTR facilitates cell survival through novel signaling cascades that result in Akt activation.

INTRODUCTION

The neurotrophins are a family of growth factors involved in the survival, development, and death of specific populations of neurons and non-neuronal cells. Nerve growth factor (NGF), the prototypic neurotrophin, is the best characterized member of this family that in mammals also includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5) (Lewin and Barde, 1996). The signal transduction systems that mediate the diverse biological functions of the neurotrophins are initiated by two categories of cell surface receptors; the Trk receptors and the p75 neurotrophin receptor (p75NTR).

One of the main survival pathways for neuronal cell survival is mediated by phosphatidylinositol 3-kinase (PI3-K) and involves activation of the Akt, serine/threonine kinase (Dudek et al., 1997). Increased PIP3 production results primarily from relocalization of PI3-K from cytosol to a juxtamembrane location that provides access to PIP substrates. This redistribution of PI3-K requires the association of the SH2 domain within the p85 regulatory subunit of PI3-K with phosphorylated tyrosines present on activated cell surface receptors or on receptor-associated adaptor proteins (reviewed in Kaplan and Miller, 2000). Accumulation of PIP_3 and its phospholipid phosphatase product phosphatidylinositol 3,4-biphosphate in the plasma membrane creates docking sites for the pleckstrein homology domains of phosphoinositide-dependent kinase 1 (PDK1) and Akt. Phosphorylation of Akt on threonine 308 by PDK1 followed by autophosphorylation on serine 473 activates Akt (Bellacosa et al., 1998; Toker and Newton, 2000) and allows the enzyme to facilitate survival by phosphorylation downstream substrates that may include Bad, Caspase 9, Forkhead family members, IKK1, and GSK-3 (Datta et al., 1997; Brunet et al., 1999; Kennedy et al., 1999; Fujita et al., 1999; Ozes et al., 1999; Romashkova and Makarov, 1999; Tang et al., 2000).

p75NTR binds all neurotrophins with similar affinity and is a member of the tumor necrosis factor receptor (TNFR) superfamily (Barker, 1998; Barret, 2000). Current data suggests that the main physiological functions of p75NTR are to regulate Trk receptor activation and signaling (Barker and Shooter, 1994; Verdi et al., 1994; Ryden et al., 1997;

Brennan et al., 1999; Bibel et al., 1999) and to activate Trk-independent signal transduction cascades involving sphingomyelinase (Dobrowsky et al., 1994, 1995; Brann et al., 1999), NF-κB (Carter et al., 1996; Bhakar et al., 1999; Khursigara et al., 1999), and JNK (Aloyz et al., 1998; Bamji et al., 1998; Yoon et al., 1998). Several findings indicate that NGF binding to p75NTR can initiate a cell death cascade in some cell types. For example, NGF treatment of embryonic retinal cells or postnatal oligodendrocytes which express p75NTR but not TrkA increases cellular apoptosis (Frade et al., 1996; Casaccia-Bonnefil et al., 1996; Frade and Barde, 1999). The precise signaling pathway(s) used by p75NTR to activate cell autonomous death cascades remain unclear but may involve activation of caspase-1, -2 and –3 (Gu et al., 1999) as well as cyclin-dependent kinases (CDKs) (Frade, 2000). A number of cytosolic proteins that interact directly with the p75NTR intracellular domain have been identified, including TRAFs (Khursigara et al., 1999; Ye et al., 1999b), Caveolin (Bilderback et al., 1997), SC-1 (Chittka and Chao, 1999), NRIF (Casademunt et al., 1999), FAP-1 (Irie et al., 1999), NADE (Mukai et al., 2000), RhoA (Yamashita et al., 1999) and NRAGE (Salehi et al.. 2000) but linking each of these to precise p75NTR signaling cascades remains a major challenge.

Activation of cell death cascades can result from suppression of signaling pathways that normally support survival. In some systems, sphingomyelinase activation results in a ceramide-dependent decrease in the generation of PIP_3 and a subsequent reduction in Akt activity (Zundel and Giaccia, 1998; Zhou et al., 1998) and in others, ceramide reduces Akt activity through specific dephosphorylation of serine 473 (Schubert et al., 2000). Since p75NTR activates sphingomyelinase in a neurotrophin-dependent manner, we have determined if p75NTR activation can suppress Akt and thereby facilitate apoptosis. Our results show that p75NTR does indeed regulate Akt but contrary to our expectations, we find that p75NTR increases Akt activation through a Trk-independent pathway that requires PI3-K and show that p75NTR expression suppresses apoptosis. Although high levels of p75NTR will mediate cell death, the p75NTR expression level required to elicit Akt phosphorylation are much lower than those required to activate the JNK pathway or mediate apoptosis. The effect of p75NTR on Akt correlates with increased tyrosine phosphorylation of the p85 regulatory subunit of PI3-K and of Shc adaptor proteins,

suggesting that PTPase inhibition may play a role in this effect. Consistent with this, p75NTR expression results in reduced cytosolic tyrosine phosphatase activity. These data indicate that a physiological role of p75NTR is to enhance cellular survival through an Akt dependent pathway.

EXPERIMENTAL PROCEDURES

Materials. NGF was purchased from Collaborative Research (Bedford, MA), cell culture reagents were from Bio-Whittaker (Walkersville, MD), and all other reagents were from either Sigma (St. Louis, MO), ICN Biochemicals (Costa Mesa, CA), or Calbiochem (San Diego, CA) unless otherwise indicated.

Preparation of recombinant adenoviruses. pAd-CMV5-F1 containing full-length rat p75NTR (Radeke et al., 1987), the p75NTR intracellular domain (Majdan et al., 1997) or the p75NTR intracellular domain modified to contain an N-terminal myristoylation tag derived from Hck, a Src-related kinase (Robbins et al., 1995), were cotransfected with replication-defective adenoviral DNA (Quantum Biotechnologies, Laval, Québec, Canada) into 293A cells. Crude viruses derived from viral plaques were used to infect 293A cells. p75NTR expression was confirmed by immunoblot analysis, and positive plaques were repurified twice by limiting dilution. Recombinant adenoviruses were amplified in 293A cells, purified on sucrose gradients, and titered by plaque assay in 293A cells. Control recombinant adenoviruses expressing b-galactosidase (LacZ) or green fluorescent protein (GFP) were generated using the same viral backbone and purification techniques as for the p75NTR viruses.

Cell culture. The rat pheochromocytoma cell lines PC12 and PC12nnr5 were maintained in 7.5% CO2 at 37 °C in Dulbecco's modified Eagle's medium (DMEM) with 5% bovine calf serum (BCS), 5% horse serum, 2 mM L-glutamine, and 100 µg/ml penicillin/streptomycin. Normal and Niemann-Pick human fibroblasts (obtained from NIGMS/Human Genetic Mutant Cell Repository, Camden, NJ), MG87-3T3, HELA, COS7 and A875 cells were maintained in DMEM containing 10% BCS. Doxycycline-inducible p75NTR-expressing MG87-3T3 fibroblasts (TIMp75-3) were produced and maintained as described previously (Bhakar et al., 1999).

Immunoblotting. Immunoblotting for total and phosphorylated proteins was performed using rabbit polyclonal antibodies from New England Biolabs (Beverly, MA) or from Upstate Biotechnology (Lake Placid, NY). p75NTR immunoreactivity was detected using

p75NTR-B1, a rabbit polyclonal antibody directed against a GST-fusion protein containing amino acids 276-425 of the intracellular domain of rat p75NTR (BABCO, Berkeley, CA) (Majdan et al., 1997; Roux et al., 1999). Protein content from cell lysates was normalized using the BCA assay (Pierce, Rockford, IL), and 10 to 25 μg of protein was solubilized in Laemmli sample buffer (Laemmli, 1970), separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted to nitrocellulose. Blocking and secondary antibody incubations of immunoblots were performed in TBST (10 mM Tris (pH 7.4), 150 mM NaCl, and 0.2% Tween 20), supplemented with 5% (w/v) dry skim milk powder. Primary antibody incubations were performed in TBST supplemented with 5% bovine serum albumin (BSA). For 4G10, 2% BSA (w/v) was used for the blocking step. HRP-conjugated donkey anti-rabbit IgG, HRP-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA), or HRP-conjugated protein-A were used at a dilution of 1:5000. Immunoreactive bands were detected using enhanced chemiluminescence (ECL) according to the manufacturer's instructions (NEN Life Science Products, Boston, MA).

Transfection and Subcellular Fractionation. COS7 cells were transfected with a control plasmid or with plasmids encoding the p75NTR intracellular domain or p75NTR intracellular domain modified to contain a myristoylation tag. Forty-eight hours after tranfection, cells were scraped from plates in cold phosphate-buffered saline (PBS), centrifuged for 5 minutes at 2000 g and then resuspended in 15 ml HES buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 255 mM sucrose, 10 μg/ml leupeptin, 25 μg/ml aprotinin, and 1 mM PMSF). Cells were homogenized in a glass on teflon homogenizer with 10 strokes at 1200 rpm and then triturated twice using a 25 gauge needle. An aliquot was set aside as initial lysate. The lysate was centrifuged at 19000g for 20 minutes and the resulting pellet was designated membrane and the supernatant was designated cytosol. All fractions were resuspended in HES containing 1% NP40, analyzed for protein concentration and equivalent amounts were analyzed by SDS-PAGE and immunoblot.

MTT survival assays. Analysis of cell survival was performed using 3(4,5-dimethylthiozol-2-yl)2,5-diphenyltetrazolium bromide (MTT), which was added at a final

concentration of 1 mg/ml for 4 hours following a 48 hour infection. The reaction was ended by the addition of 1 volume of solubilization buffer (20% SDS, 10% dimethylformamide, and 20% acetic acid). After overnight solubilization, specific and non-specific absorbance were read at 570 and 630 nm, respectively. Each condition was tested in triplicate and results were analyzed for statistical significance by multiple analysis of variance.

Immunoprecipitation. Twenty-four hours after infection, cells were washed in cold Tris-buffered saline, and lysed in NP-40 lysis buffer (10 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol, 1% NP-40, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM sodium orthovanadate). Immunoprecipitation was performed at 4 °C using polyclonal anti-panTrk 203 (gift of David Kaplan, Montreal Neurological Institute, Montreal, Canada), polyclonal anti-Shc (gift of Jane McGlade, University of Toronto, Toronto, Ontario), or polyclonal anti-p85 (Upstate Biotechnology). Complexes were precipitated using 45 μl of protein A-Sepharose (Pharmacia Amersham, Piscataway, NJ) which was added for 90 minutes at 4°C and then subjected to multiple washes. For wheat germ agglutinin (Phamacia-Amersham) precipitation, beads were added to NP-40-extracted protein samples for 2 hours at 4°C, followed by centrifugation and multiple washes. Samples were lysed in Laemmli sample buffer and analyzed by immunoblotting as described above.

Apoptotic assays. Apoptotic cell death was quantified using annexin V binding and FACS analyses. Briefly, cells were harvested using PBS with 2 mM EDTA, and washed twice in PBS supplemented with 2% BCS. After the last wash, cells were resuspended in 0.1 ml PBS containing 1 μg/ml FITC-conjugated annexin V (BD-Pharmingen, Franklin Lakes, NJ) and incubated 15 minutes in the dark at room temperature. 0.3 ml of PBS was added to each tube, and cells were analyzed on a FACScan flow cytometer (Becton Dickenson, Franklin Lakes, NJ). For FACS analysis of cells with sub-G1 DNA content, cells were harvested and resuspended in 50% ethanol/PBS, and left on ice for 15 minutes. An underlayer of 1 volume cold BCS was added and cells were spun at 250 g for 5 minutes. Cells in the resulting pellet were resuspended in blocking buffer consisting of PBS

containing 2% BSA and 2% BCS and then incubated for 30 minutes on ice. Anti-p75NTR-B1 was added at a dilution of 1:500 and the incubation continued for an additional 30 minutes. Cells were washed three times in blocking buffer, then incubated 30 minutes in blocking buffer supplemented with a 1:500 dilution of FITC-conjugated goat anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA). Cells were washed three times and resuspended in 0.1 ml PBS with 0.1 mg/ml RNase A for 15 minutes at room temperature. 0.3 ml of PBS with 25 μg/ml propidium iodide was added to the cells, incubated for 15 minutes, and then analyzed on a FACScan. Each condition was tested in triplicate and results were analyzed for statistical significance by multiple analysis of variance.

Protein-tyrosine phosphatase assays. Twenty-four hours after infection, PC12nnr5 cells were washed twice in cold HEPES-buffered saline to remove free phosphate, and then harvested in 1 ml of suspension buffer (50 mM HEPES, 150 mM NaCl, 2 mM EDTA, 0.25 M sucrose, 1 mM DTT, 10 μg/ml leupeptin, 25 μg/ml aprotinin, and 1 mM PMSF), sonicated, and spun at 1000g for 5 minutes to remove intact mitochondria and nuclei. Cytosolic and membrane fractions were separated by spinning at 100000g for 60 minutes at 4°C. The membrane pellet was resuspended for 30 minutes in 1 ml of suspension buffer supplemented with 1% Triton X-100, and then spun at 1000 g for 5 minutes to remove insoluble contaminants. Endogenous phosphate was removed from membrane and cytosolic fractions by buffer exchange in Centricon 10 columns (Amicon, Beverly, MA). Protein assays were performed on resulting fractions and the tyrosine-phosphorylated substrate END(pY)INASL was added to 25 μg of protein to a final concentration of 100 μM. The reaction was stopped after 30 minutes at room temperature by the addition of 1 volume of stopping solution (0.02% malachite green, 0.5% ammonium molybdate tetrahydrate, and 0.1% polyvinyl alcohol). Absorbance at 630 nm was read after a 60 minute incubation. As control, sodium orthovanadate was added to a parallel set of samples to ensure the presence of specific tyrosine phophatase activity. Background absorbance for parallel samples that did not receive the substrate was subtracted as contaminating free phosphate. Each condition was tested in triplicate and results were analyzed for statistical significance by multiple analysis of variance.

RESULTS

Recombinant adenoviruses encoding p75NTR activate JNK and induce cell death of PC12nnr5 cells. The actions of p75NTR are complex and not well understood. This is due in part to the lack of cellular systems in which p75NTR-dependent signaling events can be reliably observed. Previous work from our group (Majdan et al., 1997) and others (Boldin et al., 1995) has shown that overexpression of the intracellular domain of TNF receptor superfamily members will constitutively activate downstream signaling pathways. To reliably activate p75NTR signaling cascades in a variety of cellular circumstances, recombinant adenoviruses encoding either full length p75NTR (p75NTR), the intracellular domain of p75NTR (p75ICD), or a myristoylated form of the intracellular domain of p75NTR which is targeted to the plasma membrane (p75mICD; Figure 3.1B) were produced. Recombinant adenovirus encoding LacZ or GFP were used as controls for all studies. To validate signaling properties of these recombinant p75NTR viruses, we initially tested their effects on survival of PC12nnr5 cells (which express endogenous p75NTR but not TrkA). Figure 3.1A shows that LacZ virus had no effect on cell survival whereas viruses encoding p75NTR or p75ICD were cytotoxic at multiplicities of infection (MOI) of 200. Overexpression of p75mICD induced cytotoxicity at lower MOIs than p75NTR or p75ICD, suggesting that membrane localization of the intracellular domain may be important for p75NTR-dependent apoptosis. p75NTR-dependent cell death was not an artifact of protein overexpression or viral infection since equivalent quantities of LacZ adenovirus had no effect on cell survival. Several reports have shown that p75NTR activation can lead to c-Jun N-terminal kinase (JNK) activation (Aloyz et al., 1998; Bamji et al., 1998; Yoon et al., 1998) and consistent with this, we found that expression of p75mICD (Figure 3.1C), p75ICD, and p75NTR increase phosphorylation of JNK on threonine 183 and tyrosine 185 and increase c-Jun phosphorylation on serine 73. Equivalent levels of LacZ virus did not alter JNK or c-Jun phosphorylation (data not shown). The level of c-Jun protein was also elevated at high p75NTR expression levels, likely due to autoregulation of c-Jun transcription (Cochran, 1993) during the relatively long infection period (48 hours). To demonstrate the specificity of the JNK activation initiated by p75NTR, we analyzed phosphorylation of the transcription factor CREB, which lies downstream of PKA, and assessed the

phosphorylation status of MKK3/6, which activates the p38 MAPK. Neither CREB or MKK3/6 phosphorylation was altered by p75NTR expression (Figure 3.1C and data not shown). These results show that p75NTR signaling results in specific activation of the JNK pathway and promotes cell death, and therefore demonstrate that the recombinant p75NTR adenoviruses showed expected constitutive signaling properties.

p75NTR activates Akt. Recent reports have shown that ceramide inhibits PI3-K activity, reduces cellular PIP3 levels and thereby inhibits Akt activity (Zundel and Giaccia, 1998; Zhou et al., 1998). p75NTR activates SMase upon neurotrophin binding (Dobrowsky et al., 1994, 1995) and we hypothesized that p75NTR could facilitate apoptosis by attenuating PIP3 production and reducing Akt activity. To determine if p75NTR altered Akt activity, PC12nnr5 cells were infected with recombinant virus expressing each of the three p75NTR isoforms or LacZ and analyzed for Akt activation using phospho-specific antibodies directed against Akt serine 473 (Ser473), an Akt autophosphorylation site that correlates with Akt kinase activity (Toker and Newton, 2000). Surprisingly, expression of full length p75NTR, p75ICD (Figure 3.2) or p75mICD (see Figure 3.3B, 3.6B), resulted in significant increases in the phosphorylation of Akt on Ser473 whereas control adenovirus expressing LacZ had no effect on Akt Ser473 phosphorylation. As noted above, relatively high levels of p75NTR expression were required to observe JNK activation, c-Jun phosphorylation and apoptosis but much lower levels of p75NTR expression were sufficient to induce Akt phosphorylation. p75NTR-mediated reduction in Akt phosphorylation was never observed, even in cells exposed to high titers of p75NTR recombinant adenovirus. The effect of ligand binding to p75NTR on Akt activation in the presence and absence of virus was also tested; treatment of control and infected cells with NGF, BDNF or NT-3 at various concentrations and timecourses had no effect on the p75NTR adenovirus-induced phosphorylation of Akt in PC12nnr5 cells (data not shown). Therefore, these data indicate that p75NTR expression activates Akt in a ligand-independent manner.

p75NTR-induced Akt phosphorylation is Trk-independent and does not require acidic SMase. The TrkA receptor is a potent activator of Akt and previous results from ourselves

and others have shown that p75NTR can increase the response of TrkA to limiting NGF concentrations (Barker and Shooter, 1994; Verdi et al., 1994; Mahadeo et al., 1994). It was therefore possible that the p75NTR-dependent Akt phosphorylation observed is secondary to activation of low levels of TrkA that may be present in PC12nnr5 cells. To address this, PC12nnr5 cells were first examined to determine if they expressed NGF-responsive TrkA. Figure 3.3A shows that activated TrkA is not detected in PC12nnr5 cells under conditions in which trkA is readily detected in normal PC12 cells. We then tested K252a, a specific TrkA inhibitor, for its ability to block p75NTR-mediated Akt phosphorylation, in this case using recombinant virus encoding p75ICD and p75mICD which are incapable of binding ligand. LacZ, p75ICD, and p75mICD adenoviruses were used at 25 MOI for these experiments since this infection level activates Akt (Figure 3.2), but does not induce cell death (Figure 3.1). Figure 3.3B shows that K252a completely blocks NGF-mediated Akt phosphorylation in PC12 cells but has no effect on p75ICD and p75mICD-induced Akt phosphorylation in either PC12nnr5 cells or PC12 cells. Therefore, p75NTR-mediated activation of Akt occurs independently of TrkA signaling.

