# Mechanisms underlying p75 neurotrophin receptor-regulated neuronal survival and apoptosis

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*Dedicated to M.*

Bandar kya jane adhrak ka swat.

- *Hindi proverb*

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# CONTRIBUTION OF AUTHORS

## Chapter 2

I performed all experiments that contributed to the manuscript entitled "The p75 neurotrophin receptor (p75NTR) alters tumour necrosis factor-mediated NF-kB activity under physiological conditions, but direct p75NTR-mediated NF-kB activation requires cell stress", with the exception of the transcriptional assay, which was performed by Ms. Christine Zeindler and two confirmatory Western blots which was performed by Dr. Philippe Roux. Dr. Christian Lachance and David Kryl provided useful discussions and technical assistance. Both Dr. Philip Barker and I contributed to the writing of the manuscript.

Contribution of figures:

| | |
|---|---|
| A. Bhakar | Figures 2.1, 2.2, 2.3, 2.4, 2.5 |
| C. Zeindler | Figure 2.6 |
| P. Roux | Figure 2.3b bottom panel, 2.4b bottom panel |

## Chapter 3

I performed all experiments within the manuscript entitled "Constitutive NF-kB activity in the brain". The transgene cassette was constructed by Dr. Sandra McPherson. Ms. Laura-Lee Tannis and Ms. Christine Zeindler provided excellent technical assistance with the mouse colony. Drs. Maria Pia Russo, Christian Jobin, and David Park provided the dominant negative NIK recombinant adenovirus and technical support. Both Dr. Philip Barker and I contributed to the writing of the manuscript.

Contribution of figures:

| | |
|---|---|
| A. Bhakar | Figures 3.1, 3.2, 3.3, 3.4, 3.5, 3.6 |

## Chapter 4

For the manuscript entitled "Apoptosis Induced by p75NTR Requires Jun Kinase-dependent Phosphorylation of Bad", I established the conditions and experimental systems for all of the experiments performed. I performed all of the primary cultures and infections involved with the exception of the last figure. I generated all of the figures, performed all of the statistical operations and provided intellectual and hands-on training for C. Paul and J. Howell to perform the last two experiments and take over the project. A. Salehi performed the subcellular fractionation and initial PC12rtta experiment, E. Becker and A. Bonni provided the dominant-negative Bad constructs and the phospho-Bad antibody. F. Said provided technical assistance for the amplification of the p75NTR recombinant adenovirus. Both Dr. Philip Barker and I contributed to the writing of the manuscript.

Contribution of figures:

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| A. Bhakar | Figures 4.1 all, 4.2a, b, c, 4.3 all, 4.4 all, 4.5 all, 4.6b, 4.8a, b, c |
| A. Salehi | Figure 4.2 part c, 4.6 part a |
| C. Paul | Figure 4.7, 4.9 |
| J. Howell | Figure 4.8 part c, top panel only, 4.9 |
| E. Becker | Figure 4.10 |

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# ABSTRACT

Mature neurons are among the most long-lived cell types in mammals. Yet large numbers of neurons die during the early stages of development and later in life when neurodegenerative diseases set in or whenever the neurons are subjected to trauma. The processes that control neuronal survival and death are not fully understood. However, key regulators of these events include a family of proteins called the neurotrophins.

The neurotrophins are growth factors that control neuronal development and maintenance in addition to survival. Neurotrophins interact with two types of cell surface receptors, the Trk family of tyrosine kinases, and the p75 neurotrophin receptor (p75NTR). Roles for Trk receptors in neurotrophin-dependent effects are well established but the functions of p75NTR have been less clear. So far, three main functions have been determined. First, p75NTR positively or negatively modulates Trk receptor signaling. Second, p75NTR autonomously activates signaling cascades that, for the large part, regulate cellular apoptosis. Third, p75NTR cooperates with receptors that respond to myelin-based growth inhibitory signals. The signaling pathways used by p75NTR to mediate its effects are unclear, but p75NTR activates the Akt and JNK pathways, modulates RhoA activity, and interacts with several adaptor proteins.

The discovery of the TNFR (Tumour Necrosis Factor Receptor) family of related receptors that are capable of regulating cell death suggested that p75NTR might activate similar cellular responses. One well-studied effect is the activation of the transcriptional complex, NF-κB. In the first part of this dissertation, we compared p75NTR and TNF-R1-mediated NF-κB activation in a variety of cell types and found that p75NTR directly activates NF-κB only under conditions of cellular stress. Under normal growth conditions, p75NTR modulates TNF-dependent NF-κB activation to ultimately increase the levels of active NF-κB within the cell. This suggests that a significant physiological function of p75NTR is to modulate cytokine receptor signaling.

In the second study, we generated a transgenic NF-κB reporter mouse to clarify the role and the pattern of transcriptionally active NF-κB within the nervous system. We found high levels of constitutive NF-κB activity restricted to neurons of the developing and mature CNS. We also demonstrated that NF-κB activity is required for cortical neuron survival, and that NF-κB activity provides neurons with a high degree of neuroprotection through the production of anti-apoptotic proteins.

In the final study, we used recombinant p75NTR adenovirus to define the signals necessary for p75NTR-activated cell death. We found that p75NTR activation leads to JNK activation, mitochondrial release of Cytochrome c, activation of Caspases 9, 3, 6, and ultimately cell death in all p75NTR-sensitive cells. We demonstrated that this p75NTR-initiated death pathway requires JNK activity. We also identified the pro-apoptotic Bcl-2 family member, Bad, as a JNK target required to mount this death response.

Taken together, this dissertation will add to our understanding of the physiological functions of p75NTR and contribute to our knowledge of the cellular machinery that controls neuronal cell survival and death.