PC12nnr5 cells express endogenous p75NTR and it is conceivable that p75NTR overexpression may mediate an increase in Akt phosphorylation by disrupting signaling from endogenous p75NTR. To address this, the effects of p75NTR on Akt phosphorylation were determined in a variety of cell types that do not express p75NTR or TrkA. p75NTR expression in 3T3-MG87 fibroblasts (100 MOI; Figure 3.3C) or HELA cells (data not shown) resulted in increased Akt phosphorylation similar to that observed in PC12nnr5 cells. These results indicate that the effect of p75NTR on Akt phosphorylation does not involve disruption of endogenous p75NTR signaling.

Recombinant adenovirus have significant effects on cellular physiology and we therefore sought additional means to confirm that p75NTR expression increases Akt activation. TIMp75-3 is a MG87-derived cell line in which expression of p75NTR is tightly regulated through the addition of doxycycline (Bhakar et al., 1999) and therefore could be used to confirm that p75NTR overexpression results in Akt phosphorylation. Cells incubated with 2.5 μg/ml doxycycline for 24 hours showed the expected increase in

p75NTR expression and this correlated with a rise in Akt Ser473 phosphorylation (Figure 3.3D). Therefore, two independent means of p75NTR expression result in Akt activation in different cell types. The relatively modest increase in Akt phosphorylation observed likely reflects the fact that only a subpopulation of TIMp75-3 cells show robust doxycycline-induced p75NTR expression (see Figure 3.5B).

Neurotrophin binding to p75NTR increases SMase activity and one possible explanation for the effect of p75NTR on Akt is that unliganded p75NTR functionally inactivates cellular SMase, thereby reducing cellular ceramide levels and causing a consequent increase in PI3-K activity, PIP3 levels and Akt phosphorylation. To address this, we compared the effects of p75NTR overexpression on Akt activation in normal and in Niemann-Pick fibroblasts. Niemann-Pick fibroblasts are deficient in acidic SMase, which is the only form of SMase activated by p75NTR in PC12 cells (Rick Dobrowsky – personal communication). Figure 3.4 shows that p75NTR and p75ICD induce Akt phosphorylation in primary human fibroblasts derived from control and Niemann-Pick patients, suggesting that inactivation of acidic SMase activation is not involved in this p75NTR response.

Expression of p75NTR increases cellular survival. These data are consistent with the hypothesis that p75NTR produces biphasic autonomous responses. High levels of p75NTR signaling result in JNK activation, c-Jun phosphorylation and cell death, and lower levels of p75NTR signaling induce alternative pathways that include Akt activation and survival. To examine this, PC12nnr5 cells were infected with LacZ, p75NTR or p75ICD adenovirus (all at 25 MOI), exposed to 0.5 μM staurosporine for 18 hours and levels of cellular apoptosis were determined by assessing Annexin V binding by FACS analysis. Figure 3.5A shows that infection of PC12nnr5 cells with p75NTR and p75ICD viruses significantly reduces the incidence of apoptosis compared to DMSO-treated cells or cells infected with control LacZ virus.

To extend these results to other cellular models, TIMp75-3 cells were analyzed for p75NTR-mediated survival properties. TIMp75-3 cells were produced from the MG87

cell line which undergoes rapid cell death in the absence of serum-derived growth factors. TIMp75-3 cells were treated with 2.5 μg doxycycline for 36 hours (to induce p75NTR expression), and then deprived of serum for 18 hours. FACS analysis was performed to assess p75NTR expression and apoptosis. Figure 3.5B clearly shows that the TIMp75-3 population which expresses p75NTR is much less susceptible to cell death induced by serum deprivation, indicating that p75NTR promotes survival under these circumstances.

Activation of Akt by p75NTR requires active PI3-kinase. To begin to determine the mechanisms used by p75NTR to activate Akt, the effect of PI3-K inhibitors on p75NTR-induced Akt phosphorylation was examined. LY294002 and wortmannin were first tested for their ability to block Akt phosphorylation induced by NGF in PC12 cells; Figure 3.6A shows, as expected that both inhibitors efficiently reduce NGF-induced Akt activation. Both inhibitors also completely block Akt phosphorylation induced by the p75mICD (Figure 3.6B), p75ICD, or p75NTR (data not shown) recombinant adenoviruses in PC12nnr5 cells and in A875 human melanoma cells. Therefore, PI3-K activity is required for p75NTR-mediated activation of Akt.

p75NTR expression increases tyrosine phosphorylation of p85 and Shc. Activated receptor tyrosine kinases increase PIP_3 production largely by allowing PI3-K proximity to the plasma membrane through SH2-mediated interactions of the p85 regulatory subunit with receptor or with receptor-associated adaptor proteins such as Shc. To determine if Shc could contribute to the p75NTR-mediated increase in Akt phosphorylation, the tyrosine phosphorylation level of immunoprecipitated Shc was analyzed in cells expressing p75ICD or control adenovirus. Figure 3.7A shows that phosphorylation of all three Shc isoforms (p66, p52, and p46) was increased relative to controls and that this effect is potentiated when tyrosine phosphatase activity (PTPase) is inhibited by treating the cells with sodium orthovanadate for one hour prior to harvesting. A 160 kDa protein that coimmunoprecipitated with Shc also showed increased tyrosine phosphorylation in response to p75NTR overexpression; this protein is likely SHIP, a 160 kDa lipid phosphatase that binds activated Shc with high affinity (Liu et al., 1997). Experiments were also performed to determine if the p85 subunit of PI3-K showed a similar p75NTR-

dependent increase in tyrosine phosphorylation. Figure 3.7B shows that full-length p75NTR, but not GFP, strongly increases tyrosine phosphorylation of p85 (Figure 3.7B). These results indicate that p75NTR activates a PI3-K/Akt pathway by increasing tyrosine phosphorylation of adaptor proteins that relocalize PI3K to the plasma membrane. To determine if the phosphotyrosine content of any cell surface proteins are altered by p75NTR expression, PC12nnr5 cells were infected with recombinant p75ICD adenovirus and cell surface glycoproteins were precipitated using Sepharose-conjugated wheat germ agglutinin and assayed for phosphotyrosine content by 4G10 immunoblot. Figure 3.7C shows that p75NTR expression specifically increases tyrosine phosphorylation of a 120 kDa protein, particularly in cells pretreated with sodium orthovanadate.

p75NTR decreases protein-tyrosine phosphatase activity. Our results show that p75NTR expression increases tyrosine phosphorylation of several proteins, particularly in the presence of orthovanadate, a non-specific competitive PTPase antagonist. p75NTR has no intrinsic enzymatic activity and its effect on phosphorylation must be due to regulatory interactions with proteins capable of increasing kinase activity, decreasing PTPase activity or some combination of the two. A physical interaction between p75NTR and the FAP PTPase has recently been reported (Irie et al., 1999), raising the possibility that cellular tyrosine phosphatase activity could be modulated by p75NTR. To test this, PC12nnr5 cells were infected with either full-length p75NTR, p75ICD or LacZ adenovirus at 25 MOI, subjected to subcellular fractionation and analyzed for cytosolic and membrane-bound PTPase activity. Figure 3.8 shows that expression of p75NTR or p75ICD results in a significant decrease in cytosolic PTPase activity whereas membrane-associated PTPase activity was unchanged by p75NTR expression. These results indicate that p75NTR is capable of inhibiting cytosolic PTPase(s) and suggest that increases in the phosphotyrosine content mediated by p75NTR may be secondary to alterations in PTPase activity.

DISCUSSION

We have demonstrated that p75NTR expression leads to Trk-independent, PI3-K-dependent Akt phosphorylation. High levels of p75NTR expression increase JNK and c-Jun phosphorylation and promote apoptosis, yet lower p75NTR expression levels are associated with activation of Akt and suppression of apoptosis induced by distinct stressors. p75NTR expression levels that potentiate Akt phosphorylation and survival increase the phosphotyrosine content of several cellular proteins, including p85 and Shc, suggesting that p75NTR affects the activity of tyrosine kinases or PTPases. Consistent with this, we demonstrate that p75NTR expression is associated with a decrease in cytosolic PTPase activity.

Many studies have demonstrated that p75NTR can facilitate apoptosis. Our earlier work has shown that overexpression of the p75NTR intracellular domain within neurons of transgenic mice results in dramatic loss of peripheral and central neurons (Majdan et al., 1997) and Barde's group has shown that embryonic retinal cells which express p75NTR undergo cell death that can be prevented by the application of antibodies against either NGF or the p75NTR extracellular domain (Frade et al., 1996). Genetically altered mice rendered null at either p75NTR or NGF loci show deficits in developmental apoptosis within retina and spinal cord (Frade and Barde, 1999) and p75NTR can facilitate apoptosis of cultured rat oligodendrocytes (Casaccia-Bonnefil et al., 1996) and sympathetic neurons (Bamji et al., 1998). The precise pathways that p75NTR activates to induce apoptosis are unclear, but JNK, caspase activation and increased p53 levels have been observed in some systems (Aloyz et al., 1998; Bamji et al., 1998; Yoon et al., 1998; Gu et al., 1999). To reliably activate p75NTR signaling cascades, we created recombinant adenoviruses that encode either full length p75NTR, or the p75NTR intracellular domain. As expected from our earlier work in transgenic mice (Majdan et al., 1997), adenovirus mediated overexpression of p75ICD resulted in cellular apoptosis that was associated with increased JNK activity and c-Jun phosphorylation. Expression of p75NTR or myristoylated p75ICD gave similar results, with the p75mICD proving a particularly potent apoptotic inducer. These reagents will be useful for studies designed to identify specific signaling events in the p75NTR apoptotic cascade.

Neurotrophin binding to p75NTR results in the activation of sphingomyelinase and the production of ceramide (Dobrowsky et al., 1994, 1995; Casaccia-Bonnefil et al., 1996; Blochl and Sirrenberg, 1996; Brann et al., 1999). Ceramide generated by p75NTR activation may inhibit Trk receptor activation (MacPhee and Barker, 1997), activate JNK (Westwick et al., 1995; Casaccia-Bonnefil et al., 1996; Verheij et al., 1998), and affect neuronal differentiation (Brann et al., 1999). In some systems, sphingomyelinase activation results in a ceramide-dependent decrease in PIP_3 production and a subsequent reduction in Akt activity (Zundel and Giaccia, 1998; Zhou et al., 1998) and our initial hypothesis was that p75NTR-dependent ceramide accumulation would suppress PI3-K activity and thereby reduce Akt activation. However, overexpression of p75NTR resulted in ligand-independent activation of Akt in multiple cell types. Indeed, Akt was activated even at p75NTR expression levels that facilitate apoptosis, indicating that when the apoptotic pathway is activated, it can override the pro-survival effect of Akt.

The activation of Akt by p75NTR requires active PI3-K and correlates with increases in the phosphotyrosine content of several proteins, including the adaptor protein Shc, the p85 regulatory subunit of PI3-K, and a 120 kD cell surface protein. The increased phosphotyrosine content of these proteins correlates with reduced cytosolic PTPase activity in the presence of p75NTR. p75NTR-mediated inhibition of a cytosolic PTPase may therefore be a proximal event in the signaling cascade that may allow a Shc/PI3-K complex to associate with the plasmalemma and increase PIP_3 production. Additional studies will be required to identify specific phosphatase(s) inhibited by p75NTR but one candidate is FAP, which physically interacts with p75NTR when overexpressed in 293T cells (Irie et al., 1999). FAP also binds human Fas (Sato et al., 1995) and Fas-mediated apoptosis can be suppressed by FAP (Yanagisawa et al., 1997; Li et al., 2000) through an unknown mechanism. An alternative mechanism that may account for p75NTR-mediated Akt activation involves a link between TRAF proteins and Src kinases. The TRANCE receptor is a member of the TNFR superfamily that activates survival pathways in osteoclasts in part by activating Akt and a recent study has found that a complex of Src kinase and TRAF6 is required for this effect (Wong et al., 1999). p75NTR interacts with several members of the TRAF family, including TRAF6 (Khursigara et al., 1999; Ye et

al., 1999b), raising the possibility that this signaling path may also contribute to a p75NTR-mediated Akt activation. Future experiments specifically examining FAP and Src signaling will be required to reveal the relative contributions of each of these pathways to p75NTR-mediated Akt activation.

The signaling mechanisms employed by p75NTR are not well understood and the relationship of neurotrophin binding to p75NTR action remains unclear. All neurotrophins activate sphingomyelinase when bound to p75NTR (Dobrowsky et al., 1994, 1995), but only NGF is capable of inducing apoptosis and NF-κB activation in most systems (Carter et al., 1996; Casaccia-Bonnefil et al., 1996; Frade et al., 1996). Paradoxically, some studies suggest that the receptor signals apoptosis when free of ligand and that this function is suppressed by ligand binding to p75NTR (Rabizadeh et al., 1993, 1999). For our studies, we produced a group of recombinant adenovirus that would constitutively activate p75NTR signaling and thereby allow us to identify p75NTR cascades irrespective of ligand binding. All of the p75NTR constructs employed specifically activate the JNK pathway and mediate apoptosis when expressed at high levels, indicating that they are capable of activating p75NTR signaling pathways. The high expression levels required to induce apoptosis presumably forces formation of a receptor signaling complex normally obtained in the presence of appropriate ligand (Majdan et al., 1997). It is noteworthy that proximity of the intracellular domain to the plasma membrane appears important for activation of apoptosis by p75NTR since the p75mICD fragment elicits stronger apoptotic signaling than either p75NTR or p75ICD.

Cytotoxic effects are observed when p75NTR is highly overexpressed but much lower expression levels of full length p75NTR and the intracellular domain mutants elicit Akt phosphorylation and enhance survival. The canonical view of p75NTR action is that receptor signaling is activated by ligand binding but recent studies on other TNFR superfamily members suggests an alternative paradigm for p75NTR signaling. Lenardo and colleagues have recently shown that some TNFR superfamily members must pre-assemble into cell surface oligomers before binding ligand (Chan et al., 2000; Siegel et al., 2000). Many investigators have observed oligomeric p75NTR in the absence of ligand

in a variety of preparations (for an early example, see Grob et al., 1995), consistent with the possibility that p75NTR may also pre-assemble into oligomers. With Fas and TNFR1, ligand binding produces a conformational shift that enables specific signaling events, but does not alter the oligomeric nature of the receptor complex. Thus, the role of ligand is to shift the pre-assembled receptor complex to a different signaling mode. All three p75NTR constructs employed in our studies elicited Akt activation when produced at low expression levels but much higher levels of expression were required for apoptotic signaling. We favor the hypothesis that these signaling events reflect distinct p75NTR signaling complexes and that oligomeric p75NTR exists in at least two distinct signaling complexes; in the ligand-free state, p75NTR will constitutively activate survival pathways that involve Akt, and when bound by ligand, it activates signaling pathways that result in cellular apoptosis.

In conclusion, we have identified a novel p75NTR signaling pathway that results in phosphorylation of Akt and enhances cellular survival. These data suggest that the autonomous signaling role of p75NTR may be broader than previously considered, with p75NTR capable of signaling pathways that support survival and death under different cellular circumstances.

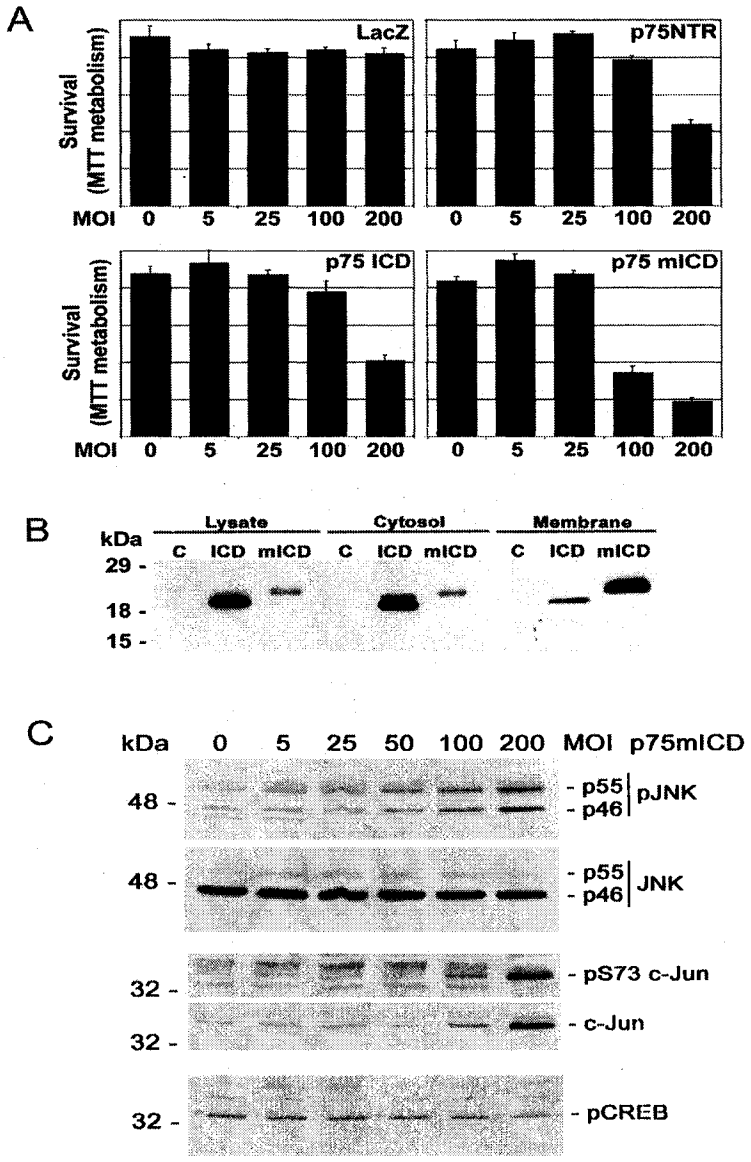

Figure 3.1. Overexpression of p75NTR in PC12nnr5 cells induces cell death. (A) PC12nnr5 cells were infected with recombinant adenoviruses expressing either LacZ, full length p75NTR, the intracellular domain of p75NTR (p75ICD), or myristoylated p75NTR intracellular domain (p75mICD) at increasing multiplicity of infection (MOI) and after 48 hours, were assayed for survival using the MTT assay as described in *Materials and Methods*. (B) The p75mICD is enriched in cell membranes but the p75ICD is cytosolic. COS7 cells were transiently transfected with plasmids encoding either p75ICD or p75mICD and after 48 hours, fractionated as described in *Materials and Methods*. (C) PC12nnr5 cells which were infected and incubated under the same conditions as in (A) were lysed and analyzed for c-Jun (phospho-Ser73), JNK (phospho-Thr183/Tyr185), and CREB (phospho-Ser133) phosphorylation by immunoblotting. Results shown in (A) are represented as the mean (± SEM) of three independent experiments, and the experiment in (C) was repeated twice with similar results.