# RÉSUMÉ

Les neurones matures sont parmi les cellules avec la durée de vie la plus longue chez les mammifères. Cependant, un nombre important de neurones meurt pendant les phases initiales du développement et plus tard, lors de l'apparition de maladies neurodégénératrices ou quand les neurones subissent un trauma. Les processus qui contrôlent la survie et la mort neuronale ne sont pas complètement maitrisés. Toutefois, parmi les régulateurs clés de ces évènements existe une famille de protéines appelées les neurotrophines.

Les neurotrophines sont des facteurs de croissance qui contrôlent le développement et l'entretien des neurones, en plus de leur survie. Les neurotrophines interagissent avec deux types de récepteurs, les tyrosine kinases de la famille Trk, et le récepteur de neurotrophines p75 (p75NTR). Les fonctions des récepteurs Trk dans les effets neurotrophinaux sont bien etablis, mais les fonctions de p75NTR ont été moins bien identifiées. A ce jour, seuls trois fonctions majeures ont été déterminées. Premièrement, p75NTR peut moduler l'activité des récepteurs Trk d'une façon positive ou négative. Deuxièmement, p75NTR peut activer des signaux de façon indépendante pour principalement réguler l'apoptose cellulaire. Troisièmement, p75NTR est un co-récepteur de signaux inhibitoires de croissance dérivé de myelin. Les mécanismes exacts utilizes par p75NTR sont encore peu connus, mais il a été démontré que l'activation de p75NTR mène à l'engagement des voies de signalisation Akt et JNK, aux modulations de l'activité de RhoA, et à l'interaction du récepteur avec plusieurs proteins cytosoliques.

La découverte de tels récepteurs de la famille TNFR, capables de réguler la mort de cellules, a suggeré que p75NTR pourrait activer les memes réponses cellulaires. Un effet bien étudié est l'activation du complexe transcriptionel, NF-κB. Dans la première partie de cette dissertation, nous avons comparé l'activation de NF-κB par p75NTR et TNF-R1 dans une variété de cellules, et nous avons trouvé que p75NTR n'active NF-κB que sous conditions de stress de la cellule. Sous des conditions de croissance normale, p75NTR peut moduler l'activation de NF-κB par TNF pour que le montant de NF-κB activé dans

la cellule soit élevé. Ces effets suggèrent qu'une des fonctions physiologiques de p75NTR est de moduler l'activité des récepteurs cytokines.

Dans la deuxième étude, nous avons généré une souris trangénique avec un reporteur de NF-kB pour clarifier le rôle et les régions de NF-κB actif dans le système nerveux central (SNC). Nous avons trouvé une intense activité constitutive de NF-κB limitée aux neurones du SNC mature et en développement. Nous avons aussi démontré que l'activité de NF-κB est nécessaire pour la survie des neurones corticaux, et que l'activité NF-κB élèvé produit des neurones avec un haut niveau de neuroprotection en produisant les protéines anti-apoptoses.

Dans la dernière étude, nous avons utilisé un adenovirus recombiné de p75NTR pour établir les signaux nécessaires pour activé l'apoptose par p75NTR. Nous avons trouvé que l'activation de p75NTR amène l'activation de JNK, une libération de Cytochrome c du mitochondrie, l'activation des Caspases 9, 3 et 6, et finalement la mort de cellules dans toutes les cellules sensibles. Nous avons montré que l'activité de JNK est nécessaire pour l'apoptose initiée par p75NTR. Nous avons aussi identifié Bad, un member pro-apoptic de la famille Bcl-2, comme cible de JNK nécessaire pour accomplir cette réponse de mort de la cellule.

En conclusion, les résultats dans cette thèse apportent une meilleure connaissance des rôles physiologiques de p75NTR, et contribuent à notre savoir sur les mécanismes cellulaires impliqués dans la survie et la mort des neurones.

# LIST OF ABBREVIATIONS

| | |
|---|---|
| **Akt** | Protein kinase B, or protein kinase related to A and C |
| **ALS** | Amyotrophic lateral sclerosis |
| **AMPA** | alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| **APP** | Amyloid precursor protein |
| **ARMS** | Ankyrin-rich membrane spanning |
| **ATF-2** | Activating transcription factor-2 |
| **BCA** | Bicinchoninic acid |
| **Bcl-2** | B cell lymphoma-2 protein |
| **BDNF** | Brain-derived neurotrophic factor |
| **BH3** | Bcl-2 homology-3 |
| **$Ca^{2+}$** | Calcium |
| **CARD** | Caspase recruitment domain |
| **CDK** | Cyclin-dependent kinase |
| **CNS** | Central nervous system |
| **$cPLA_2$** | Cytosolic phospholipase $A_2$ |
| **CRD** | Cysteine-rich domain |
| **CREB** | cAMP response element-binding protein |
| **CRNF** | Cysteine-rich neurotrophic factor |
| **DAG** | Diacylglycerol |
| **DD** | Death domain |
| **DISC** | Death-inducing signaling complex |
| **DNA** | Deoxyribonucleic acid |
| **DOC** | Deoxycholate |
| **DR** | Death receptor |
| **DTT** | Dithiothreitol |
| **ECD** | Extracellular domain |
| **EGF** | Epidermal growth factor |
| **EMSA** | Electrophorectic mobility shift assay |
| **ER** | Endoplasmic reticulum |
| **ERK** | Extracellular signal-regulated kinase |
| **FADD** | Fas-associated death domain-containing protein |
| **FAP-1** | Fas-associated phosphatase-1 |
| **FKHR** | Forkhead |
| **FRS2** | Fibroblast growth factor receptor substrate-2 |
| **GAP** | GTPase-activating protein |
| **Gab-1** | Grb-associated binder-1 |
| **GDI** | GTP-dissociation inhibitor |
| **GEF** | Guanine nucleotide exchange factor |
| **GFP** | Green fluorescent protein |
| **GSK-3** | Glycogen synthase kinase-3 |
| **GTP** | Guanine triphosphate |
| **HIV** | Human immunodeficiency virus |
| **IAP** | Inhibitor of apoptosis |

| | |
|---|---|
| **ICD** | Intracellular domain |
| **Ig** | Immunoglobin |
| **IKK** | IkB kinase |
| **IL-1** | Interleukin-1 |
| **iNOS** | Inducible nitric oxide synthase |
| **$IP_3$** | Inositol triphosphate |
| **IRAK** | IL-1 receptor-associated kinase |
| **IRS** | Insulin receptor substrate |
| **JNK** | c-Jun amino-terminal kinase |
| **JXM** | Juxtamembrane domain |
| **kb** | Kilobase pair |
| **KCl** | Potassium chloride |
| **Kd** | Dissociation constant |
| **kDa** | KiloDalton |
| **LTP** | Long-term potentiation |
| **LTR** | Long terminal repeats |
| **MAG** | Myelin-associated glycoprotein |
| **MAGE** | Melanoma antigen |
| **MAPK** | Mitogen-activated protein kinase |
| **MEFs** | Mouse embryonic fibroblasts |
| **MEKK** | MAPK kinase kinase |
| **MHD** | MAGE homology domain |
| **MKK** | MAPK kinase |
| **MLK** | Mixed lineage kinase |
| **mRNA** | Messenger ribonucleic acid |
| **MTT** | 3(4,5-dimethylthiozol-2-yl)2,5-diphenyltetrazolium bromide |
| **NADE** | Neurotrophin receptor-associated death effector |
| **NF-κB** | Nuclear factor-kappaB |
| **NGF** | Nerve growth factor |
| **NgR** | Nogo receptor |
| **NIK** | NF-κB inducing kinase |
| **NLS** | Nuclear localization signal |
| **NMDA** | N-methyl-D-aspartate |
| **NMJ** | Neuromuscular junction |
| **Nnr5** | Neurotrophin non-responsive cell line 5 |
| **NRH** | Neurotrophin receptor homologue |
| **NRIF** | Neurotrophin receptor-interacting factor |
| **NT-3, NT-4/5** | Neurotrophin-3, neurotrophin-4/5 |
| **NRAGE** | Neurotrophin receptor-interacting Mage homolog |
| **OMgp** | Oligodendrocyte-myelin glycoprotein |
| **ONPG** | O-nitrophenyl-β-galactopyranoside |
| **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 |
| **PLAD** | Pre-ligand binding assembly domain |
| **PLAIDD** | P75NTR-like apoptosis-inducing DD protein |
| **PLCγ** | Phospholipase C gamma |
| **PMSF** | Phenylmethylsulfonyl fluoride |
| **PNS** | Peripheral nervous system |
| **PrP** | Prion protein fragment |
| **PTPase** | Protein tyrosine phosphatase |
| **RIP-2** | Receptor-interacting protein-2 |
| **RSK** | Ribosomal S6 kinase |
| **SC-1** | Schwann cell factor-1 |
| **SDS-PAGE** | Sodium dodecyl sulfate-polyacrylimide gel electrophoresis |
| **SH2** | Src homology domain-2 |
| **SIIK** | Serine-, threonine-specific innate-immunity kinase |
| **SODD** | Silencer of death domain |
| **SOS** | Son of sevenless |
| **TM** | Transmembrane |
| **TNF** | Tumor necrosis factor |
| **TNFR** | TNF receptor |
| **TNFRSF** | TNFR superfamily |
| **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 |
| **UNC5** | Uncoordinated 5 |

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# CHAPTER 1

# INTRODUCTION AND LITERATURE REVIEW

# 1.0 GENERAL INTRODUCTION

## 1.0.1 The Neurotrophic Hypothesis and Apoptosis within the Nervous System

During the development of the nervous system, immature neurons are initially produced in excess. Only a portion of these is maintained while others are removed by a natural process of cell death. This selection process begins when newly born neurons extend their axons toward targets, which secrete limited amounts of support signals. Neurons that are unsuccessful in establishing correct synaptic connections will lack the trophic factor support necessary for their survival and thus 'die off'. This phenomenon was first described by Hamburger and Levi-Montalcini (Hamburger and Levi-Montalcini, 1949) and later coined as the Neurotrophic Hypothesis (Purves et al., 1986; Oppenheim, 1991).

The Neurotrophic Hypothesis predicts a positive survival signal. It does not, however, provide a concrete explanation as to how neurons die in the absence of trophic support. For many years, it was assumed that neurons die simply of passive starvation in the absence of trophic factors. But in 1988, Johnson and colleagues (Martin et al., 1988) established that the death of sympathetic neurons caused by neurotrophic factor removal could be prevented by transcription and translation inhibitors. These experiments provided the first evidence that neurons might instigate their own demise. Soon after, cell death gene products, identified in lower species, were found to participate in trophic deprivation-induced death (Sadoul et al., 1996; Schwartz et al., 1994; Troy et al., 1996). This result confirmed that vertebrate neurons can activate a cellular suicide program called apoptosis.

Although apoptosis is necessary for proper neuronal development, too much apoptosis is a feature of the neuronal damage in several acute and chronic neurodegenerative diseases. For example, in laboratory rats given experimental strokes, a significant percentage of neurons die in an apoptotic manner (Linnik et al., 1993). This death entails protein synthesis and activation of the cell death gene products identical to that found in developmental death. In fact, in the mid to late 90's, inhibiting protein synthesis or these

gene products in stroke-induced animals resulted in a smaller damaged area and left animals with fewer neuronal impairments (Hara et al., 1997a, b).