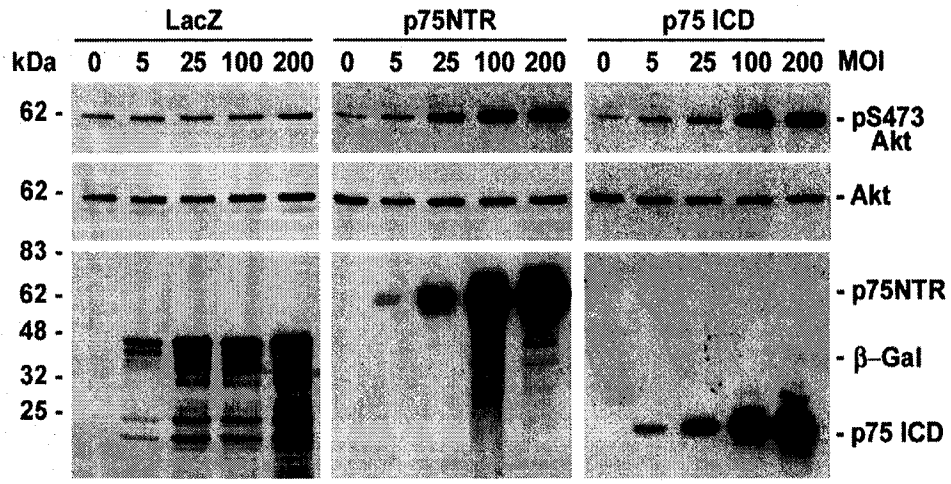

Figure 3.2. Expression of p75NTR in PC12nnr5 cells increases Akt Ser473 phosphorylation. PC12nnr5 cells were infected with recombinant adenovirus expressing either LacZ, full length p75NTR, or the intracellular domain of p75NTR (p75ICD) at different multiplicity of infection (MOI). Cells were harvested 24 hours after infection and analyzed for Akt levels, Akt serine 473 phosphorylation, LacZ expression and p75NTR expression by immunoblotting. Results shown were repeated three times with similar results.

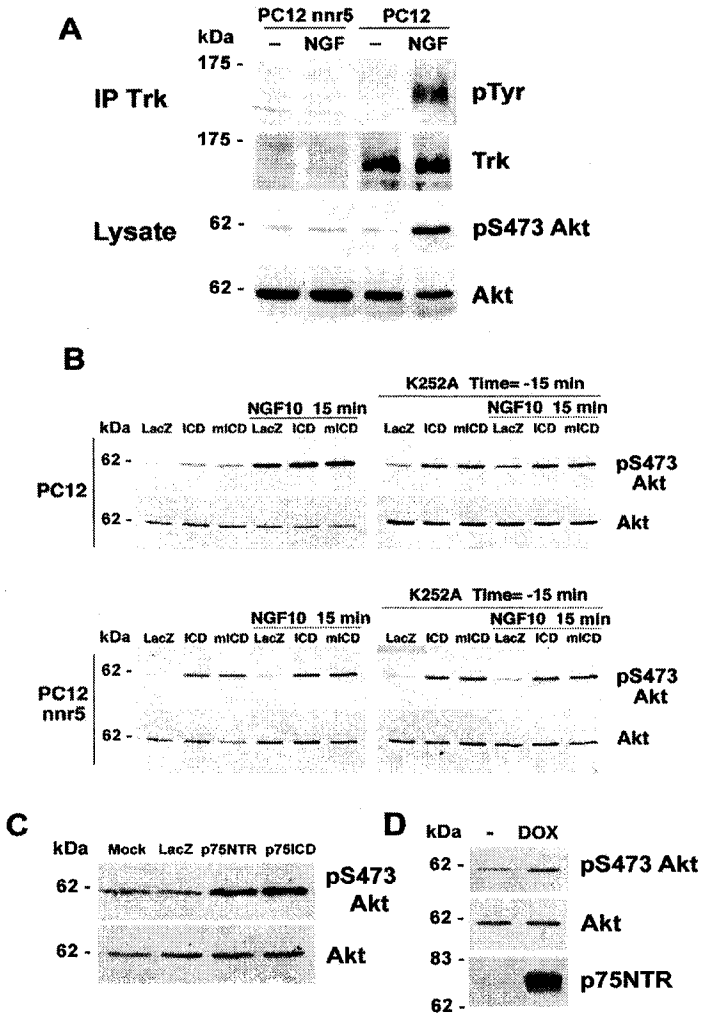

Figure 3.3. p75NTR-induced Akt phosphorylation is Trk-independent. (A) PC12 or PC12nnr5 cells were treated for 15 minutes with 10 ng/ml NGF, lysed in NP40-containing buffer, and Trk receptors were immunoprecipitated using the anti-panTrk 203 antibody. Protein lysates were analyzed by immunoblot for levels of total and phosphorylated Akt, for phosphotyrosine content and TrkA levels within immunocomplexes. (B) PC12 and PC12nnr5 cells were each infected with 25 moi of LacZ, p75ICD or p75mICD adenovirus for 24 hours, then treated with the Trk inhibitor K252a (200 nM) for 30 minutes followed by 50 ng/ml NGF for 15 minutes before harvest. Comparison between the two cell lines shows that the Trk inhibitor K252a effectively inhibits Akt phosphorylation induced by NGF in PC12 cells but has no effect on p75NTR-induced activation of Akt in PC12 cells or in PC12nnr5 cells. (C) 3T3-MG87 cells were infected with 100 moi of recombinant adenovirus encoding p75NTR or p75ICD harvested 24 hours later and analyzed for Akt levels and Akt Ser473 phosphorylation. (D) TIMp75-3 cells were treated with 2.5 μg/ml doxycycline for 24 hours, harvested, and analyzed for Akt levels, Akt Ser473 phosphorylation, and p75NTR expression. Results shown in (A) and (B) were repeated twice, and those in (C) and (D) were repeated three times, all with similar results.

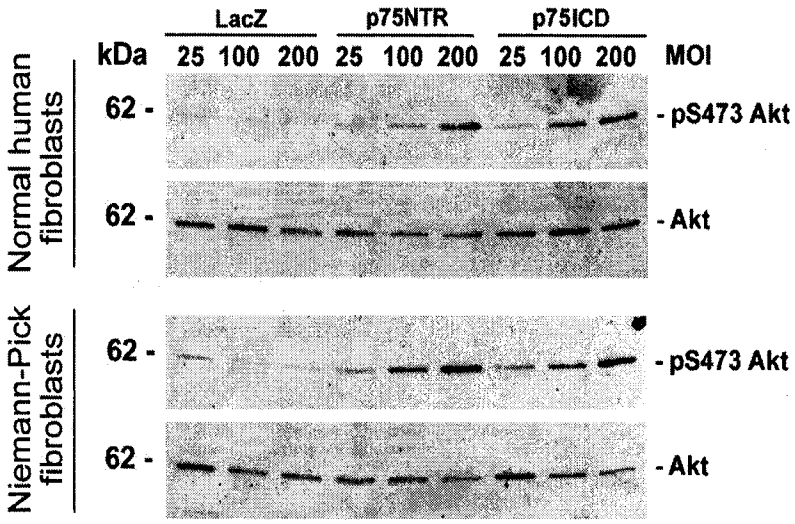

Figure 3.4. p75NTR-mediated Akt phosphorylation does not require acidic sphingomyelinase activity. Overexpression of p75NTR or p75ICD in control human (A) or Niemann-Pick (B) fibroblasts induces Akt phosphorylation. High viral MOIs (100-200) were due to the low infectability of these lines (data not shown). Results shown were repeated twice with similar results.

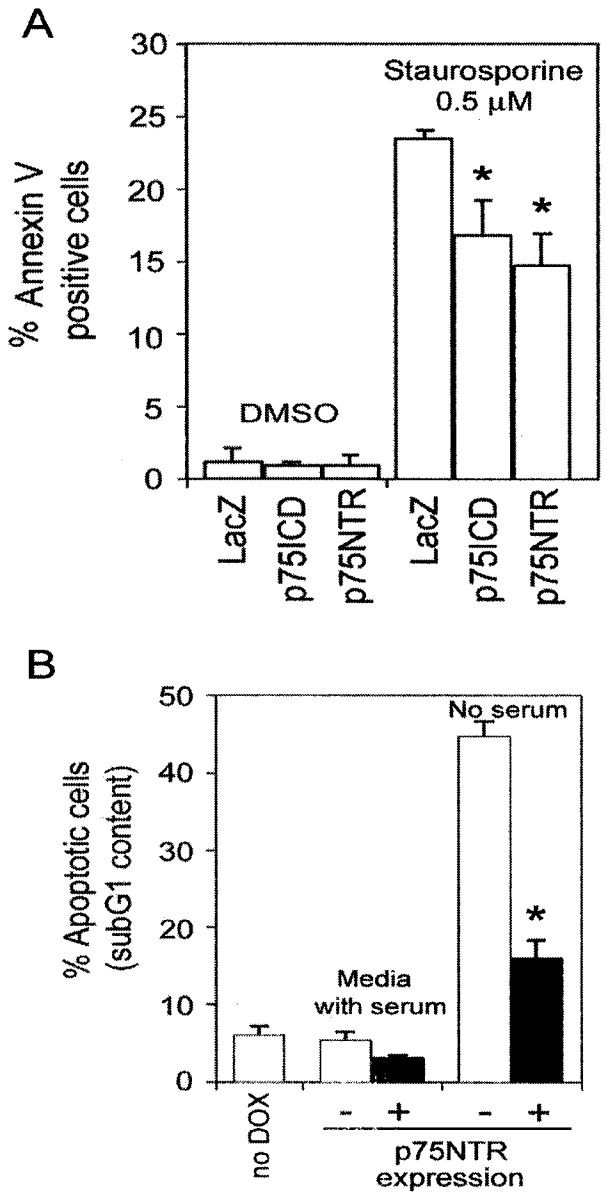

Figure 3.5. Expression of p75NTR facilitates cell survival. (A) PC12nnr5 cells were infected with 25 MOI recombinant adenovirus encoding LacZ, p75ICD or p75NTR, incubated for 24 hours, then treated with 0.5 µM staurosporine or vehicle for 18 hours and analyzed for Annexin V binding. A minimum of 20 000 cells were analyzed for each condition shown. (B) TIMp75-3 cells were treated with 2.5 µg/ml doxycycline for 36 hours, and serum-deprived for 18 hours. Flow cytometry was used to determine p75NTR expression levels (using antibody p75NTR-B1) and to determine the proportion of cells with sub-G1 DNA content using propidium iodide staining. A minimum of 30 000 cells were analyzed by flow cytometry for each condition. Statistically significant differences determined by MANOVA are indicated with asterisks in panel A and B ('*' = p <0.05; '**' = p <0.001) . Data in Panels A and B represents the mean (± SEM) of three independent experiments.

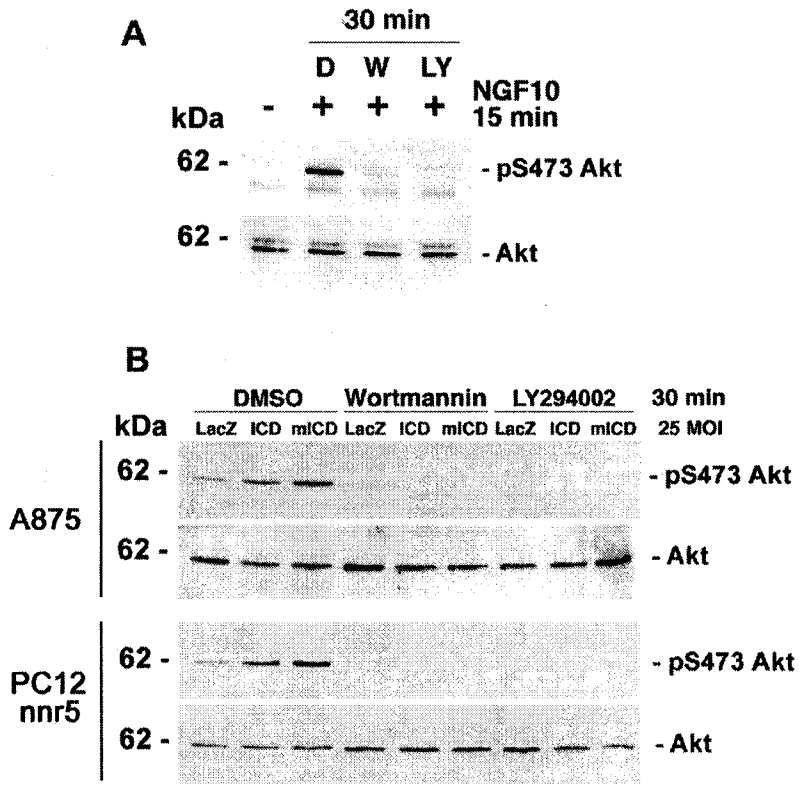

Figure 3.6. PI3-kinase activity is required for p75NTR-induced phosphorylation of Akt. (A) PC12 cells were treated with 50 ng/ml NGF and with wortmannin (100 nM) or LY294002 (20 μM) for 15 minutes prior to lysis and analyzed by immunoblotting for Akt levels and Akt Ser473 phosphorylation. (B) A875 human melanoma cells and PC12nnr5 cells were infected with adenovirus encoding for LacZ, p75ICD, or p75mICD at 25 MOI for 24 hours, and then treated 30 minutes with wortmannin (100 nM) or LY294002 (20 μM) prior to lysis and analysis by immunoblotting for Akt levels and Akt Ser473 phosphorylation. Results shown were repeated three times with similar results.

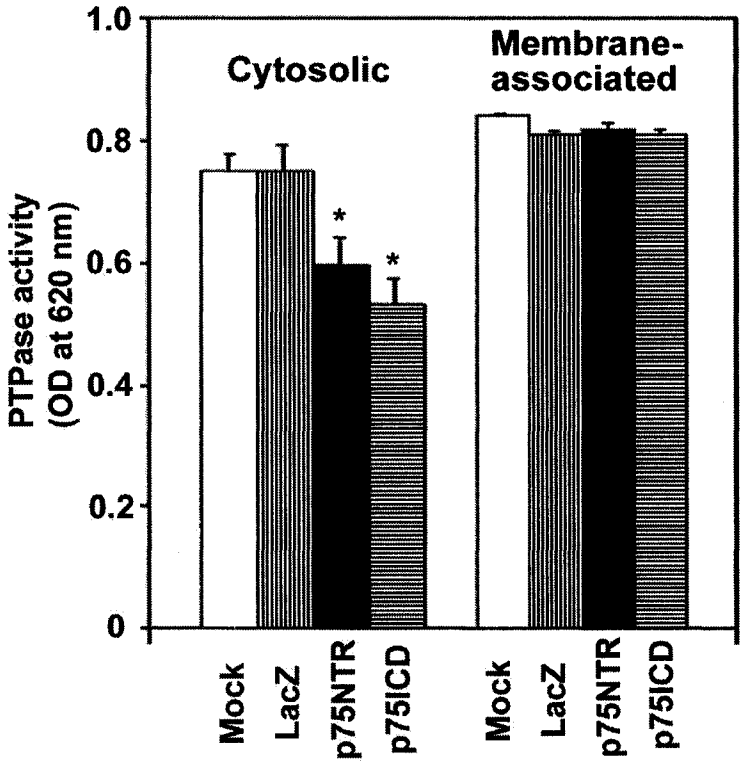

Figure 3.8. p75NTR expression decreases cytosolic protein-tyrosine phosphatase activity. PC12nnr5 cells were infected with LacZ, p75NTR, or p75ICD adenovirus for 24 hours and then harvested in high salt buffer. Cytosolic and membrane compartments were separated by centrifugation and analyzed for phosphatase activity as described in *Materials and Methods*. Conditions that are statistically different from controls ($p<0.001$) are indicated with an asterisk. Data shown represents the mean (± SEM) of three independent experiments.

PREFACE TO CHAPTER 4

As described in Chapter Three, we have found that p75NTR can autonomously regulate survival and death, using pathways that are independent of Trk receptor signaling. We confirmed the latter by showing that the Trk inhibitor K252a had no effect on p75NTR-mediated Akt activation. Intriguingly, we have found that prolonged exposure of PC12 cells and primary neurons to K252a alone induced the activation of the Akt and ERK survival pathways. K252a has known survival promoting activities, which suggested that its neuroprotective activities may results from the activation of Akt and ERK. Therefore, in Chapter Four we characterized the regulation of these pathways by K252a, and determined if they were required for survival mediated by the compound. We also analyzed the neuroprotective effects of the K252a derivative, CEP1347, a compound currently in clinical trials for the treatment of Parkinson's and Alzheimer's diseases.

CHAPTER 4

K252A AND CEP1347 MEDIATE SURVIVAL BY ACTIVATING PI3-K AND MEK

K252a and CEP1347 mediate survival by activating PI3-K and MEK

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ABSTRACT

K252a and its derivative CEP1347/KT7515 promote neuronal survival and growth. CEP1347 blocks MLK activation in several neuronal systems but it is not known if inhibition of the JNK pathway accounts for the neuroprotective and neurotrophic properties of these compounds. To address this, the effect of K252A and CEP1347 on stress pathways and survival- and growth-promoting signaling pathways were compared. Kinase assays and *in vivo* analyses show that K252A inhibits MLK3 with an *in vitro* IC50 in the low nanomolar range and therefore is similar to CEP1347 in this regard. Intriguingly, K252a and CEP1347 are also potent activators of Akt and ERK in primary neurons and PC12 cells. Activation of Akt and ERK by K252a and CEP1347 is not secondary to MLK3 inhibition but represents an independent action of these compounds. PI3-K and MEK inhibitors block Akt and ERK activation mediated by either K252a or CEP1347 and block their survival promoting effect. These results show that the neurotrophic effects of K252A and CEP1347 require modulation of several signaling molecules that include Akt and ERK and suggest that additional direct neurotrophic targets of these compounds remain to be identified.

INTRODUCTION

Apoptosis plays a crucial role during neuronal development but in the adult, apoptotic cascades may contribute to neurodegenerative disease (Oppenheim, 1981; Cowan et al., 1984; Raff et al., 1993; Jacobson, 1998; Rubin, 1998). It is well established that neuronal apoptosis can be triggered by a loss of survival factors and subsequent activation of the caspase family of proteases but the precise signaling mechanisms that regulate apoptotic cascades in neurons remain elusive. One of the best-studied systems is NGF-dependent peripheral sympathetic neurons subjected to neurotrophin-withdrawal where activation of the Jun kinase (JNK) signaling cascade and subsequent AP-1-dependent transcription have been shown to be required for caspase-dependent cell death (Estus et al., 1994; Ham et al., 1995, 2000; Mesner et al., 1995; Eilers et al., 2001). The initial events that allow stressed or damaged neurons to activate JNK are not certain but distal signaling elements include mitogen-activated protein kinase kinase kinases (MAPKKK) such as GCK, MEKK1-4, and MLK2-3 (Fan et al., 1996; Hirai et al., 1996; Rana et al., 1996; Tibbles et al., 1996; Sakuma et al., 1997) which phosphorylate and activate the MAPKK family members MKK4/MKK7 and MKK3/MKK6. Activated MKK4/MKK7 and MKK3/MKK6 in turn phosphorylate and activate JNK and p38 MAPK, respectively (Tibbles et al., 1996; Davis, 2000). The JNK signaling pathway has emerged as a key element in several central neuron trauma models that involve hypoxic and excitotoxic injury and this has raised the possibility that strategies which reduce activation of the JNK signaling cascade may have therapeutic value in treatment of neurodegenerative disorders (Ham et al., 2000).