### 1.0.2 Key Regulators of Neuronal Survival and Apoptosis

Identifying the trophic factors that mediate survival-promoting effects on neurons has been the focus of intensive research for many years (reviewed in Hendersen, 1996). The physiologically relevant factors are numerous and belong to a number of gene families including transforming growth factor βs, several cytokines, and the neurotrophins. The neurotrophins, initially identified as target-derived survival factors, are the best characterized survival factors for mammalian neurons. In fact, in the 1950s, following their prediction of a positive target-derived survival factor, Levi-Montalcini, in collaboration with Stanley Cohen and Vicktor Hamburger, identified and isolated the first secreted trophic factor which is the neurotrophin, NGF (nerve growth factor) (for review see Marx, 1986). Moreover, in 1986, Levi-Montalcini and Cohen received the Nobel prize in Physiology and Medicine for this work along with the discovery and isolation of another growth factor, EGF (epidermal growth factor).

Even today work continues in this vital area of research. However, recent research has found that neurotrophins are also able to regulate additional biological responses, including the induction of cell death (reviewed in Bibel and Barde, 2000; Huang and Reichardt, 2001). Regardless, all neurotrophin functions are mediated by interactions with two structurally unrelated receptor types, the tropomyosin-related kinase (Trk) tyrosine kinase receptors and the p75 neurotrophin receptor (p75NTR). These receptors can act independently to activate different signaling pathways. However, collaboration of these receptors does occur and in many instances is necessary to mediate appropriate neurotrophin effects. In the following thesis review, the functions of neurotrophins and their receptors will be described, emphasizing evidence supporting a role for p75NTR in the regulation of neuronal survival and death.

## 1.1 THE NEUROTROPHINS

### 1.1.1 Neurotrophin Structure and Expression

The mammalian neurotrophin family includes four members. The first identified member, nerve growth factor (NGF), was isolated as a factor able to support the survival and growth of peripheral neurons in culture (Levi-Montalcini, 1987). However, few central neuron populations are dependent on NGF for survival, and the isolation of the second factor (Leibrock et al., 1989), brain-derived neurotrophic factor (BDNF), was key in establishing the importance of these trophic cues for the entire vertebrate nervous system (reviewed in Hofer and Barde, 1988). Homologies between BDNF and NGF provided the impetus for cloning and characterizing the rest of the family that now includes neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) (Ernfors et al., 1990; Hohn et al., 1990; Jones et al., 1990; Maisonpierre et al., 1990).

All neurotrophins are generated as 30-35kDa precursor proteins containing a signal peptide, glycosylation sites, and basic amino acid pairs for cleavage by Furin and prohormone convertases. Cleavage of the precursors releases mature 12-14kDa proteins that associate into noncovalent homodimers. Within each neurotrophin subunit, an ordered array of six disulfide bonds creates an essential cysteine knot. This three dimensional fold has since been found in other trophic factors including transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) (reviewed in McDonald and Hendrickson, 1993).

Both processed and unprocessed neurotrophins are secreted from a number of tissues and cell types. Consistent with the neurotrophic hypothesis, neurotrophins are synthesized and released by sympathetic and sensory target organs including the iris, the pineal gland, the submandibular gland, skin, and blood vessels (reviewed in Korsching, 1993). Neurotrophins are also synthesized and released by neurons, and both their synthesis and secretion can be constitutive or depend on neuronal activity (Thoenen, 1995). Nerve lesion sites are another major source of neurotrophins. Activated autoimmune T or mast cells summoned to injury sites release neurotrophin as well as cytokine-activated

neighbouring Schwann cells and fibroblasts within the injury site (reviewed in Korsching, 1993; Levi-Montalcini, 1996).

Once secreted, neurotrophins act locally, in both autocrine and paracrine fashions, by binding to the neurotrophin cell surface receptors, p75NTR and TrkA, B, or C (see below) (Acheson et al., 1995). In some circumstances, neurotrophin/receptor complexes are then internalized and transported in one of two ways. They can be transported back from nerve terminals toward the cell body (Grimes et al., 1996, 1997), or down neuronal axons to act trans-synaptically on afferent projections (Fawcett et al., 1998; Altar et al., 1997; Brady et al., 1999; von Bartheld et al., 1996).

### 1.1.2 Neurotrophin Functions

The biological roles of neurotrophins are broad, but they are best known for their effects on cell growth and survival. During embryonic development, neurotrophins promote the survival of peripheral neurons before and during target innervation (Henderson, 1996; Lewin and Barde, 1996; Davies, 1997). Neurotrophins function as survival signals for central neurons (Alcantara et al., 1997; Schwartz et al., 1998) and regulate other cell fate decisions including cell proliferation (Sieber-Blum et al., 1991; Barres et al., 1994) and neuronal differentiation (Jones et al., 1994; Smeyne et al., 1994; Vicario-Abejon et al., 1995; Altar et al., 1997; Ghosh et al., 1995). Paradoxically, in some cases neurotrophins can regulate survival by inducing apoptotic responses (see below). Also, motility growth events including neurite extension, branching, growth cone guidance, and cell migration, are promoted by neurotrophins, particularly in neuronal populations whose survival they can support (Albers et al., 1994; Edwards et al., 1989; Guidry et al., 1998; Patel et al., 2000; Schnell et al., 1994; Cohen-Cory et al., 1995; Cabelli et al., 1997; Shieh et al., 1997; Brunstrom et al., 1997; reviewed in Thoenen, 1991).

In the mature nervous system, neurotrophins function to modulate synaptic properties, regulating both short-term synaptic transmission and long-term potentiation (LTP) (Thoenen, 1995; Sheih et al, 1997). This includes dramatic and sustained enhancement of synaptic strength through presynaptic terminal modification (Kang et al., 1995), and via

direct, neurotrophin-mediated induction of action potentials within postsynaptic cells (Kafitz et al., 1999). Therefore, in addition to regulating survival, neurotrophins are important regulators of neural development, function and plasticity (reviewed in Lewin and Barde, 1986; Bibel and Barde, 2000; Huang and Reichardt, 2001).

### 1.1.3 Neurotrophins in Disease

Neurotrophin expression is often mis-regulated in nervous system diseases and disorders. Dramatic changes in neurotrophin expression have been found in neurological disorders including Alzheimer's (Mufson et al., 1989; Phillips et al., 1991), Huntington's (Canals et al., 1998), Parkinson's (Hyman et al., 1991), peripheral neuropathies (Fressinaud et al., 2003; Cai et al., 1999; Yiangou et al., 2002; Kennedy et al., 1998; Apfel, 1999), epilepsy (Gall and Isackson, 1989; Isackson et al., 1991), and a variety of CNS cancers (Kogner et al., 1993; Segal et al., 1994; Ryden et al., 1996). Upregulation of neurotrophins is also observed under pathological inflammatory conditions, particularly after nerve injury and damage to the vascular system (Donovan et al., 1995). More recently, genetic polymorphisms in neurotrophin prodomains have been linked to deficits in learning and memory (Egan et al., 2003) and sometimes neurological disease (Ribases et al., 2003; Kanemoto et al., 2003; Riemenschneider et al., 2002). In one example, a single amino acid change in proBDNF results in disrupted intracellular trafficking of BDNF. This disruption is believed to substantially decrease the amount of synaptically released BDNF, but this remains to be shown (Egan et al., 2003).

Accordingly, the therapeutic use of neurotrophins has proved successful in certain experimental models of these diseases. Neurotrophins have been used to treat symptoms of traumatic nervous system lesions, ischemic damage, and ALS within mice (reviewed in Thoenen and Sendtner, 2002). Similarly, intraventricular injection of NGF has successfully reversed basal forebrain cholinergic-dependent memory deficits and degenerative changes in rats (Winkler et al., 1998). Clearly then, understanding neurotrophin action may provide insight into the mechanisms, and therefore treatment, of nervous system disease.

## 1.2 THE TRK RECEPTORS

### 1.2.1 Trk Structure and Neurotrophin Binding

Many effects of neurotrophins are mediated by Trk receptor signaling (reviewed in Kaplan and Miller, 2000). Three genes in mammals give rise to the three major Trk receptor forms (TrkA, TrkB, TrkC). Each of these highly homologous, Type-I transmembrane receptor tyrosine kinases is composed of about 800 amino acids (Martin-Zanca et al., 1989; Ip and Yancopoulos, 1994; Barbacid, 1995). All Trks have an N-terminal signal peptide, several extracellular N-glycosylation sites, a transmembrane domain, and an intracellular portion (ICD) containing the tyrosine kinase catalytic region followed by a short carboxy tail. The Trk extracellular domains (ECDs) contain two cysteine-rich clusters (domains 1 and 3) separated by three leucine-rich motifs (domain 2), and these three domains precede two C2 type immunoglobin(Ig)-like domains (domains 4 and 5) (Schneider et al., 1991; Windish et al., 1995) (Figure 1.1).

Trk gene products can be alternatively spliced to produce truncated receptors. TrkA and TrkB have receptor isoforms that are missing short amino acid sequences in their extracellular juxtamembrane domains. These deletions affect neurotrophin binding affinity and specificity (Strohmaier et al., 1996; Clary and Reichardt, 1994). TrkB has isoforms lacking the intracellular kinase domain. TrkC isoforms contain an amino acid insert within the kinase domain or lack the kinase domain all together (Klein et al., 1990; Middlemas et al., 1991; Okazawa et al., 1993; Tsouflas et al., 1993; Fryer et al., 1996; Valenzuela et al., 1993). The physiological functions of these products are not clear. However, roles in modifying substrate specificity, aiding ligand presentation, inhibiting normal Trk signaling, or even activating independent signaling events have been suggested (Baxter et al., 1997; Eide et al., 1996; Hapner et al., 1998; Kryl et al., 2000; Meakin et al., 1997).

Radio-labelled neurotrophin binding experiments have demonstrated that Trk receptors bind each neurotrophin with differential affinity. TrkA preferentially binds NGF (Kaplan et al., 1991a, b), TrkB prefers BDNF and NT-4/5 (Ip et al., 1993; Klein et al, 1991, 1992; Squinto et al, 1991), and TrkC binds to NT-3 (Lamballe et al., 1991). NT-3 can also bind