In healthy neurons, apoptosis is suppressed by specific signaling pathways. One of the main pro-survival pathways is phosphatidylinositol 3-kinase (PI3-K) dependent activation of Akt, a serine/threonine kinase (Dudek et al., 1997). Phosphatidylinositol 3, 4, 5-triphosphate (PIP_3) produced by PI3-K mediates the association of Akt with the plasma membrane and thereby facilitates phosphorylation of PDK1 on threonine 308 and subsequent autophosphorylation on serine 473 (Bellacosa et al., 1998; Toker and Newton, 2000). Activated Akt promotes survival by phosphorylating downstream substrates that may include Bad, Caspase 9, Forkhead family members, IκB kinase, GSK-3, and Ask1

(Datta et al., 1997; Brunet et al., 1999; Fujita et al., 1999; Kennedy et al., 1999; Ozes et al., 1999; Romashkova and Makarov, 1999; Tang et al., 2000; Kim et al., 2001). The ERK MAPKs also mediate neuronal cell survival. Activation of these proteins through the Ras/Raf/MEK pathway is mediated by cell surface tyrosine kinases, such as the Trks, and their activation increases cell survival in sympathetic neurons (Anderson and Tolkovsky, 1999), retinal ganglion cells (Meyer-Franke et al., 1998), cerebellar granule neurons (Bonni et al., 1999), and cortical neurons (Hetman et al., 1999).

K252 compounds are glycosylated indolocarbazole alkaloids which share the same polyaromatic aglycone as staurosporine (Kase et al., 1986, 1987). K252a is widely used as a Trk kinase inhibitor but paradoxically, K252a has neurotrophic effects on a variety of neuronal cells. K252a supports both neurite growth and survival of primary sensory neurons, neuroblastoma cells and PC12 cells (Hashimoto and Hagino, 1989; Borasio, 1990; Tischler et al., 1991) and induces ChAT activity in neurons derived from embryonic spinal cord, basal forebrain, and striatum (Glicksman et al., 1993, 1995). Related compounds such as staurosporine and K252b also have neurotrophic and neuroprotective properties on hippocampal, septal, and cortical neurons (Cheng et al., 1994). CEP1347 (also known as KT7515), a semisynthetic derivative of K252a, lacks Trk-inhibitory activity (Maroney et al., 1998) and inhibits cell death of motoneurons (Borasio et al., 1998; Glicksman et al., 1998; Maroney et al., 1998), cortical neurons (Namgung and Xia, 2000), and auditory hair cells (Pirvola et al., 2000). The targets of K252a which mediate neuroprotection are not known but CEP1347 inhibits JNK activation mediated by NGF withdrawal or p75NTR activation (Maroney et al., 1998; Friedman, 2000; Namgung and Xia, 2000; O'Ferrall et al., 2000; Wagner et al., 2000). A recent study indicates that suppression of JNK activation by CEP1347 results from direct inhibition of members of the mixed lineage kinase (MLK) family of MAPKKK (Maroney et al., 2001).

K252a and CEP1347 do not simply inhibit apoptosis but also activate neurotrophic pathways that result in somal hypertrophy, neurite extension and neurotransmitter synthesis. It is unlikely that the full neurotrophic effects of these compounds are due

simply to inhibition of the JNK pathway and therefore we compared pro-survival and anti-apoptotic signaling pathways activated by K252a and CEP1347 in primary cortical neurons and PC12nnr5 cells. We show that K252A is a potent inhibitor of MLK3 activity *in vitro* (IC_{50} ~5 nM) and that cellular JNK activation induced by MLK3 overexpression, serum withdrawal or staurosporine treatment is blocked by both K252a and CEP1347. In addition, we show that nanomolar concentrations of K252a and CEP1347 induce activation of Akt and ERK. Chemical inhibition of PI3-K or MEK activity blocks K252a and CEP1347-dependent induction of Akt and ERK, respectively, and ablates their neuroprotective effects. Finally, we show that activation of Akt and ERK by K252a or CEP1347 is not secondary to MLK3 inhibition, indicating that K252a and CEP1347 activate neurotrophic pathways through proximal targets distinct from MLK3.

EXPERIMENTAL PROCEDURES

Materials. BDNF was purchased from Collaborative Research (Bedford, MA), CEP1347 was supplied by Aegera Therapeutics Inc. (Montreal, QC), K252a was purchased from Calbiochem (San Diego, CA), and cell culture reagents were from Bio-Whittaker (Walkersville, MD) or Life Technologies (Burlington, ON). Primary antibodies directed against total and phospho-Akt, phospho-ERK and phospho-JNK were from New England Biolabs (Beverly, MA), the anti-phosphotyrosine antibody 4G10 was from Upstate Biotechnology (Lake Placid, NY), the monoclonal anti-haemaglutinin (HA) 12CA5 antibody was from Roche Diagnostics (Laval, QC), and the polyclonal anti-TrkB (directed against the extracellular domain) was a generous gift of L. Reichardt (University of California, San Francisco, CA). Horseradish peroxidase-conjugated donkey anti-rabbit, anti-mouse IgG, or protein-A were obtained from Jackson ImmunoResearch (West Grove, PA). All other reagents were from either Sigma (St. Louis, MO), ICN Biochemicals (Costa Mesa, CA), or Calbiochem (San Diego, CA) unless otherwise indicated.

Cell culture. The rat pheochromocytoma cell lines PC12 and PC12nnr5 were maintained in 7.5% CO2 at 37∞C in Dulbecco's modified Eagle's medium (DMEM) with 5% bovine calf serum (BCS), 5% horse serum, 2 mM L-glutamine, and 100 µg/ml penicillin/streptomycin. 293A cells were cultured in DMEM supplemented with 10% BCS, 2 mM L-glutamine, and 100 µg/ml penicillin/streptomycin. Primary cortical cultures were prepared from E15-16 CD1 mouse telencephalon as described (Gloster et al., 1999) and maintained 3-5 days *in vitro* (DIV) in Neurobasal media supplemented with a final concentration of 0.5X B27 supplement, 0.5X N2 supplement, 2 mM L-glutamine, and 100 µg/ml penicillin/streptomycin.

Preparation of recombinant adenoviruses. N-terminal HA-tagged wild-type MLK3 was subcloned in pAdTrack-CMV and viruses were generated by homologous recombination in bacteria and packaged in 293A cells as described in He et al. (1998). Crude viruses derived from viral plaques were used to infect 293A cells, and HA-MLK3 expression was confirmed by immunoblot analysis. Recombinant adenoviruses were amplified in 293A

cells, purified on sucrose gradients, and titered by plaque assay in 293A cells. Control recombinant adenovirus expressing green fluorescent protein (GFP) was generated using the same viral backbone and purification techniques as for the MLK3 virus.

Immunoblotting. To produce lysates for immunoblots, cell cultures were washed twice with cold phosphate-buffered saline (PBS), lysed in NP-40 lysis buffer (10 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol, 1% NP-40, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM sodium orthovanadate) and centrifuged to remove insoluble material. Protein content of supernatants was determined using the BCA assay (Pierce, Rockford, IL) and 25 μg of lysate was combined with sample buffer (Laemmli, 1970) and separated on SDS-polyacrylamide gels (SDS-PAGE) and electroblotted to nitrocellulose. Blocking, primary antibody, and secondary antibody incubations were all performed in 10 mM Tris (pH 7.4), 150 mM NaCl, and 0.2% Tween 20 with 5% (w/v) dry skim milk powder using recommended dilutions of commercially available antibodies, or 1:2000 of rabbit polyclonal anti-TrkB. For the phospho-specific antibodies, 5% (w/v) bovine serum albumin (BSA) was used instead of milk during the primary antibody incubation. For anti-phosphotyrosine immunoblotting using 4G10, 2% bovine serum albumin (w/v) was used for the blocking step instead of milk powder, and no blocking agents were used during the primary antibody incubation. Secondary antibodies were used at a dilution of 1:10000 and immunoreactive bands were detected using enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Dupont, ON).

Immunoprecipitation. After treatment, cells were washed in cold PBS, and lysed in NP-40 lysis buffer. Immunoprecipitations were performed at 4 °C using polyclonal anti-TrkB antibodies. Complexes were precipitated using 45 μl of protein A-Sepharose (Pharmacia Amersham, Piscataway, NJ) which was added for 90 minutes at 4 °C and then subjected to multiple washes. Samples were lysed in Laemmli sample buffer and analyzed by immunoblotting as described above.

MTT survival assays. Analysis of cell survival was performed using 3(4,5-dimethylthiozol-2-yl)2,5-diphenyltetrazolium bromide (MTT), which was added at a final

concentration of 1 mg/ml for 4 hours following a 24 hour incubation. The reaction was ended by the addition of 1 volume of solubilization buffer (20% SDS, 10% dimethylformamide, and 20% acetic acid). After overnight solubilization, specific and non-specific absorbance were read at 570 and 630 nm, respectively. Each condition was tested six times and results were analyzed for statistical significance by multiple analysis of variance.

Immune complex kinase assay. Recombinant adenovirus encoding human HA-tagged MLK3 was overexpressed in 293A cells and harvested in NP-40 lysis buffer. Immunoprecipitations were performed using the anti-HA monoclonal antibody at 1 μg/ml for 3 hours at 4 °C followed by incubation with 45 μl of protein A-Sepharose for 90 minutes at 4 °C. Beads were washed three times in NP-40 lysis buffer, followed by two washes in kinase buffer (50 mM HEPES (pH 7.4) and 10 mM MgCl2). Prior to the last wash, the lysate was divided into several equal aliquots and 30 μl of kinase buffer was added to the beads together with K252a, CEP1347, or DMSO such that DMSO concentration was constant across all conditions tested. Complexes were pre-incubated with compounds for 10 min at 30 °C and the reaction was initiated by addition of 10 μl of kinase buffer containing 40 μM ATP, 5 μCi of [g-32P]ATP (3000 Ci/mmol, Pharmacia-Amersham). After 20 minutes at 30 °C, kinase reactions were terminated by addition of Laemmli sample buffer, boiled for 5 minutes, separated by SDS-PAGE, dried, and autoradiographed. Levels of total MLK3 were assessed by immunoblotting for the HA epitope using 12CA5.

RESULTS

Nanomolar concentrations of K252a promotes survival of primary mouse cortical neurons and PC12nnr5 cells. Two systems amenable to biochemical analysis were established to analyze K252A survival-promoting activity. The first involves primary cortical neurons which when treated with staurosporine for 24 hours show 40% reduction in survival and increased phosphorylation of JNK. To determine if K252A confers neuroprotection from staurosporine, primary cortical neurons derived from E15-E16 mice were treated with 0.5 μM staurosporine for 24 hours in the presence of increasing concentrations of K252A and then assayed for survival and JNK phosphorylation. Figure 4.1A shows that K252a treatment results in significantly improved survival that was accompanied by a sharp reduction in JNK activation. K252a was also tested for its protective properties on PC12nnr5 cells, a variant PC12 line that lacks TrkA receptors. PC12nnr5 cells deprived of serum for 24 hours normally show 70% reduction in viability and increased JNK phosphorylation but in the presence of K252A, viability is increased and JNK activation is suppressed (Figure 4.1B).

K252a activates Akt and ERK in primary neurons, and PC12nnr5 cells. Neurotrophic signals, such as those induced by TrkB, mediate neuronal survival through PI3-K-dependent activation of Akt and Ras-dependent activation of MEK/ERK. To begin to define K252a-induced survival pathways, primary cortical neurons maintained for 4 days *in vitro* (DIV) were treated with BDNF (100 ng/ml) with or without K252A (100 nM) and then assayed for TrkB and Akt activation. Figure 4.2 shows that BDNF treatment resulted in robust phosphorylation of both TrkB and Akt and shows that these phosphorylation events were suppressed by K252A, as expected given the Trk inhibition conferred by the compound. Intriguingly, in the absence of BDNF, K252a was a potent activator of Akt in primary cortical neurons (Figure 4.2A – compare lanes 1 and 2). This raised the possibility that neuroprotective properties of K252a may involve PI3-K dependent Akt activation and therefore prompted investigation of Akt and MEK/ERK activation by K252A and related compounds.

To address this, primary cortical neurons were incubated with increasing amounts of K252a for one hour to establish the dose-response for maximal Akt activation, and to determine whether K252A affected the MEK/ERK pathway (Figure 4.2B – left). K252a-dependent Akt phosphorylation was observed at concentrations as low as 10-50 nM, with maximal effect observed at 200 nM. At high concentrations of K252A (2 μM), Akt phosphorylation dropped to baseline levels. ERK phosphorylation was also increased by K252A, with a similar dose-response to that for Akt phosphorylation. The phosphorylation of Akt and ERK induced by K252 (100 nM) was consistently observed within 10 minutes. Maximal levels were obtained by 30-60 minutes and showed no diminution after 2 hours exposure (Figure 4.2B - right). Akt and ERK phosphorylation were also analyzed in serum-deprived PC12nnr5 cells exposed to 100 nM K252a for one hour. Figure 4.2C shows that K252a produced robust phosphorylation of Akt and ERK (~5 fold and ~25 fold, respectively) which was potentiated by co-incubation with sodium orthovanadate, a tyrosine phosphatase inhibitor. In all experiments, DMSO was used as vehicle at concentrations up to 0.2%; control experiments showed that DMSO concentrations as high as 1% had no effect on Akt or ERK phosphorylation (data not shown). K252a was originally identified as an inhibitor of protein kinase C (PKC) and therefore the role of PKC inhibition on Akt and ERK phosphorylation was examined. Akt and ERK phosphorylation was not increased in primary cortical neurons incubated with chelerythrine, a PKC inhibitor at concentrations ranging from 10 nM to 2 μM (data not shown).

CEP1347 activates Akt and ERK in primary cortical neurons and PC12nnr5 cells. CEP1347 is a simple ethylthiomethyl derivative of K252a that is protective in several types of neurons (Glicksman et al., 1995; Maroney et al., 1998; Namgung and Xia, 2000; Pirvola et al., 2000). The shared structural and functional properties of K252a and CEP1347 suggest that they have shared cellular targets. To compare effects of CEP1347 and K252A on Akt and ERK, PC12nnr5 cells or primary cortical neurons were incubated with increasing concentrations of the compounds and lysates were examined by immunoblot. Figure 4.3 shows that CEP1347 induces robust Akt and ERK phosphorylation in primary cortical neurons (Figure 4.3A) and in PC12nnr5 cells (Figure

4.3B), with maximal phosphorylation observed at 500 nM. Using these same conditions, K252a produced similar activation levels and maximal Akt and ERK phosphorylation levels were attained using 100 nM of the compound. These data suggest that neuroprotective properties of K252a and CEP1347 may involve activation of Akt and ERK pathways.

PI3-K and MEK inhibitors repress K252a-induced Akt and ERK phosphorylation, respectively. Inhibitors of MEK and PI3-K were then used to examine upstream signaling events controlled by K252a and CEP1347 in PC12nnr5 cells. Figure 4.4A shows that the MEK inhibitor PD98059 completely blocks the activation of ERK phosphorylation induced by K252a, but has no effect on Akt phosphorylation. Conversely, the PI3-K inhibitors LY294002 and wortmannin had no effect on K252a-induced ERK phosphorylation, but effectively inhibited K252a-dependent Akt phosphorylation. These results show that PI3-K activity is required for the activation of Akt by K252a, and suggest that K252a is acting upstream of PI3-K. These data also indicate that K252a acts upstream of MEK to activate the ERK pathway.

To test whether PI3-K and MEK activity are necessary for the neuroprotective properties of K252A and CEP1347, PC12nnr5 cells were serum deprived and exposed to K252a or CEP1347 in the presence or absence of 25 μM LY294002 or 25 μM PD98059. Survival assays revealed that the protective effect of K252a and CEP1347 were lost in the presence of either PI3-K or MEK inhibition (Figure 4.4B). These results indicate that active PI3-K and MEK are required for K252a- and CEP1347-induced survival.

K252a and CEP1347 inhibit MLK3 with similar dose dependency. CEP1347 reduces JNK activation induced by NGF withdrawal (Maroney et al., 1998; Namgung and Xia, 2000; O'Ferrall et al., 2000; Wagner et al., 2000), probably by inhibiting members of the mixed lineage kinase (MLK) family (Maroney et al., 2001). K252A also reduces stress-induced JNK activation (see Figure 4.1) and, given its close structural relationship to CEP1347, might also inhibit MLK family members. To gain insight into K252a and CEP1347 actions, the compounds were directly compared for their ability to inhibit TrkB and

MLK3 activity. Figure 4.5A shows TrkB immunoprecipitated from cortical neurons stimulated with 100 ng/ml BDNF in the presence of 200 nM of K252A or CEP1347. TrkB phosphorylation induced by BDNF is completely blocked by 200 nM K252a but is not affected by CEP1347. However, *in vitro* kinase autophosphorylation assays using immunoprecipitated MLK3 reveal that K252a and CEP1347 have equivalent MLK3 inhibitory activity (Figure 4.5B). Both compounds display similar dose-profiles, with IC50 for MLK3 inhibition of approximately 5 nM.

Activation of MEK/ERK and PI3-K/Akt pathways by K252a or CEP1347 is not secondary to MLK3 inhibition. Since MLK3 is a direct target of both K252a and CEP1347, it is possible that the effects of the compounds on ERK and Akt activation may be secondary to MLK3 inhibition. Alternatively, activation of ERK and Akt could represent an action of K252A and CEP1347 on independent pathways involving target(s) distinct from MLK3. To determine if there is crosstalk between MLK3, Akt and ERK, PC12 cells were infected with recombinant adenovirus expressing MLK3, or as a control, infected with equivalent titers of recombinant adenovirus expressing GFP. Figure 4.6 shows that, as expected, JNK phosphorylation is specifically increased in cells overexpressing MLK3. However, MLK3 overexpression had no effect on basal Akt or ERK phosphorylation (compare lanes 1 and 4) or on Akt or ERK phosphorylation induced by NGF (compare lanes 2 and 5, 3 and 6).

To directly compare the *in vivo* effect of K252a and CEP1347 on MLK3-dependent JNK activation, ERK activation and Akt activation, PC12nnr5 cells were infected with MLK3 adenovirus for 24 hours, treated with 200 nM K252a and CEP1347 for 1 hour then lysed and analyzed by immunoblot (Figure 4.7). Overexpression of MLK3 in the PC12nnr5 line resulted in JNK activation but had no effect on basal Akt and ERK phosphorylation (compare lanes 1 and 2), consistent with results from PC12 cells (above). K252a and CEP1347 were both potent inhibitors of MLK3-dependent JNK activation and a one hour treatment completely inhibited p54 JNK phosphorylation. Importantly, under conditions where K252a and CEP1347 clearly inhibit JNK activation mediated by MLK3 overexpression, both compounds induce Akt and ERK phosphorylation in both the

presence or absence of exogenous MLK3. Together, these data indicate that K252a and CEP1347 are potent inhibitors of the MLK3/JNK pathway *in vivo*, and that activation of Akt and ERK by CEP1347 and K252a occurs through an MLK-independent pathway.

DISCUSSION

Neuronal damage resulting from trauma or from neurodegenerative disease is an area of large unmet medical need and there is a critical necessity for therapeutic agents that help prevent neuronal loss in acute and chronic conditions. The recognition that neuronal cell loss can occur by apoptosis has raised the possibility that therapeutics which target regulatory proteins in the neuronal apoptotic cascade may have clinical benefit. These pathways have been most extensively studied in peripheral neurons, especially within sympathetic neurons undergoing apoptosis in response to nerve growth factor (NGF) withdrawal. Apoptosis in these cells involves activation of JNK, phosphorylation of c-Jun and ATF-1 transcription factors, and increased AP-1 transcriptional activity (reviewed in Ham et al. 2000). Indeed, activation of c-Jun-dependent transcriptional activity is a crucial step in NGF withdrawal mediated neuronal apoptosis and is required for release of Cytochrome c from mitochondria and subsequent Caspase 9 activation (Eilers et al., 2001).