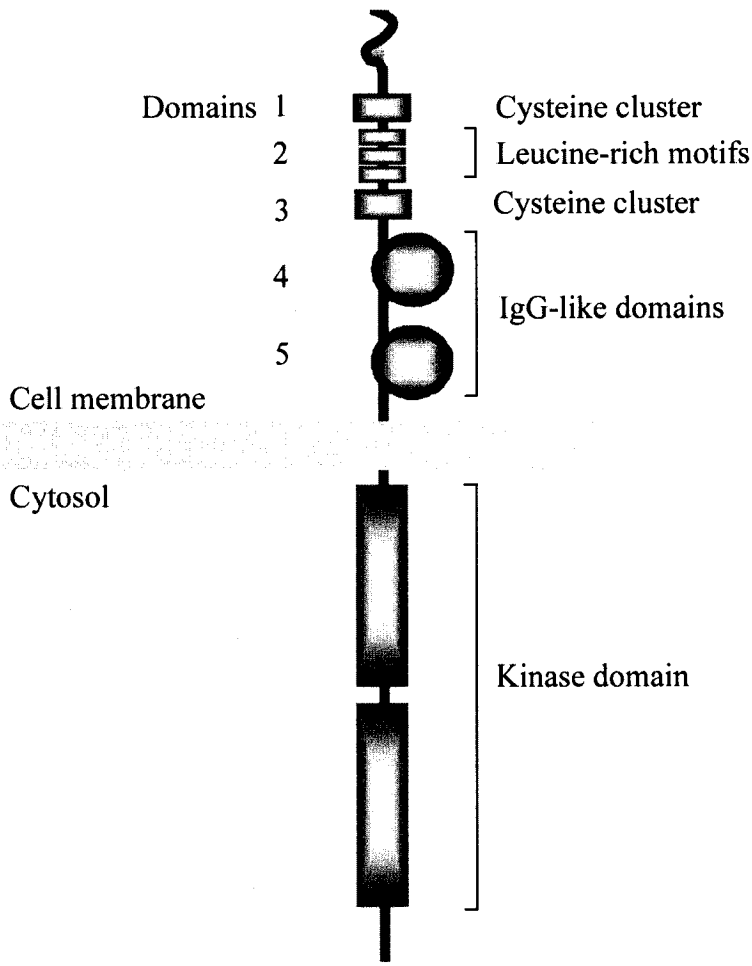


**Figure 1.1.** Structure of a Trk receptor. Trk receptors are Type I transmembrane receptor tyrosine kinases (intracellular kinase domaine). The Trk extracellular domains contain several N-linked glycosylation sites, two cysteine-rich clusters (domains 1 and 3), three leucine-rich motifs (domain 2), and two C2 type immunoglobin(Ig)-like domains (domains 4 and 5).

TrkA and TrkB at high concentrations and similarly NT-4/5 has reduced affinity for TrkA (Urfer et al., 1994; Segal and Greenberg, 1996). These non-preferred ligand interactions, however, are most physiologically relevant in cell types where p75NTR is not expressed (see section 1.3.6.2).

The affinity and specificity of neurotrophin binding is largely determined by the Trk Ig-like domain proximal to the membrane [domain 5] (Urfer et al., 1995, 1998; Perez et al., 1995; Ultsch et al., 1999). Crystal structure analysis of neurotrophin in complex with domain 5 of a Trk receptor has shown a conserved, two-patch ligand-receptor interface (Wiesmann et al., 1999, Banfield et al., 2001). The first patch, common to all neurotrophins, consists of interactions between the neurotrophin dimer core and loops in the carboxy-terminal pole of Trk domain 5. The second patch determines neurotrophin/Trk specificity and occurs through the ordering of helical N-terminal residues of Trk domain 5. The spatial arrangement of these residues is unique for the interaction between each neurotrophin and its preferred Trk receptor.

Trk receptors, when bound by their preferred neurotrophin ligands, transphosphorylate key intracellular tyrosine residues (Y) to increase kinase activity and to provide a scaffold to which various signaling proteins are recruited and activated (Segal and Greenberg, 1996). Adaptor proteins containing phosphotyrosine-binding (PTB) domains or src-homology-2 (SH2) motifs couple activated Trks to their main intracellular signals and this occurs largely through Y490 and Y785 (based on human TrkA nomenclature). Additionally, interactions between Trk receptors and intracellular signaling molecules are not only dictated by phosphotyrosine motifs but also by the cellular location of phosphorylated receptors.

### 1.2.2 Trk Receptor Function

Genes coding for neurotrophins or their receptors are not found in most invertebrates or in the nematode genome. Clearly then, development of a nervous system can occur regardless of the presence of neurotrophins. However, long-lasting, activity-dependent synaptic changes (learning and memory) and increased neuron numbers essential for the

development and functioning of higher and more complex vertebrate nervous systems, suggests an evolutionary need for additional regulators such as the neurotrophins. In support of this, Lymnaea stagnalis, an invertebrate with a relatively complex nervous system, expresses a Trk-like transmembrane protein (van Kesteren et al., 1998).

Mice with the neurotrophin genes or the Trk receptor genes knocked out by homologous recombination also demonstrate a requirement for these regulators in the proper construction of a complex nervous system. In fact, Trk knockout defects parallel the phenotypes found in animals lacking the corresponding neurotrophin ligand and confirm the dependencies of neurons on neurotrophin availability. Moreover, most mutant mice die postnatally and display additional trophic complexities in the CNS (Crowley et al., 1994; Smeyne et al., 1994; Ernfors et al., 1994a, b; Jones et al., 1994; Liebl et al., 1997; Conover et al., 1995; Kahn et al., 1999; Fagan et al., 1996; Francis et al., 1999; Klein et al., 1993; Airaksinen et al., 1996a, b). For example, neurotrophin mutant animals can suffer from dramatic LTP deficits due to impaired synaptic function, resulting in poor memory and learning. Mutant mice can also develop ataxia, balance disorders, and grow slowly (Sendtner et al., 1992; Knusel et al., 1992; Xie et al., 2000; Minichiello et al., 1999; Xu et al., 2000).

### 1.2.2.1 Major mechanisms of Trk-mediated Cell Survival and Growth

The phenotypic results of Trk receptor deficiency are largely attributed to defects in cell survival and differentiation. These are the primary Trk-activated cellular responses and the pathways that regulate these responses include the Ras/MEK/ERK (extracellular signal-regulated kinase) pathway, the phosphoinositol-3-kinase (PI3-K)/Akt kinase pathway, and activation of phospholipase C (PLC)-γ1 (Segal and Greenberg, 1996, Kaplan and Miller, 1997).