The kinase pathways that result in c-Jun phosphorylation in neurons is becoming well defined and therapeutics targeting this pathway may have utility as neuroprotectants. K252a was originally identified as a protein kinase C inhibitor but is now most widely used as a Trk tyrosine kinase inhibitor (Berg et al., 1992; Nye et al., 1992; Tapley et al., 1992). However, despite its inhibitory effect on Trk, K252a is a potent neurotrophic molecule and can facilitate survival in primary neurons and neural cell lines (Hashimoto and Hagino, 1989; Borasio, 1990; Tischler et al., 1991). CEP1347 is a semisynthetic derivative of K252a which does not exhibit Trk inhibitory activity and which exerts potent neuroprotection *in vitro* and *in vivo* (Glicksman et al., 1998; Maroney et al., 1998; Namgung and Xia, 2000; Pirvola et al., 2000). Several studies have shown that CEP1347 inhibits activation of the JNK pathway (Friedman, 2000; Namgung and Xia, 2000; O'Ferrall et al., 2000; Wagner et al., 2000) and recent work has revealed that CEP1347 is a potent inhibitor of the MLK family of MAPKKK (Maroney et al., 2001). However, the possibility that this compound may affect other targets that do not lie on the JNK pathway has not been explored and it is unclear whether K252a or CEP1347 affect identical neurotrophic targets.

Our data show that K252a efficiently blocks JNK activation induced by serum withdrawal or by staurosporine treatment and that K252a is an effective MLK3 inhibitor *in vivo* and *in vitro*. Indeed, the IC_{50} of both K252a and CEP1347 for MLK3 inhibition are essentially identical (~ 5 nM) and both compounds completely block JNK activation mediated by MLK3 overexpression. Together, these data suggest that CEP1347 and K252a exert similar effects on members of the MLK3 family and that some of the neuroprotective properties of K252a may be explained by its potent MLK inhibition.

Both K252a and CEP1347 exert effects on primary neurons that go beyond maintenance of survival. Neurons that are rendered null for bax or maintained in caspase inhibitors do not undergo apoptosis in response to neurotrophin withdrawal, but undergo neuritic degeneration and somal atrophy (Deckwerth et al., 1996; Deshmukh et al., 1996). However, in cells treated with K252a, neurite outgrowth occurs and cellular atrophy is prevented (Glicksman et al., 1995; Maroney et al., 1995). CEP1347 exerts similar neurotrophic effects and has been shown capable of rescuing metabolic activity and somal size in neurons deprived of neurotrophic factor and maintained in caspase inhibitors (Borasio et al., 1998; Glicksman et al., 1998; Harris et al., 2000). It is difficult to explain the neurotrophic properties of K252a and CEP1347 solely in terms of JNK pathway inhibition and we therefore examined the effects of CEP1347 and K252a on neuronal signaling pathways involved in growth and survival. Our results show that low concentrations of K252a and CEP1347 rapidly activate Akt and ERK signaling in primary cortical neurons as well as in PC12nnr5 cells. The activation of Akt and ERK mediated by these compounds was blocked by inhibitors of PI3-K and MEK, respectively, indicating that the primary target(s) for K252a and CEP1347 lie upstream of PI3-K and MEK. Using survival assays on primary cortical neurons and PC12 cells, we have found that inhibitors of PI3-K and MEK block the survival response elicited by K252a and CEP1347, indicating that the MLK-independent properties of K252a and CEP1347 are essential to promote survival.

There is extensive cross regulation of survival and death signaling pathways. For example, Akt facilitates cellular survival in part by phosphorylating and inactivating an array of pro-apoptotic proteins that include Ask1, Bad, Caspase 9, and Forkhead family members (Datta et al., 1997; Brunet et al., 1999; Fujita et al., 1999; Kennedy et al., 1999; Ozes et al., 1999; Romashkova and Makarov, 1999; Tang et al., 2000; Kim et al., 2001). One conceivable explanation for increased Akt and ERK activity induced by K252a and CEP1347 is that MLK normally phosphorylates and thereby negatively regulate a common upstream activator of Akt and ERK pathways. In this scenario, K252a- and CEP1347-mediated inhibition of MLK would indirectly result in activation of Akt and ERK. However, MLK3 overexpression had no effect on basal or induced Akt or ERK activity in cells in which the enzyme dramatically increased JNK activation. Furthermore, K252a and CEP1347 clearly increase Akt and ERK activity under conditions where the compounds simultaneously block MLK3-mediated JNK activation. Therefore, K252a and CEP1347 appear capable of activating Akt and ERK activity through unique targets distinct from MLK3. Identification of the upstream targets of CEP1347 and K252a which give rise to increased ERK and Akt activity may provide novel insights into aspects of neurotrophic signaling which are amenable to therapeutic intervention.

K252a-mediated tyrosine phosphorylation events have been described. Treatment of the human neuroblastoma cell line, SH-SY5Y, results in the tyrosine phosphorylation of the pp125 focal adhesion protein tyrosine kinase (Fak) (Maroney et al., 1995). The induction of Fak phosphorylation by K252a was also observed in LA-N-5 cells and primary cultures of rat embryonic striatal cells, but not in PC12 cells and under conditions where Fak phosphorylation was induced, PI3-K or MAPK activity was reportedly unchanged. However, in a kinase screen designed to identify targets of K252a, we have observed an increase in Fak tyrosine phosphorylation under conditions where Akt and ERK are activated (data not shown). The identification of direct targets of K252a and CEP1347 which result in Akt and ERK activation should prove an interesting challenge, particularly given recent technological advances in target identification.

In recent years, K252a has been used as a Trk tyrosine kinase inhibitor, with the oft stated assumption that K252a, at 100-200 nM, is a specific Trk inhibitor. The data presented here shows that this assumption is incorrect. K252a at concentrations as low as 50 nM acts not only as a very efficient MLK inhibitor but also activates PI3-K and MEK signaling pathways. It is possible that with appropriate controls, K252a may be useful for analyzing proximal molecular signaling events regulated by Trk receptors and MLKs. However, K252a has become widely used for examining Trk signaling in complex cellular events in intact cells, hippocampal slices and even intact animals (reviewed in Knusel and Hefti, 1992). It is clear that even low nanomolar concentrations of K252a and CEP1347 will alter activity of several intracellular signaling pathways and therefore, effects of these compounds on distal signaling events or cellular processes must be interpreted with caution.

In closing, we have shown that activation of Akt and ERK is an important component of the neuroprotective properties of K252a and CEP1347 and that activation of Akt and ERK by these compounds occurs through pathways independent of MLK3 activity.

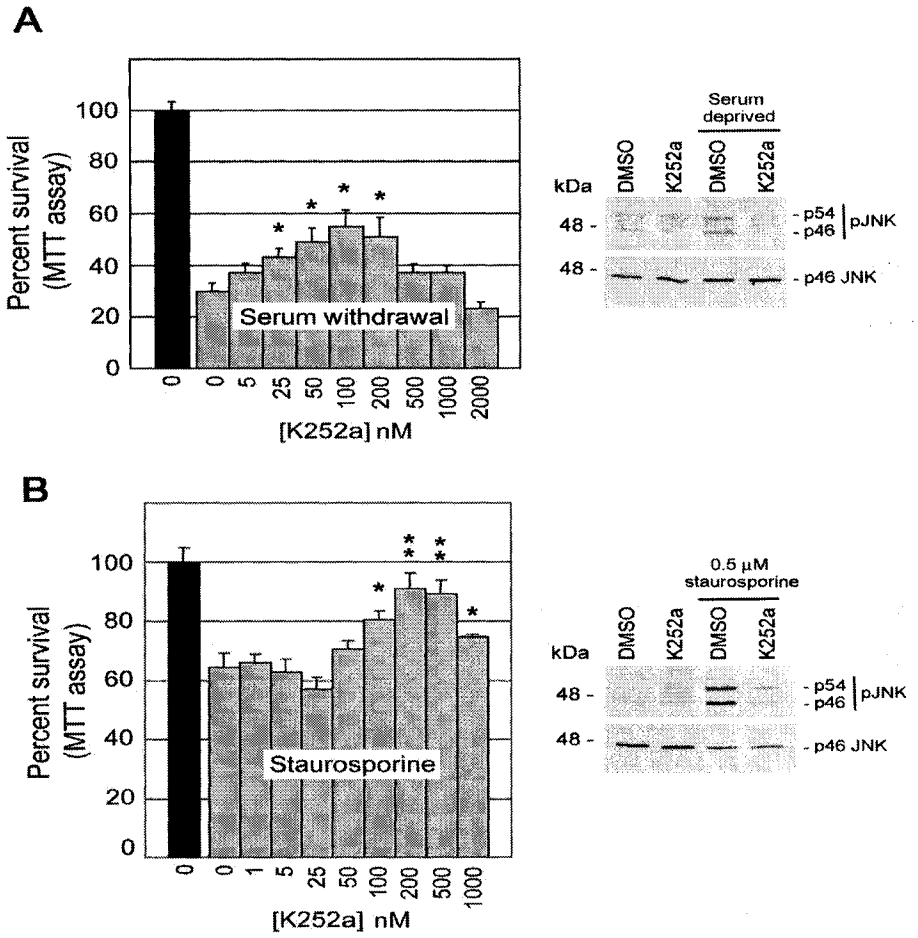

Figure 4.1. K252a promotes survival of PC12nnr5 cells and primary cortical neurons at nanomolar concentrations. (A – left) Primary mouse cortical neurons maintained 4 DIV were exposed to 500 nM staurosporine for 24 hours together with increasing concentrations of K252a, and assayed for survival by MTT assay. Data is expressed as percent survival of control untreated cells (black column). (A – right) Primary mouse cortical neurons exposed to 500 nM staurosporine for 24 hours in the absence or presence of 200 nM K252A were lysed and analyzed for phospho-JNK and total JNK levels by immunoblot. (B – left) PC12nnr5 cells were deprived of serum, incubated with increasing concentrations of K252a for 24 hours, and cell survival was determined by MTT assay. Data is expressed as percent survival of cells grown in serum-containing media (black column). (B – right) PC12nnr5 cells deprived of serum for 24 hours in the absence or presence of 200 nM K252a for 24 hours were lysed and analyzed for phospho-JNK and total JNK levels by immunoblot. All experiments were repeated at least three times and the final DMSO (vehicle) concentration was identical in all conditions. For MTT assays, results represent the mean of three separate experiments. Statistically significant differences were detected by multiple analysis of variance and are indicated by one ($p<0.05$) or two ($p<0.001$) asterisks.

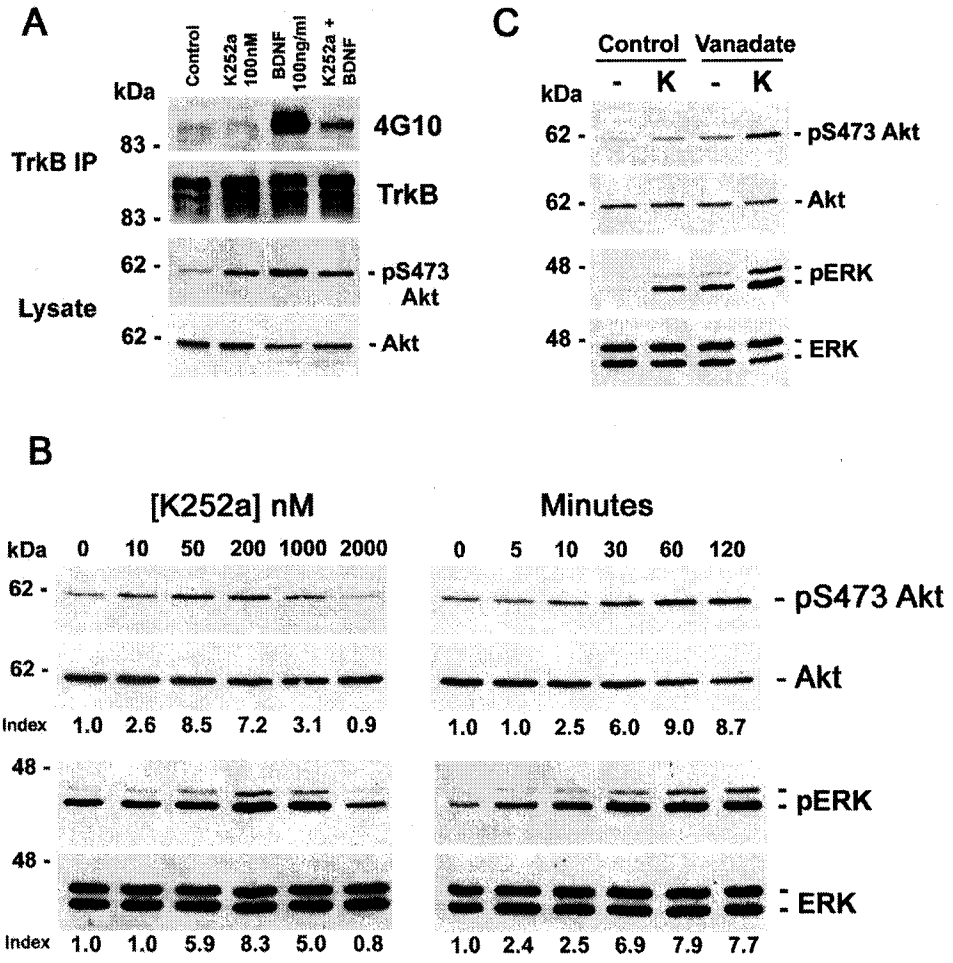

Figure 4.2. K252a induces Akt and ERK phosphorylation in primary cortical neurons and PC12nnr5 cells. (A) Primary mouse cortical neurons maintained 4 DIV were incubated with 100 nM K252a for 45 minutes, exposed to BDNF (100 ng/ml) for an additional 15 minutes, and lysed for immunoprecipitation. TrkB immunoprecipitates were immunoblotted for phosphotyrosine levels using the 4G10 monoclonal antibody, and TrkB protein levels as indicated. Akt phosphorylation was monitored in the total cell lysate. (B) Dose curve and time course of K252a-induced Akt and ERK phosphorylation in primary cortical neurons. For the dose curve, cells were exposed to K252a for one hour; 200 nM of K252a was used in the time course experiment. For quantitation, autoradiographs were scanned and images analyzed using NIH Image. (C) Serum-deprived PC12nnr5 cells were treated with 100 nM K252a for one hour (K) and phosphorylation levels of Akt and ERK were analyzed by immunoblot. All experiments were repeated at least four times with similar results.

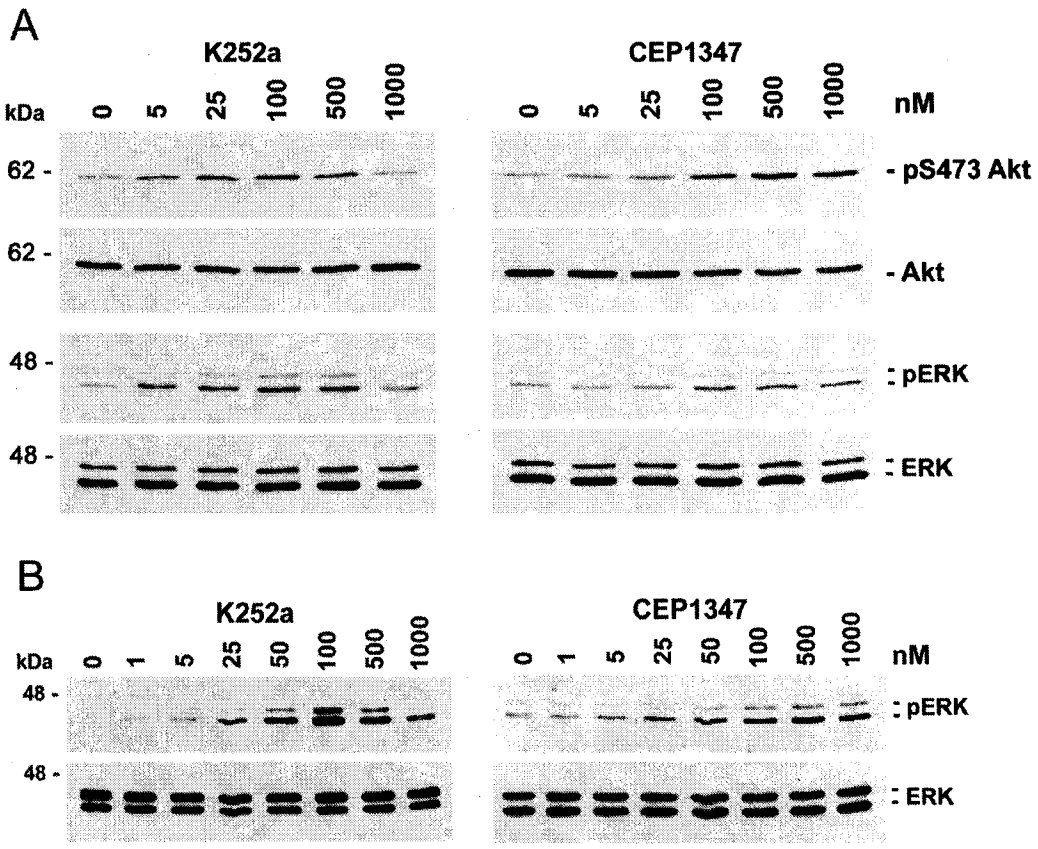

Figure 4.3. CEP1347 activates Akt and ERK in primary cortical neurons and PC12nnr5 cells. (A) Mouse primary cortical neurons were exposed for one hour to increasing concentrations of K252a and CEP1347, and assayed for Akt and ERK phosphorylation. (B) PC12nnr5 cells were serum deprived for 18 hours and exposed to increasing concentrations of K252a and CEP1347 for one hour. ERK phosphorylation and protein levels were analyzed by immnoblotting. Experiments were repeated three times with similar results.

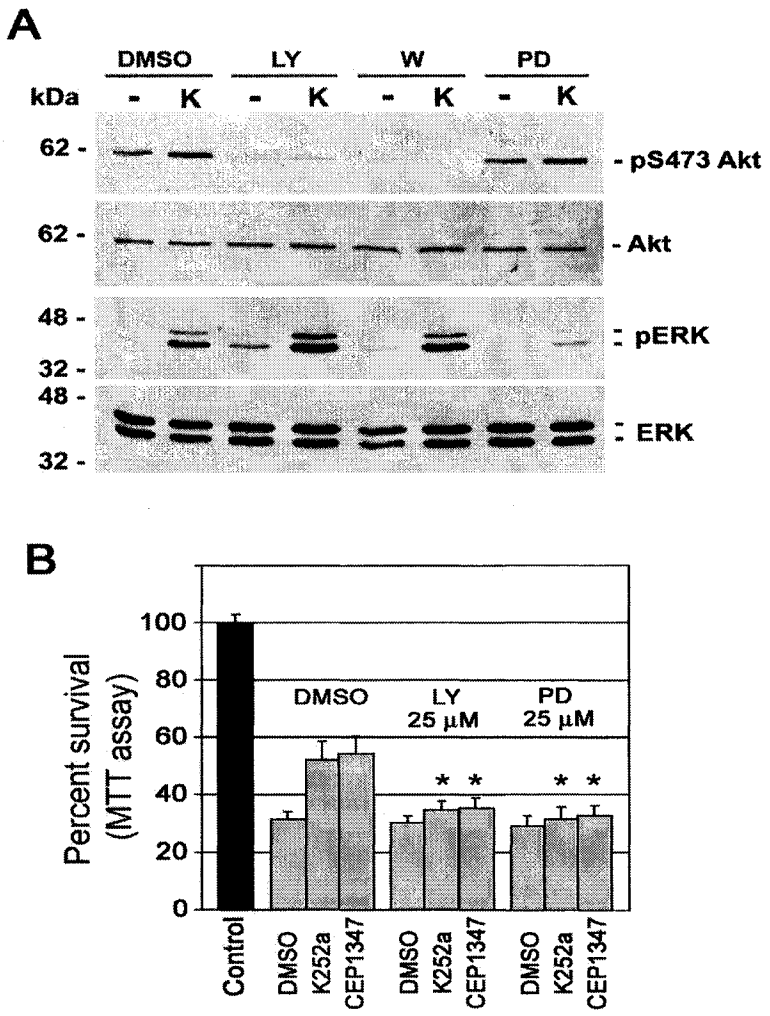

Figure 4.4. PI3-K and MEK inhibitors suppress K252a-induced Akt and ERK phosphorylation, respectively. (A) Serum deprived PC12nnr5 cells were exposed to 100 nM K252a for one hour in the presence of the PI3-K inhibitors, LY294002 (20 µM) and wortmannin (100 nM), or the MEK inhibitor PD98059 (30 µM). Cells were harvested and assayed for Akt and ERK phosphorylation by immunoblot. Experiments were repeated twice with identical results. (B) PC12nnr5 cells were deprived of serum and incubated for 24 hours with 100 nM of K252a or CEP1347 in the presence of 25 µM LY294002 (LY), 25 µM PD98059 (PD), or DMSO, and mitochondrial activity (MTT) was assayed as a marker for cell survival. Data is expressed as percent survival of cells grown in serum-containing media (black column). Conditions that are statistically different from cells deprived of serum with K252a or CEP1347 are indicated with an asterisk ($p<0.001$). All experiments were repeated at least four times with similar results.