#### 1.2.2.1.1 Trk-regulated survival signals

For Trk-activated survival events, the mechanisms are well understood and are now known to require the Y490 site for Shc binding, PI3K activity and to a lesser extent MEK/ERK activity (Atwal et al., 2000; Borasio et al., 1993; Datta et al., 1997; Kaplan

and Miller, 2000; Klesse et al., 1998; Minichiello et al., 1998; Nobes et al., 1996; Yao et al., 1995). Briefly, phosphorylation of Y490 recruits two main mutually exclusive adaptors to the receptor, namely Shc or FRS2. Binding and phosphorylation of the Shc adaptor assembles a complex containing Grb2 and the guanine nucleotide exchange factor (GEF) SOS (son of sevenless). Membrane recruitment of this complex leads to activation of the small GTP-binding protein Ras. Ras subsequently binds to and activates PI3-K and the serine/threonine kinase Raf for initiation of the PI3-K/Akt and MEK/ERK pathways, respectively (Figure 1.2) (Atwal et al., 2000; Klesse and Parada, 1998; Meakins et al., 1999; Rodriguez-Viciana et al., 1994; Stephens et al, 1994; Xing et al., 1996).

Activation of PI3-K, the main regulator of Trk-dependent survival, can occur through direct interaction with Ras and through Ras-independent pathways. Ras-independent pathways require PI3-K association with docking proteins, which bind directly to Shc-Grb2 complexes. Docking proteins include the insulin receptor substrates IRS-1 and -2, and the Grb2-associated binder-1 (Gab1) (Holgado-Madruga et al., 1997; Miranda et al., 2001; Nguyen et al., 1997; Yamada et al., 1997). Once PI3-K is brought to the plasma membrane, it phosphorylates phosphatidylinositols (PIs) and generates lipid products PIP2 and PIP3, to recruit and activate the serine/threonine kinases PDK-1 (phosphoinositide-dependent kinase-1) and Akt (Bellacosa et al., 1991; Coffer and Woodgett, 1991; Jones et al., 1991; Vanhaese-Broeck and Alessi, 2000). Survival is then facilitated by the phosphorylation of critical survival regulators (reviewed in Brunet et al., 2001). For Akt, this includes the Forkhead (FKHR) family members (Biggs et al., 1999; Brunet et al., 1999; Guo et al., 1999; Kops et al., 1999), the Bcl-2 family member BAD (Datta et al., 1997, del Peso et al., 1997), Caspase 9 (Cardone et al., 1998; Rohn et al., 1998), IkB kinase (Kane et al., 1999), and glycogen synthase-3 (GSK-3) (Cross et al., 1995; Shaw et al., 1997; Pap et al., 1998; van Weeren et al., 1998).

Raf-activated MEK/ERK activity, on the other hand, phosphorylates and activates Rsk (the pp90 ribosomal S6 kinase) family members to promote survival (Blenis et al., 1993; Xia et al., 1995). Rsks in turn, function by phosphorylating and inactivating pro-apototic proteins such as BAD (serine 112) (Shimamura et al., 2000) or by phosphorylating and