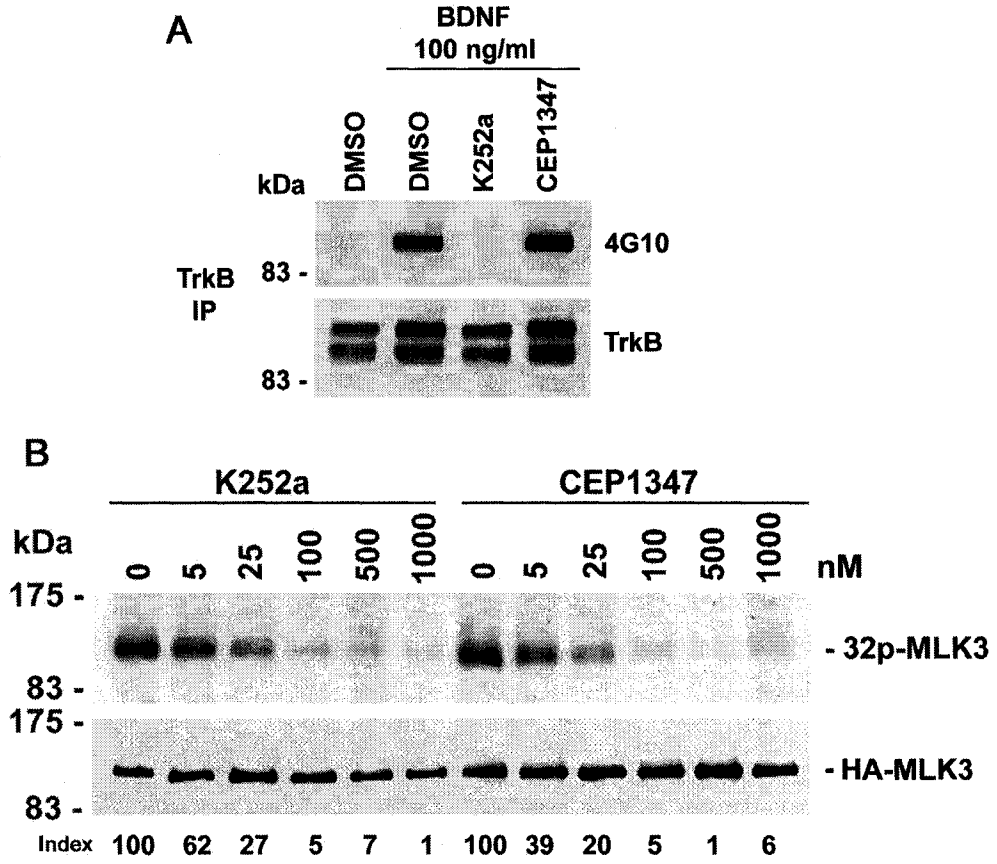

Figure 4.5. K252a and CEP1347 inhibit MLK3 activity with similar potency. (A) Primary mouse cortical neurons maintained 4 DIV were incubated with either 100 nM K252a or CEP1347 for 45 minutes, exposed to BDNF (100 ng/ml) for an additional 15 minutes, and lysed for immunoprecipitation. TrkB immunoprecipitates were assayed by immunoblot for phosphotyrosine levels (4G10), and TrkB protein levels as indicated. (B) HA-tagged MLK3 was overexpressed in 293 cells, immunoprecipitated and incubated with increasing concentrations of K252a and CEP1347. Kinase assays were initiated by the addition of 32P-ATP and proceeded at 30°C for 30 minutes prior to SDS-PAGE and autoradiography. The same samples were subjected to anti-HA immunoblotting to confirm equivalent MLK3 protein levels in each condition. Autoradiographs were scanned and quantified using NIH Image. Experiments were repeated at least four times with similar results.

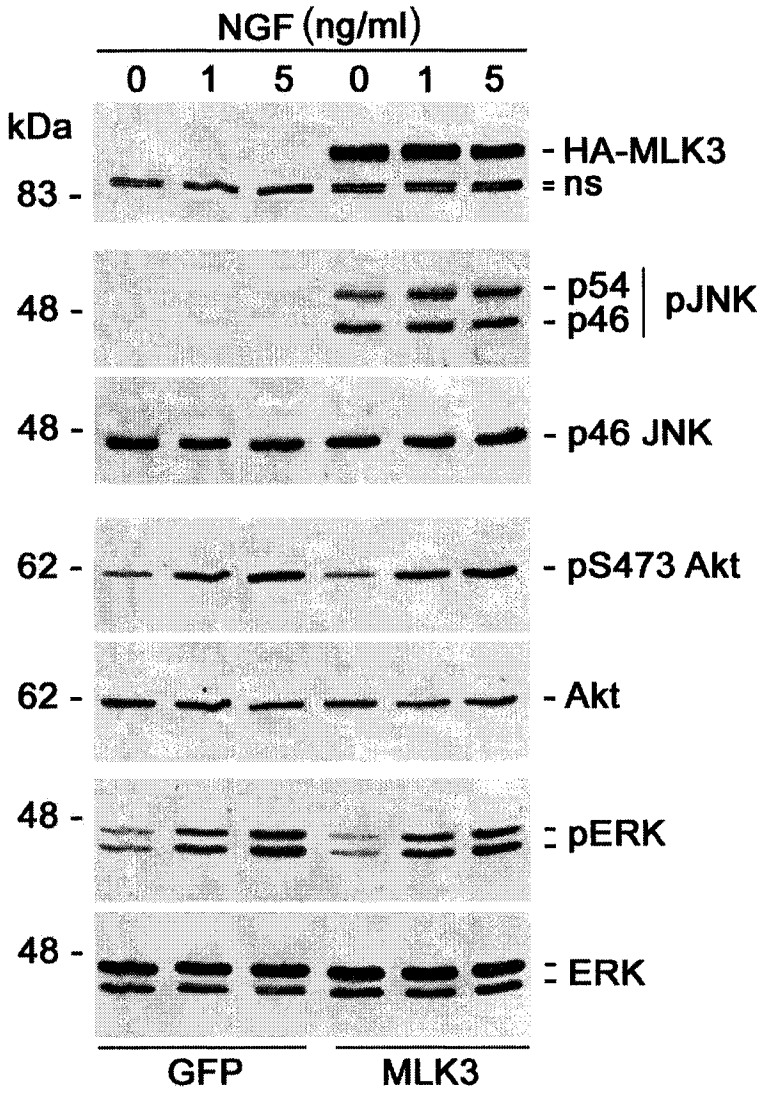

Figure 4.6. Expression of active MLK3 does not affect basal or NGF-induced Akt and ERK phosphorylation in PC12 cells. PC12 cells were infected with recombinant adenovirus expressing either GFP or human HA-tagged MLK3 at 100 MOI for 24 hours. Cells were treated with 1 or 5 ng/ml NGF for 10 minutes, harvested, and analyzed by SDS-PAGE immunoblotting. The presence of MLK3 was revealed using anti-HA antibodies (ns: non-specific bands). Akt, ERK and JNK activation levels were assayed using phospho-specific antibodies. This experiment was repeated three times with similar results.

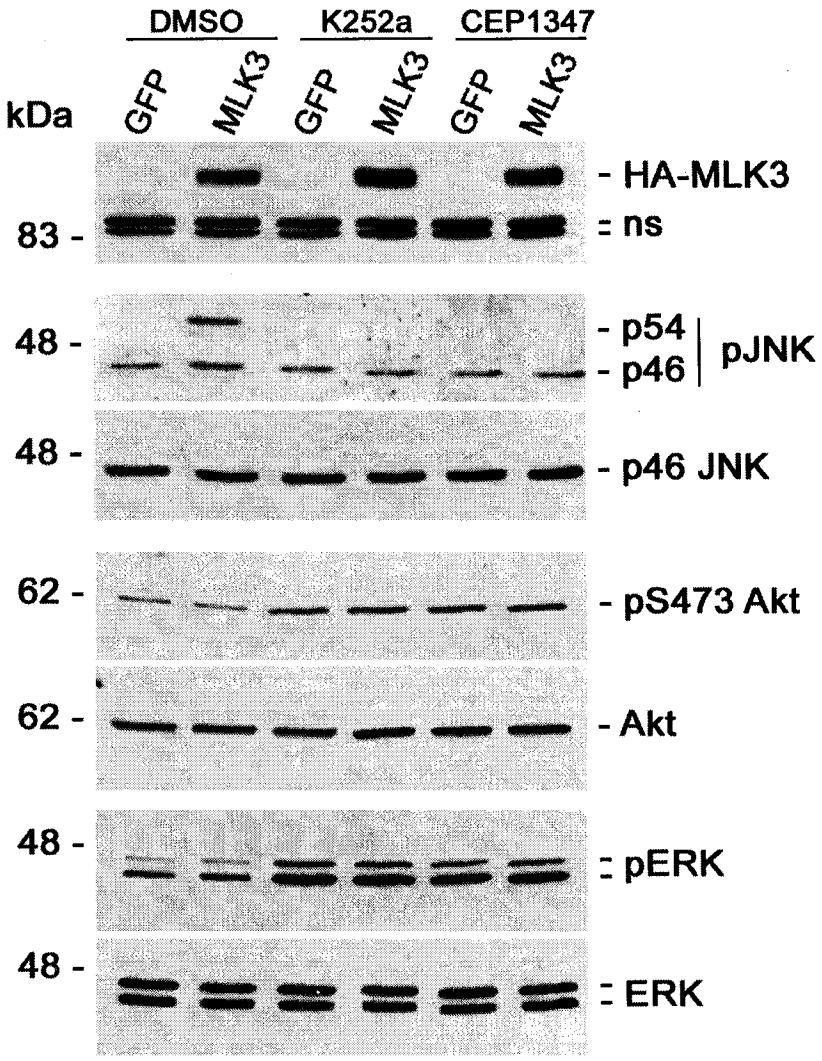

Figure 4.7. K252a and CEP1347 inhibit MLK3-induced JNK activation in PC12nnr5 cells. PC12nnr5 cells were infected with GFP or MLK3 adenoviruses (100 MOI) for 24 hours and then exposed to 200 nM K252a and CEP1347 for one hour prior to harvesting. The presence of MLK3 was shown using anti-HA antibodies (ns: non-specific bands). Akt, ERK and JNK activation levels were assayed using phospho-specific antibodies. This experiment was repeated three times with similar results.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

5.1 MAJOR FINDINGS

1. We have demonstrated for the first time that pilocarpine-induced seizure produces a large increase in neuronal apoptosis in the rat hippocampal, entorhinal, and piriform cortices (Roux et al., 1999). TUNEL positive neurons were seen in the rat central nervous system up to 14 days after seizure, indicating that apoptotic mechanisms are important mediators of cell death after seizure. While these findings are important to understand the pathophysiology of epilepsy, this injury paradigm also represents a good model for the study of novel compounds having neuroprotective properties (Barker et al., 2000).

2. We have implicated p75NTR in seizure-induced neuronal cell death. Specifically, the apoptotic cell death that occurs following pilocarpine-induced seizure was found to be accompanied by a large increase in levels of p75NTR mRNA and protein (Roux et al., 1999). The incidence of apoptosis within individual neurons correlated tightly with p75NTR expression, with over 85% of the cells with apoptotic nuclei showing induced p75NTR expression. Apoptotic cells and p75NTR expression were still observed many days after pilocarpine administration, indicating that p75NTR-mediated signaling may contribute to long-term cell loss that occurs after seizure.

3. We have identified a novel survival promoting role for p75NTR. Specifically, we found that expression of p75NTR promotes survival, and increases PI3-K-mediated Akt activity (Roux et al., 2001a). These effects were accompanied by increased tyrosine phosphorylation of the p85 PI3-K subunit and the adaptor protein Shc, suggesting that p75NTR may modulate tyrosine kinase or phosphatase activity. Consistent with the latter, we demonstrated that p75NTR expression reduced cytosolic tyrosine phosphatase activity.

4. We have demonstrated that the related compounds, K252a and CEP1347, stimulate survival by activating the PI3-K and MEK pathways (Roux et al., 2001b). Specifically, we have found that K252a and CEP1347 are both potent MLK3 inhibitors, yet both simultaneously activated Akt and ERK through MLK3-independent mechanisms. These

activations appeared essential for the neuroprotective effects of K252a and CEP1347, suggesting that these compounds may inhibit a common target, distinct from MLK3, that is involved in the regulation of the Akt and ERK pathways.

5.2 SEIZURE-INDUCED NEURONAL CELL DEATH

5.2.1 The relative contribution of apoptotic and necrotic mechanisms

Neuronal cell death has been well documented in both human epilepsy and experimental seizure models (reviewed in Represa et al., 1995; Sloviter, 1996; Treiman, 1996; Morrison et al., 1996), and is thought to contribute to the pathophysiology characteristic of epilepsy (Aicardi and Chevrie, 1983; Cavalheiro et al., 1991; Priel et al., 1996). However, the specific contribution of necrotic versus apoptotic mechanisms remains unclear. Necrotic cell death was shown to occur very early after pilocarpine-induced seizure, with some brain regions being affected within twenty minutes of pilocarpine injection (Turski et al., 1983, 1987; Olney et al., 1986; Fujikawa, 1996). As described in Chapter Two, we have found that pilocarpine-induced seizure leads to a dramatic increase in apoptotic cell death, as detected by *in situ* TUNEL and DNA fragmentation assays (Roux et al., 1999). We have observed the presence of apoptosis as late as fourteen days after seizure, suggesting that apoptotic cell death might account for a large proportion of the neuronal cell loss after seizure.

Necrosis and apoptosis are defined on the basis of morphological criteria. Nuclear and cytoplasmic condensation and intranucleosomal DNA cleavage (characteristic of apoptosis) results from the activation of an intracellular signaling cascade leading to cellular suicide, whereas swelling of mitochondria and endoplasmic reticulum, and subsequent loss of membrane integrity (characteristic of necrosis) occurs when the cell is challenged by external insults, not necessarily involving the activation of an intracellular program (Wyllie, 1980). It is impossible to determine with accuracy the contribution of each death mechanism *in vivo*, because some dying cells can show morphological features of both necrosis and apoptosis; for example, cells showing morphological hallmarks of necrotic death can sometimes also be TUNEL positive (Charriaut-Marlangue and Ben-Ari, 1995). Because apoptotic cell death is a relatively slow process, it is possible that some cells that are already engaged an intracellular signal leading to apoptosis are lost through necrosis, which is a relatively fast process, therefore leading to the dual morphology of the dying cell. Nonetheless, we have demonstrated that pilocarpine-induced seizure results in DNA fragmentation which correlated with the

region specific increase in TUNEL staining, DNA cleavage, and pyknotic nuclei, indicating that pilocarpine-induced seizure results in widespread activation of intracellular apoptotic cascades (Roux et al., 1999). These findings suggest that anti-apoptotic compounds may prove useful for prevention of seizure-induced neuronal cell loss and degeneration. Therefore, the pilocarpine-induced seizure model might represent a useful system to study compounds with potential neuroprotective properties (Barker et al., 2000).

5.2.2 A role for p75NTR after seizure

Although p75NTR has been reported to induce numerous effects that run the gamut from activation of survival responses, to modulation of Trk receptors and facilitation of apoptosis, the physiological function of p75NTR remains elusive (reviewed in Barker, 1998; Barrett, 2000). *In vivo* injury paradigms represent interesting models to study the function of p75NTR, because levels of p75NTR expression are increased in many injured tissues (Dusart et al., 1994; Martinez-Murillo et al., 1998; Kokaia et al., 1998; Nataf et al., 1998; Roux et al., 1999; Hu et al., 1999; Oh et al., 2000; Wang et al., 2000; Bagum et al., 2001; Casha et al., 2001; Giehl et al., 2001; Stark et al., 2001), and intriguingly, p75NTR expression often tightly correlates with injury-induced apoptosis (Roux et al., 1999; Syroid et al., 2000; Wang et al., 2000; Casha et al., 2001). Therefore, using the pilocapine-induced seizure model of central nervous system injury, we determined if p75NTR was expressed in the rat brain following this type of injury, and the nature of its function.

We found that pilocarpine-induced seizure produces a dramatic increase in p75NTR mRNA and protein in the rat cortex and hippocampus (Roux et al., 1999). The presence of mRNA for p75NTR within cell bodies of the entorhinal cortex indicated that p75NTR was produced locally. The transcriptional regulation of the *p75ntr* gene is still unknown, but it has been shown that p75NTR expression is increased by KCl treatment of cultured Purkinje cells (Cohen-Cory et al. 1993), suggesting that depolarization and/or excitotoxicity may activate transcription of the p75NTR gene after seizure. Interestingly, seizure-induced cell loss can be inhibited by NMDA receptor antagonists (Rice and

DeLorenzo, 1998), suggesting that excitotoxic mechanisms are responsible for seizure-induced cell death; however, it is unknown whether NMDA antagonists also inhibit seizure-induced p75NTR expression. Nonetheless, the pilocarpine model of seizure represented a good system for studying p75NTR, since both p75NTR expression and apoptotic cell death were induced in this injury paradigm.

Analysis of the cellular localization of p75NTR after seizure revealed an expression pattern that followed very closely the tissue distribution of the induced apoptotic cell death (Roux et al., 1999). Cell counts within the seized entorhinal and hippocampal cortices demonstrated a strong correlation (85%) between neuronal cell apoptosis and p75NTR expression (Table 2.1). These results suggested that p75NTR may be involved in seizure-induced apoptotic cell death, however, p75NTR has been shown in other systems to enhance survival in some circumstances and to facilitate apoptosis in others. Therefore, to determine the exact function of p75NTR, we transferred the pilocarpine model of seizure to mice and tested the outcome of seizures in $p75NTR^{exonIII-/-}$ animals. We quickly realized, however, that certain mouse strains are completely resistant to excitotoxic cell death (Table 5.1). Although Balb/c, C57/bl6, or sv129 mice responded to pilocarpine injection identically to CD1 mice, no apoptotic cells could be detected following one hour of *status epilepticus* in these strains. Therefore, we started to inbreed the $p75NTR^{exonIII-/-}$ mouse (C57BL/6), into a susceptible strain (CD1), and are currently at the third generation of breeding, and intend to subject these mice to pilocarpine-induced seizure in the near future.

We cannot rule out that p75NTR might play a pro-survival function after seizure, but on the basis of the strong correlation between induced p75NTR expression and apoptosis following seizure, we favor the hypothesis that p75NTR is induced following seizure through an activity-dependent mechanism and is then capable of activating apoptotic signaling cascades in response to bound neurotrophin. While the use of the $p75NTR^{exonIII-/-}$ mouse in a proper background will be useful to determine the function of p75NTR after seizure, administration of compounds that selectively inhibit molecules in the p75NTR signaling pathway or antagonizes neurotrophin binding to p75NTR may help determine

the contribution of p75NTR-mediated effect in seizure-induced cell death. However, since the regulation of p75NTR is not well understood, we generated an adenoviral expression system to study p75NTR function *in vitro*.

Table 5.1

Differential susceptibility of certain mouse strains to pilocarpine-induced seizure-mediated apoptotic cell death[a].

Susceptible[b]	**Non-susceptible[c]**
CD1	Balb/c
	C57/bl6
	sv129

[a] Apoptotic cell death was quantified using an *in situ* cell death detection kit (TUNEL). Animals were analyzed two days following pilocarpine-induced *status epilepticus.*

[b] Mouse strains were found susceptible when pilocarpine-induced seizure resulted in increased TUNEL staining in brain sections of the piriform and entorhinal cortices of the seized mice. [c] Mouse strains were found non-susceptible when mice that responded normally to pilocarpine did not display induced apoptotic cell death.

5.2.3 The involvement of Trk receptors after seizure

As discussed in the first chapter, p75NTR acts as a Trk co-receptor that can enhance or suppress neurotrophin-mediated Trk receptor activity, resulting in increased or reduced Trk-mediated survival signals (Ip et al., 1993; Benedetti et al., 1994; MacPhee et al., 1997; Bibel et al., 1999; Mischel et al., 2001). In the adult cortical and hippocampal cortices, TrkB and TrkC are both present, and their expression is also regulated by seizure. A transient increase in mRNA for TrkB and TrkC, but not for TrkA, has been demonstrated in the hippocampus and neocortex during kindling, and after chemically-induced seizure (Bengzon et al., 1993; Merlio et al., 1993; Persson and Ibanez, 1993; Dugich-Djordjevic et al., 1995; Mudo et al., 1996; reviewed in Gall, 1993). It is possible that seizure-induced apoptosis results from direct p75NTR signaling, but the presence of increased levels of Trk receptors after seizure suggest that p75NTR may also promote apoptosis by suppressing Trk-mediated regenerative survival signals.

In contrast, Trk receptors can also modulate p75NTR signaling (Dobrowsky et al., 1995), and inhibit p75NTR-mediated cell death (Yoon et al., 1998; Salehi et al., 2000). Therefore, the role of Trk receptors after seizure may be to promote survival of stressed neurons, and to repress p75NTR-mediated apoptosis in cells expressing the two receptors. It will be interesting to determine if the cells undergoing apoptosis after seizure have insufficient levels of Trk receptors to survive, which would allow p75NTR-dependent apoptosis to occur. A precise analysis of the expression patterns of all neurotrophin receptors after seizure will be required to determine their autonomous and transmodulatory effects.

5.2.4 Ligand-dependent or independent mechanisms

Ligand-dependency remains a confusing issue in p75NTR research. Various signaling activities have been ascribed to p75NTR in both its unliganded and in its ligand-bound form (Barker, 1998; Bredesen et al., 1998; Barret, 2000). Moreover, p75NTR-mediated cell death has been reported to occur in response to ligands (Barker, 1998), or in a ligand-independent manner (Bredesen et al., 1998). The latter seems unlikely, however, because it has not been demonstrated *in vivo*, and most of the experimental paradigms used to

demonstrate ligand-independent p75NTR-mediated apoptosis have employed very high expression levels of p75NTR, which bear little resemblance to the *in vivo* situation (Rabizadeh et al., 1993; Bunone et al., 1997; Lievremont et al., 1999; Roux et al., 2001a). Therefore, while p75NTR may be capable of some level of constitutive signaling, it appears more likely that p75NTR-mediated apoptosis require ligand interaction.

Many p75NTR ligands are induced by seizure; a transient increase in mRNA for NGF and BDNF in the hippocampus and neocortex has been demonstrated during kindling, and after kainic acid or bicuculline-induced seizure (Zafra et al., 1990; Ballarin et al., 1991; Gall et al., 1991b; Isackson et al., 1991; Ernfors et al., 1991; Bengzon et al., 1992; Dugich-Djordjevic et al., 1992; Humpel et al., 1993; Wetmore et al., 1994). Consistent with this, we have found that pilocarpine-induced seizure leads to increase BDNF protein levels in the rat neocortex and hippocampus (P. Roux, unpublished results). The presence of BDNF, and possibly NGF, after pilocarpine-induced seizure suggests that these ligands may activate p75NTR in the injured tissues, thereby leading to p75NTR-dependent apoptotic cell death. It will be interesting to determine if the administration of neurotrophins or inhibitors of ligand binding to p75NTR will result in increased or reduced neuronal apoptosis after seizure.

5.2.5 Signaling pathways involved in seizure-induced apoptosis

The signaling pathways involved in seizure-induced apoptosis are still unknown, but studies using knock out mice have suggested the involvement of JNK3, an isoform of JNK enriched in the CNS, and the p53 tumor suppressor protein. Kainic acid-induced seizure of $JNK3^{-/-}$ or $p53^{-/-}$ mice resulted in decreased excitotoxicity-induced neuronal damage, compared to wild-type animals, suggesting an important role for these molecules in seizure-induced cell death (Morrisson et al., 1996; Yang et al., 1997). Following pilocarpine-induced seizure, we have found a correlation between JNK activation and apoptotic cell death (Figure 5.1), and consistent with a role for JNK in excitotoxicity-induced cell death, it has been shown that brain ischemia increases c-Jun phosphorylation (Herdegen et al., 1998), and interestingly, ischemia also leads to increased p75NTR expression (Kokaia et al., 1998; Bagum et al., 2001). While there is good evidence for the

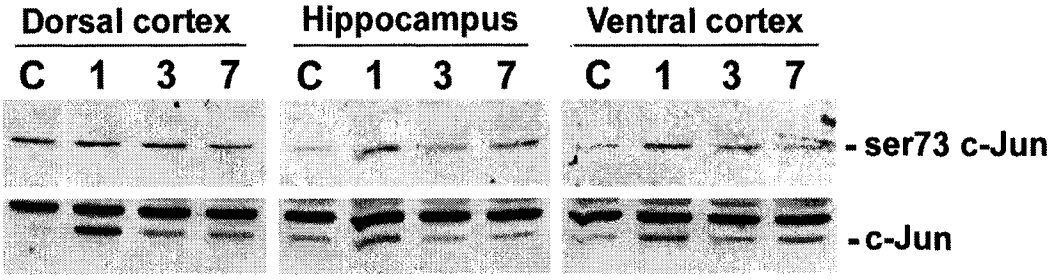

Figure 5.1. Pilocarpine-induced seizure promotes the accumulation of c-Jun and its phosphorylation in the rat brain. Protein homogenates from the dorsal cortex (neocortex), hippocampus, and ventral cortex (piriform and entorhinal cortices) of control, one, three, and seven days post-seizure rats were analyzed for levels of c-Jun (lower panels) and phosphorylated c-Jun on serine 73 (upper panels).

involvement of JNK in seizure-induced apoptosis, we did not find any correlation between apoptosis and p53 activation, measured by the level of protein and by its level of phosphorylation on serine 15 and 20 (P. Roux, unpublished results).

Signaling pathways activated by p75NTR remain elusive, but likely candidates that may be involved in p75NTR-mediated apoptosis include JNK and p53. JNK is induced following p75NTR activation in sympathetic (Yoon et al., 1998; Bamji et al., 1998) and hippocampal neurons (Friedman, 2000), and oligodendrocytes (Casaccia-Bonnefil et al., 1996), suggesting that p75NTR requires JNK to promote cell death. In sympathetic neurons, p75NTR-dependent apoptosis also correlated with accumulation of p53 (Aloyz et al., 1998), suggesting that p75NTR activation may require the JNK and/or p53-dependent pathways to promote apoptosis. Interestingly, p75NTR-mediated apoptosis of hippocampal neurons was blocked by the JNK pathway inhibitor CEP1347, suggesting that at least JNK may be essential for p75NTR-dependent apoptosis (Friedman, 2000). It will be interesting to test if p75NTR acts as an apoptotic receptor which mediates JNK and/or p53 activation in adult neurons and to determine if p75NTR expression constitutes the first step of a death process triggered by seizure.

5.3 THE DUAL SIGNALING FUNCTION OF P75NTR

Analysis of the p75NTR^{exonIII} mutant mouse (Lee et al., 1992), and the recently reported p75NTR^{exonIV} mutant mouse (von Shack et al., 2001), have shown that p75NTR is important during development for survival of several neuronal populations and for Schwann cells, while also being involved in the developmental cell death of sympathetic, motor, and retinal neurons (Frade et al., 1996; Bamji et al., 1998; Frade and Barde, 1999). While these developmental outcomes may result from a p75NTR-mediated modulation of Trk receptor activity, it is also possible that p75NTR regulates survival and death using Trk receptor-independent mechanisms. Interestingly, of the cytosolic adaptor proteins that have been shown to interact with p75NTR, some contribute to apoptosis, such as NRIF (Casademunt et al., 1999), NADE (Mukai et al., 2000), and NRAGE (Salehi et al., 2000), and others promote survival, including FAP-1 (Irie et al., 1999), TRAF6 (Khursigara et al., 1999), and RIP2 (Khursigara et al., 2001). These interactions suggest that p75NTR may induce apoptosis using NRIF, NADE, or NRAGE, and promote survival through binding to FAP-1, TRAF6, or RIP2. It remains a major challenge to link each of these to specific p75NTR signaling pathways, but the identification of these adaptor proteins suggest that p75NTR may collaterally regulate neuronal survival and death depending on the available network of cytosolic interactors.

5.3.1 p75NTR signals apoptosis

The ligand regulation of p75NTR is still poorly understood, and to circumvent this problem, we have generated recombinant adenoviruses encoding wild-type p75NTR, or truncated forms of the receptor (Roux et al., 2001a). The intracellular portion of p75NTR have been shown in transgenic mice to constitutively activate apoptosis during development and after injury (Majdan et al., 1997), and therefore, we have used recombinant adenoviruses expressing the intracellular domain of p75NTR to constitutively activate p75NTR signaling events. This approach has allowed us to precisely define the p75NTR signaling transduction pathway in a ligand-independent manner.

Death receptors of the TNFR superfamily, such as TNFR1 and Fas, induce apoptosis by recruiting TRADD or FADD, which lead to the activation of Caspase 8-dependent cell death (Baud and Karin, 2001). p75NTR was originally thought to activate similar pathways, but was later shown to be incapable of TRADD or FADD binding. Moreover, p75NTR-mediated apoptosis was found to correlate with activation of Caspase 9, and not Caspase 8 (P. Barker, unpublished results; Gu et al., 1999; Wang et al., 2001), suggesting that p75NTR promotes apoptosis through the mitochondrial apoptotic pathway. Consistent with this, we have found that p75NTR-mediated apoptosis results in mitochondrial release of Cytochrome c (A. Salehi, unpublished results).

As described in Chapter One, p75NTR can activate JNK in several cell types (Casaccia-Bonnefil et al., 1996; Yoon et al., 1998; Bamji et al., 1998; Aloyz et al., 1998; Friedman, 2000; Roux et al., 2001a), and mitochondrial cell death is tightly regulated by the JNK pathway (Tournier et al., 1999; Eilers et al., 2001; Bruckner et al., 2001; Harding et al., 2001; Whitfield et al., 2001). This raises the possibility that p75NTR-mediated activation of JNK results in Cytochrome c release and Caspase 9 activation, but the actual requirement of the JNK pathway in p75NTR-mediated apoptosis is still unknown. The JNK pathway inhibitor CEP1347 has been shown to inhibit p75NTR-mediated hippocampal cell death (Friedman, 2000), but as shown in Chapter Four, this inhibitor also promotes survival through JNK-independent pathways. Therefore, loss of function experiments, using JNK knock out cells, or dominant negative forms of proteins in the JNK pathway, will be useful to ascertain the role of JNK in p75NTR-mediated apoptosis. Ultimately, identification of genes activated by c-Jun-dependent transcription will be helpful in understanding the exact mechanisms of cell death.

TNFR1 and Fas are known to activate JNK though interaction with TRAF2. Extensive TRAF binding analysis revealed that only TRAF4 and TRAF6 interact with the intracellular domain of p75NTR (Khursigara et al., 1999; M. Grapes, unpublished results). These two TRAF proteins can activate NF-κB, but not JNK, suggesting that p75NTR must use other means to activate the JNK pathway. Several adaptor proteins of p75NTR have been identified, that represent potential links to the cell death machinery.

The recently identified adaptor protein NRAGE (Salehi et al., 2000), may link p75NTR with JNK activation. NRAGE has been found to facilitate p75NTR-dependent apoptosis (Salehi et al., 2000), and promotes JNK activation upon overexpression (A. Salehi, unpublished results). Moreover, NRAGE-mediated death have been found to correlate with Cytochrome c release, and Caspase 9 activation (A. Salehi and P. Barker, unpublished results). These results suggest that NRAGE is part of a p75NTR-mediated apoptotic pathway leading to JNK activation, and subsequent Cytochrome c release and Caspase 9-dependent cell death. Because NRAGE binds the juxtamembrane region of p75NTR, it will be interesting to determine if a mutant p75NTR protein lacking this region will still be capable of activating JNK and cell death. Conversely, upon determining the region of NRAGE responsible for p75NTR binding and JNK activation, expression of an isoform of NRAGE lacking one of these regions may block p75NTR-mediated effects.

5.3.2 p75NTR activates survival pathways

As discussed in Chapter One, neurotrophin binding to p75NTR results in the activation of sphingomyelinase and the production of ceramide (Dobrowsky et al., 1994, 1995; Casaccia-Bonnefil et al., 1996; Blochl and Sirrenberg, 1996; Brann et al., 1999). In some systems, sphingomyelinase activation results in a ceramide-dependent decrease in PIP_3 production and a subsequent reduction in Akt activity (Zundel and Giaccia, 1998; Zhou et al., 1998), and our initial hypothesis was that p75NTR-dependent ceramide accumulation would suppress PI3-K activity and thereby reduce Akt activation. However, using the same recombinant adenoviruses as above to constitutively activate p75NTR signaling pathways, we found that expression of p75NTR resulted in ligand-independent activation of Akt, which correlated with increased survival of several cell types exposed to distinct stressors (Roux et al., 2001a). High levels of p75NTR expression increased JNK activation and apoptosis, yet lower p75NTR expression levels were associated with activation of Akt and suppression of apoptosis induced by distinct stressors. These results indicated that p75NTR may promote apoptosis or survival depending on the cellular circumstances.

Other groups have also reported prosurvival functions for p75NTR in a variety of cell types (de Freitas et al., 2001; Khursigara et al., 2001), but the signaling mechanisms are still poorly understood. We have found that p75NTR-mediated Akt activation correlated with increased tyrosine phosphorylation of the p85 regulatory subunit of PI3-K, and the Shc adaptor proteins, suggesting that activation of a tyrosine kinase, or inhibition of a tyrosine phosphatase may play a role in this effect. The recent finding that the tyrosine phosphatase FAP-1 interacts with p75NTR has prompted us to analyse tyrosine phosphatase activity in cells expressing p75NTR isoforms. Interestingly, expression of p75NTR was found to reduce cytosolic tyrosine phosphatase activity, suggesting that p75NTR-mediated inhibition of a cytosolic tyrosine phosphatase may be a proximal event in the signaling cascade that allows a Shc/PI3-K complex to activate Akt-dependent survival. In contrast to these results, however, the phosphatase activity of FAP-1 has been shown to be necessary for protection against Fas-mediated death (Sato et al., 1995; Yanagisawa et al., 1997; Li et al., 2000), suggesting that inhibition of another tyrosine phosphatase may be necessary for p75NTR-mediated survival. It will be interesting to determine the functional interaction between p75NTR and FAP-1, or another cytosolic phosphatase, and to determine if a p75NTR mutant lacking the FAP-1 binding region is still capable of promoting survival and Akt activation.

An alternative mechanism that may account for p75NTR-mediated Akt activation involves a link between TRAF proteins and Src kinases. RANK and CD40, both TNFR superfamily members, have recently been shown to activate PI3-K and Akt through a signaling pathway involving TRAF6 and Src kinases (Wong et al., 1999; Arron et al., 2001; Xing et al., 2001). TRAF binding studies revealed that TRAF4 and TRAF6 can interact with p75NTR (Krajewska et al., 1998; Khursigara et al., 1999; M. Grapes, unpublished results), and interestingly, TRAF4 activates Akt (M. Grapes, unpublished results). Thus, the interaction of TRAF4 and TRAF6 with p75NTR, and the findings that these adaptor proteins are capable of activating Akt, raise the possibility that they may contribute to p75NTR-mediated Akt-dependent survival. Future experiments specifically examining Src signaling will be required to reveal the relative contribution of this

pathway in p75NTR-mediated Akt activation, and it will be important to determine which domain of p75NTR is necessary for this prosurvival function.

Several studies have demonstrated that p75NTR can activate the NF-κB pathway (Carter et al., 1996; Ladiwala et al., 1998; Maggirwar et al, 1998; Yoon et al., 1998; Bhakar et al., 1999; Hamanoue et al., 1999; Gentry et al., 2000; Hughes et al., 2001), and it has been suggested that RIP2 and TRAF6 binding to p75NTR are responsible for activation of NF-κB and survival (Khursigara et al., 1999, 2001). It is noteworthy that several TNFR family members activate NF-κB through a Akt-dependent pathway (Ozes et al., 1999; Romashkova and Makarov, 1999; Reddy et al., 2000), suggesting that p75NTR-mediated NF-κB and Akt activation may be interrelated. The mechanisms involved in TNFR1-mediated Akt activation are still unclear, but it has been found that Akt can phosphorylate and activate the IκB kinase (Ozes et al., 1999; Romashkova and Makarov, 1999; Reddy et al., 2000), providing a possible mechanism for the dual activation of Akt and NF-κB by p75NTR. Experiments that specifically ablate the Akt or NF-κB pathways will be helpful to understand the requirement of each signaling pathway in p75NTR-mediated survival.

5.3.3 Resolving the ligand-independent, survival versus death paradox

p75NTR can induce effects that run the gamut from activation of survival responses to facilitation of apoptosis (Roux et al., 2001a). The physiological significance of these effects is uncertain in the absence of a molecular mechanism that can explain these diverse actions. The canonical view of most receptor-ligand systems is that ligand binding initiates signal transduction cascades by altering the ability of receptors to interact with proximal signaling elements. In the case of Type I transmembrane proteins such as p75NTR, receptor oligomerization induced by ligand binding is thought to be the primary means by which this is achieved. However, various signaling activities have been described to p75NTR in both its unliganded and in its ligand-bound form (reviewed by Barker, 1998; Bredesen et al., 1998; Casaccia-Bonnefil et al., 1998; Barret, 2000), and clear consensus on the precise ligand-dependent signaling mechanisms of p75NTR has not emerged.

Recent studies have provided a new framework for considering the relationship of p75NTR signaling to ligand binding. Lenardo's group has recently shown that several TNF receptor superfamily members must actually pre-assemble as oligomers before they interact with ligand (Chan et al., 2000; Siegel et al., 2000). Ligand binding to these pre-assembled complexes was shown to mediate a conformational shift that alters the spatial relationship of the receptor's intracellular domain and thereby presumably affect signaling.

There are at least three reasons to believe that p75NTR may form an oligomeric complex that is required for ligand binding. First, the extracellular region necessary for oligomerization of Fas and TNFR1 receptors has been identified within the amino-terminal cysteine-rich domain 1 (CRD1) (Chan et al., 2000; Siegel et al., 2000). This region, termed the pre-ligand assembly domain (PLAD), shows very high conservation with the CRD1 of p75NTR (Locksley et al., 2001). Second, even in the absence of chemical crosslinking, p75NTR consistently migrates on SDS-PAGE as both monomeric (75-80 kDa) and oligomeric (160-200 kDa) forms (Grob et al., 1985), and recent findings indicate that p75NTR can be immunoprecipitated as a homodimer (Wang et al., 2001). Third, although CRD2-4 of p75NTR are responsible for neurotrophin binding to p75NTR (Yan and Chao, 1991; Baldwin et al., 1992), mutations in CRD1 ablate neurotrophin binding to the receptor (Baldwin and Shooter, 1995), consistent with the possibility that an assembly event mediated by CRD1 may be a prerequisite for ligand binding. The possibility that p75NTR exists as a pre-assembled oligomer may help explain its diverse signaling roles, assuming that both conformations may have increase affinity toward certain adaptor proteins over others, leading to increase survival or death signaling (Figure 5.2).

Another reason explaining the diverse biological function of p75NTR reside in its ability to modulate Trk receptor signaling. The cooperative actions of these receptors imply that the possibilities for extracellular signaling are greatly expanded. Moreover, the *in vivo* regulation of cellular differentiation and proliferation is likely determined by the

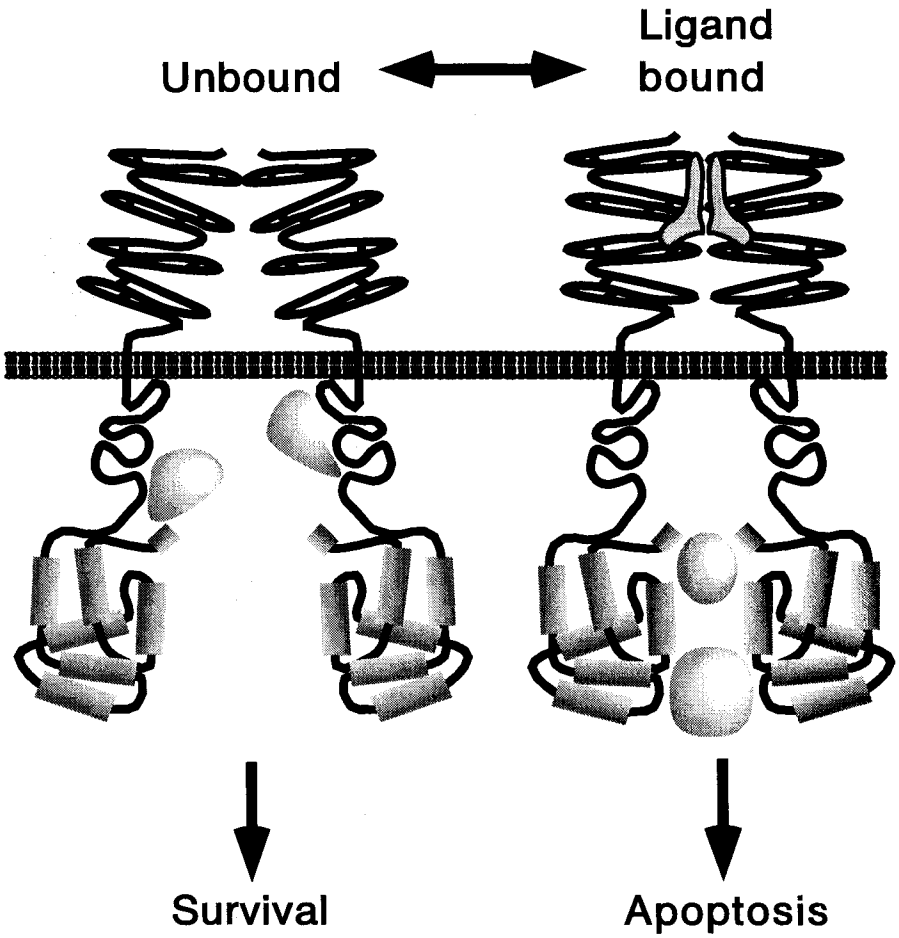

Figure 5.2. Hypothetical model explaining the dual mode of signaling of p75NTR. In the absence of ligand, p75NTR forms a homodimer through putative association between the first cysteine-rich domains. This conformation potentially promotes monomer-like association between p75NTR and adapter proteins, leading to increase survival. Neurotrophin binding to p75NTR would lead to a conformational change that results in the interaction of different adapter proteins, involved in apoptosis.

summation of signaling originating from different growth factor receptors. Careful analysis of the p75NTR^{exonIV} mutant mouse will be helpful to unveil the direct signaling mechanisms employed by p75NTR (von Shack et al., 2001). The generation of a conditional p75NTR knock out will also be useful in understanding the function of the receptor following injury. Undoubtedly, the recent identification of p75NTR interactors has sparked new directions in p75NTR research, but it will be a challenge to determine which signaling pathways are physiologically relevant. The identification of novel interactor families, and novel signaling cascades will be useful to better understand this enigmatic receptor.

5.4 KINASE INHIBITORS WITH NEUROTROPHIC EFFECTS

The K252 compounds were originally identified as potent protein kinase C inhibitors (Sezaki et al., 1985; Kase et al., 1986, 1987), but K252a has been widely used as a Trk kinase inhibitor (Berg et al., 1992; Nye et al., 1992; Tapley et al., 1992). Paradoxically, K252a also has neurotrophic effects on a variety of neuronal cells. K252a supports neurite outgrowth and survival of primary neurons and neural cell lines (Hashimoto and Hagino, 1989; Borasio, 1990; Tischler et al., 1991) and consistent with this, we have found that K252a promotes survival of primary cortical and cerebellar neurons, and PC12 cells (Roux et al., 2001b). CEP1347 is a derivative of K252a (Figure 5.3) that lacks Trk and protein kinase C-inhibitory activity (Figure 4.5)(Maroney et al., 1998), but retains its neuroprotective properties (Borasio et al., 1998; Glicksman et al., 1998; Maroney et al., 1998; Namgung and Xia, 2000; Pirvola et al., 2000). CEP1347 is currently used in clinical trial for the treatment of Parkinson's and Alzheimer's diseases. The neuroprotective mechanisms of K252a and CEP1347 are still incompletely understood, but appear to involve two independent pathways, the inhibition of the JNK pathway, and the activation of MEK and PI3-K (Roux et al., 2001b).

5.4.1 Inhibitors of the JNK pathway

As described in the first chapter, activation of the JNK signaling cascade is a general response to stress that links environmental changes to apoptotic pathways (reviewed in Davis, 2000). Sympathetic neurons deprived of NGF have been shown to undergo JNK-dependent cell death (Deshmukh et al., 1996; Deshmukh and Johnson, 1998; Putcha et al., 1999; Martinou et al., 1999; Tournier et al., 2000), through a process that requires c-Jun-dependent transcription (Bruckner et al., 2001; Eilers et al., 2001; Harding et al., 2001). The JNKs are activated by the dual specificity MAPKK MKK4/MKK7. Upstream of the MAPKKs, there are multiple MAPKKK families that regulate JNK activity, including the mixed lineage kinases (MLK). Many MAPKKKs are regulated by small GTPases via a Cdc42/Rac-interactive binding motif. Therefore, many distal kinases and small GTPases regulate the activation of the JNK pathway.

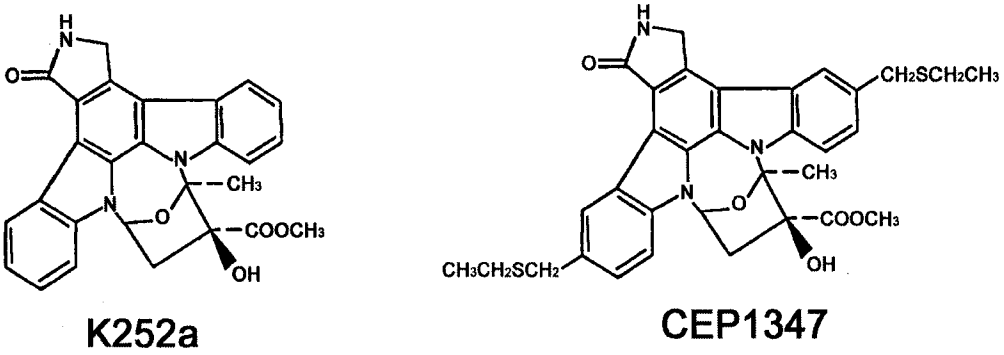

Figure 5.3. Chemical structure of the K252a and CEP1347 compounds.

CEP1347 has been shown to inhibit JNK activation in cell culture systems *in vitro* (Maroney et al. 1998; Wagner et al., 2000; Pirvola et al., 2000; Troy et al., 2001), and for many years, the mechanisms involved in this inhibition were unknown. Recent reports have shown that CEP1347 can directly inhibit members of the MLK family (Maroney et al., 2001; Xu et al., 2001), and consistent with this, we have found that CEP1347 inhibits MLK3 *in vitro* with an IC_{50} of approximately 5 nM (Roux et al., 2001b). Surprisingly, we have also found that the related compound K252a can also inhibit JNK activation (Figure 4.1), and inhibits MLK3 *in vitro* with an IC_{50} identical to CEP1347 (Figure 4.5)(Roux et al., 2001b). These results suggest that the neuroprotective activity of both K252a and CEP1347 may reside in their ability to suppress JNK activation through direct inhibition of the MLK. However, the use of MLK null cells will be required to determine whether CEP1347 promote survival solely through the inhibition of MLK enzymes.

5.4.2 Activators of survival pathways

Interestingly, K252a and CEP1347 do not simply inhibit apoptosis, but also activate neurotrophic pathways that result in somal hypertrophy, neurite extension and neurotransmitter synthesis (Glicksman et al., 1995; Maroney et al., 1995; Borasio et al., 1998; Glicksman et al., 1998; Harris et al., 2000). It is unlikely that the full neurotrophic effects of these compounds is due simply to inhibition of the JNK pathway, and consistent with this, we found that low concentrations of K252a and CEP1347 rapidly activate Akt and ERK signaling in primary cortical and cerebellar neurons, as well as in PC12 cells (Roux et al., 2001b). Intriguingly, chemical inhibition of PI3-K and MEK suppressed K252a and CEP1347-mediated activation of Akt, ERK, and survival, suggesting that K252a and CEP1347 require these pathways to promote survival. These results also indicated that the primary target(s) for K252a and CEP1347 lie upstream of PI3-K and MEK.

One conceivable explanation for increased Akt and ERK activity induced by K252a and CEP1347 is that the MLKs normally phosphorylate and thereby negatively regulate a common upstream activator of Akt and ERK pathways. In this scenario, K252a- and CEP1347-mediated inhibition of MLKs would indirectly result in activation of Akt and

ERK. If MLK3 was part of a pathway that normally negatively regulates a common upstream activator of Akt and ERK pathways, one would expect that overexpression of MLK3 would lead to decreases in the activation levels of Akt and ERK. However, we have found that MLK3 overexpression had no effect on basal or induced Akt or ERK activity in cells in which the enzyme dramatically increased JNK activation (Figure 4.6)(Roux et al., 2001b). Furthermore, K252a and CEP1347 clearly increased Akt and ERK activity under conditions where the compounds simultaneously blocked MLK3-mediated JNK activation. Therefore, K252a and CEP1347 appeared capable of activating Akt and ERK activity through unique targets distinct from MLK3. To clearly prove this hypothesis, future experiments must include the use of primary neurons derived from MLK1, 2 and 3 knock out mice. The presence of a MLK-independent pathway could be confirmed by showing that K252a and CEP1347 are still capable of supporting survival and activating Akt and ERK in these cells lacking MLK expression.

5.4.3 New therapeutic targets?

Identification of the upstream targets of CEP1347 and K252a which give rise to increased Akt and ERK activity may provide novel insights into aspects of neurotrophic signaling which are amenable to therapeutic intervention. Aside from the MLKs, there are no known inhibitory targets of both K252a and CEP1347 that could mediate their neuroprotective effects. However, some findings support the hypothesis that Src family members may be involved in K252a and CEP1347-mediated survival. The first piece of evidence comes from the finding that K252a-mediated activation of Akt and ERK can be blocked by the Src specific inhibitor, PP1 (Figure 5.4), indicating that Src kinase activity is required for K252a-mediated activation of Akt and ERK. Secondly, K252a has been reported to activate the tyrosine kinase Fak (Maroney et al., 1995), and consistent with this, we have observed an increase in Fak tyrosine phosphorylation using a kinase screen designed to identify targets of K252a (P. Roux, unpublished data). Fak binds Src kinases, and induces the PI3-K/Akt pathway upon Src activation (Jones et al., 2000). Thirdly, Src family members are known to be inhibited by the carboxyl-terminal Src kinase (Csk), a

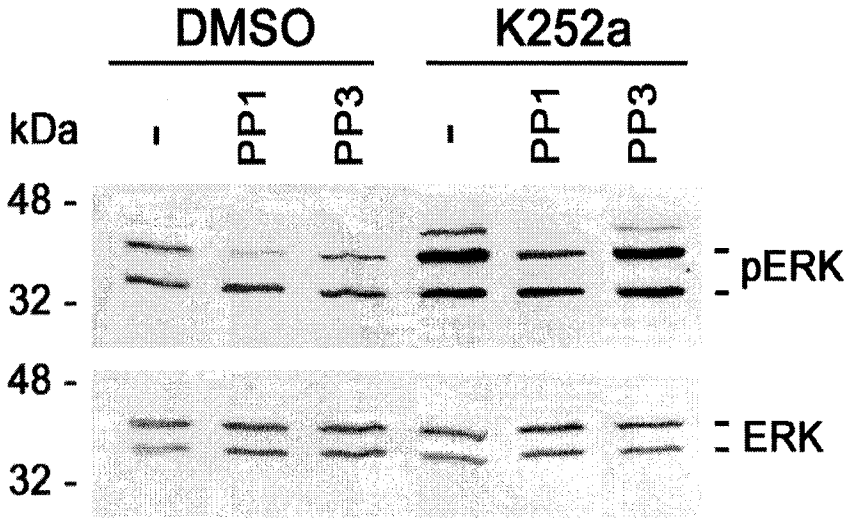

Figure 5.4. Inhibition of K252a-mediated activation of ERK by the Src-specific inhibitor PP1. PC12nnr5 cells were serum-deprived for 18 hours, and treated with 100 nM K252a for 60 minutes, with or without 5 μM PP1 or PP3. Protein lysates were then analyzed for ERK protein levels and ERK phosphorylation content.

protein tyrosine kinase that phosphorylates and thereby negatively regulates Src kinases such as c-Src and Lck (Superti-Furga and Courtneidge, 1995; Xu et al., 1997; Sicheri et al., 1997). K252a inhibits protein kinase C and Trk by competing with ATP at the ATP binding site of the enzymes (Angeles et al., 1998), and CEP1347 appears to function similarly (Maroney et al., 2001). Therefore, it is possible that K252a and CEP1347 inhibit Csk, which in turn would lead to a loss of Src inhibition, leading to Src-mediated Akt and ERK activation. Therefore, Csk represents a potential target of K252a and CEP1347, and may represent a novel class of enzyme with therapeutic value.

5.5 CONCLUSIONS

In the first part of this study, we clearly demonstrated induced expression of p75NTR in apoptotic neurons of the seized rat brain, which suggested that p75NTR may regulate cell fate following traumatic injuries of the central nervous system. In the second part of the study, we found that p75NTR was capable of promoting cell death and survival depending on the cellular circumstances, and have described potential signaling mechanisms utilized by the receptor. Finally, we have discovered the mechanism of action of two known neuroprotective compounds, and suggested the importance of these novel signaling pathways for the facilitation of neuronal survival.

The most striking finding of this study comes from the demonstration that p75NTR can function in a least two ways. On the one hand, p75NTR can induce JNK activation and apoptosis, and on the other, it can mediate Akt activation and survival. These results suggest that p75NTR may have a dual purpose during development or in the adult nervous system, and may be capable of promoting survival of some cells, while inducing apoptosis of others. The mechanisms regulating p75NTR are still unknown, but the presence of ligands, adaptor proteins, or Trk receptors, may contribute to the differential effects mediated by p75NTR. Our finding that p75NTR have dual functions may be very helpful to explain some of the ligand and cell type specific effects reported for p75NTR. It remains a major challenge to understand the regulation of p75NTR, but the recent discovery of novel families of adaptor proteins will no doubt provide new insights into the intricacies of p75NTR activity.

Understanding the full complement of functional interactions between each components of the survival and apoptotic pathways will help our understanding of the roles these molecules play in the regulation of neuronal fate. This, in turn, will aid our understanding of the intricate mechanisms that make up these complicated cellular processes. Ultimately, the goal of such research is to obtain a more complete mechanistic picture of the inner workings of the cell, eventually allowing us to synthesize ways to modulate these processes.

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APPENDIX

Copyright waivers from the co-authors and the publisher for:

Philippe P. Roux, Michael A. Colicos, Philip A. Barker, and Timothy E. Kennedy. P75 NEUROTROPHIN RECEPTOR EXPRESSION IS INDUCED IN APOPTOTIC NEURONS AFTER SEIZURE. **Journal of Neuroscience 1999; 19: 6887-6896**.

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Philippe P. Roux, Matthieu Boudreau, and Philip A. Barker. K252A AND CEP1347 MEDIATE SURVIVAL BY ACTIVATING PI3-K AND MEK. In preparation.

CURRICULUM VITAE

RESEARCH RECORD

(a) Publications

Philippe P. Roux, Asha L. Bhakar, Timothy E. Kennedy, and Philip A. Barker. THE P75 NEUROTROPHIN RECEPTOR (P75NTR) ACTIVATES AKT (PROTEIN KINASE B) THROUGH A PHOSPHATIDYLINOSITOL 3-KINASE-DEPENDENT PATHWAY. **Journal of Biological Chemistry** 2001; 276: 23097-23104.

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Matthew GR. Grapes, Asha L. Bhakar, **Philippe P. Roux**, and Philip A. Barker. STRUCTURAL AND FUNCTIONAL RELATIONSHIP OF TRAF4 AND ITS ASSOCIATION WITH THE P75 NEUROTROPHIN RECEPTOR. In preparation.

(b) Patents

Philip A. Barker, **Philippe P. Roux**, and Timothy E. Kennedy. A METHOD FOR DETECTING NEURONAL CELL SURVIVAL. **Patent pending** 60/108.375 «US».

Jerome E. Tanner, Caroline Alfieri and **Philippe Roux**. VPR-DRIVEN DNA OR RNA CONSTRUCT AND THERAPEUTIC USES THEROF. **Patent pending** 12667-14 «US».

(c) Abstracts

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