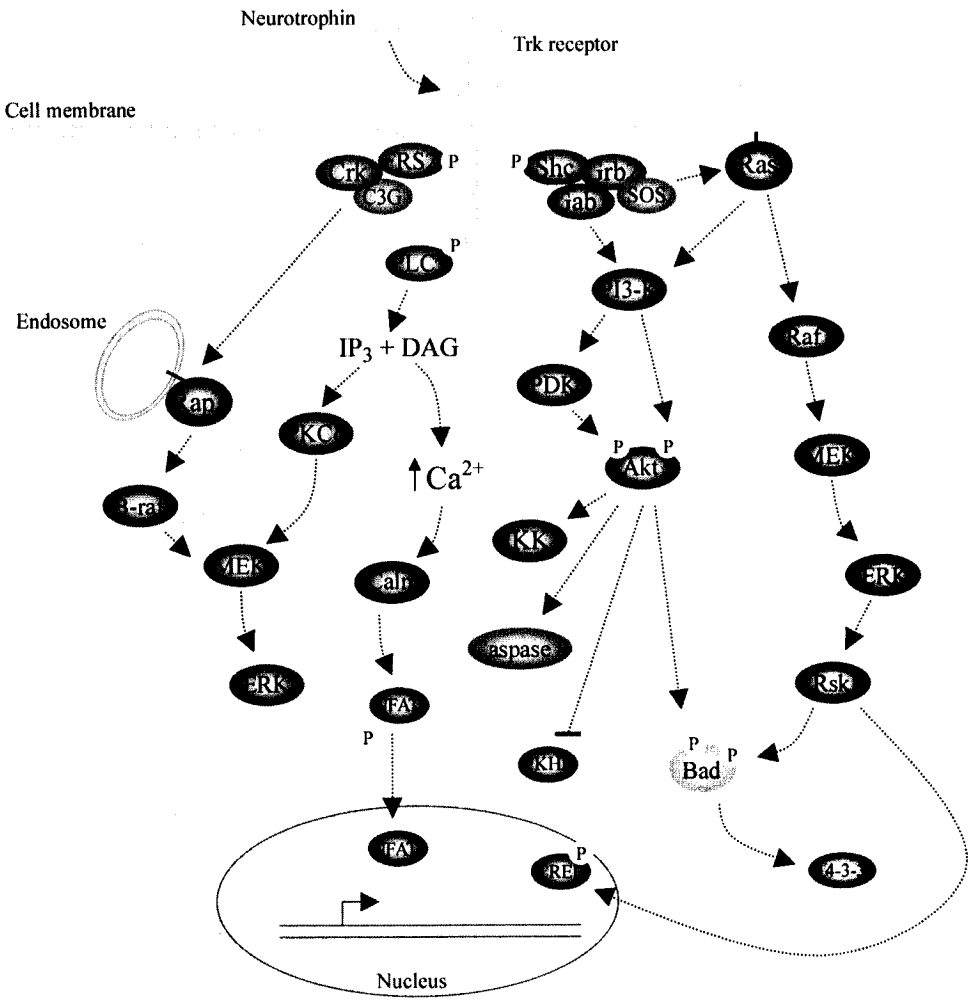


**Figure 1.2.** Major mechanisms of Trk-mediated cell survival and growth. Binding of neurotrophins to Trk receptors leads to the recruitment of proteins that interact primarily with phospho-tyrosine residues 490 and 785 (human TrkA nomenclature). These interactions lead to the activation of signaling pathways that include Ras/MEK/ERK, PI3-K/Akt, and PLC-γ (see text for details). Adaptor proteins are in grey, small G proteins in brown, kinases in red, transcription factors in blue, guanine exchange factors in green, phosphatases in black, and Bcl-2 proteins in orange. Some phospho-groups are indicated in yellow.

activating pro-survival transcription factors such as CREB (cAMP response element-binding protein (Riccio et al., 1999; Bonni et al., 1999; Xing et al., 1996).

#### 1.2.2.1.2 Mechanisms of Trk-mediated growth

Unlike most mitogens, neurotrophin stimulation of proliferating precursor cells often results in their differentiation into a neuronal phenotype (Greene et al., 1976). This second Trk-initiated outcome is also thought to require MEK/ERK and PI3-K activities (Qui et al., 1992). However, unlike survival signals, the initiation of Trk-mediated differentiation seems to involve PI3-K-dependent receptor internalization followed by sustained activation of Ras/MEK/ERK (York et al., 2000).

Maintenance of MEK/ERK activity depends upon the binding of lipid-anchored FRS2 to phosphorylated Y490. Bound FRS2 forms a stable, long-lived complex with the adaptor protein Crk and the GEF, C3G. This allows for small G protein Rap1 activation and subsequent activation of B-Raf to prolong MEK/ERK activity (Kao et al., 2001) (Figure 1.2). Therefore, differentiation responses are initiated by receptor internalization and prolonged MEK/ERK activity, whereas survival responses require activation of receptors at the cell surface to induce prolonged activation of Akt (Zhang et al., 2000).

Both MEK and PI3-K activities are also needed for Trk-initiated changes in axon morphology (Atwal et al., 2000; Borasio et al., 1989; Cowley et al., 1994). Morphological changes can range from developmental and regenerative control of neurite extension to regulation of growth cone guidance at distal axon tips (Kuruvilla et al., 2000; Ming et al., 1999; Namikawa et al., 2000). The specific Trk effector pathways necessary for different NGF-induced axon morphological features are beginning to emerge. Ras is necessary and sufficient for NGF-mediated axon growth, the Ras effector Raf-1 mediates axon lengthening whereas the PI3-K dependent effector Akt increases axon caliber and branching (Snider et al., 2002).

Trk-mediated differentiation and morphological changes can also depend upon the docking of PLC-$\gamma$ to phospho-Y785 (Canossa et al., 1997; Loeb et al., 1994; Obermeier et

al., 1994). Once docked, PLC-γ becomes phosphorylated and hydrolyses phosphatidyl inositides ($PIP_2$) to generate inositol triphosphate ($IP_3$) and diacylglycerol (DAG) (Vetter et al., 1991). $IP_3$ and DAG then typically initiate biological changes through $Ca^{2+}$ store release, activation of PKC isoforms, and ERK activation (Corbit et al., 1999).

Interestingly, recent work has shown that Trk-dependent axon outgrowth also requires the serine/threonine phosphatase calcineurin and activation of NFAT transcriptional complexes (Graef et al., 2003). More specifically, mice deficient in calcineurin-NFAT signaling have dramatic defects in axonal outgrowth, including no neurotrophin-dependent outgrowth in vitro. The signal transducing mechanisms are thought to include increased intracellular $Ca^{2+}$ for activation of calcineurin (Caln) (Klee et al., 1979) followed by rapid dephosphorylation of cytoplasmic NFATc subunits (Clipstone et al., 1992; Flanagan et al., 1991). Dephosphorylation of serines in the N-termini of NFATc proteins by calcineurin exposes nuclear localization sequences leading to their rapid import. Once in the nucleus, NFATc subunits require other transcription factors for DNA binding and these factors are often regulated by the PKC and Ras/MEK/ERK pathways (Flanagan et al., 1991). Accordingly, mutating either the PLCγ-1 or SHC binding site on Trk receptors attenuates neurotrophin-dependent activation of NFAT transcriptional activity.

## 1.3 THE P75 NEUROTROPHIN RECEPTOR

The other type of neurotrophin receptor, p75NTR, was first cloned in 1986 by virtue of its low affinity NGF binding properties (Chao et al., 1986; Johnson et al., 1986; Radeke et al., 1987). These experiments identified a receptor lacking obvious catalytic activity, and the discovery of the Trk tyrosine kinase receptors in the early 90's removed p75NTR from the limelight. Now, after many years of study, p75NTR emerges as a unique receptor, capable of both signaling independently and modifying the binding and signaling capabilities of its co-receptors.

### 1.3.1 p75NTR is an atypical TNFRSF member

Not only was p75NTR the first cloned neurotrophin receptor, but p75NTR was also the first cloned member of a larger protein family called the TNF Receptor Superfamily (TNFRSF). The TNFRSF comprises roughly 25 members including Fas, CD40, TNFR, and RANK receptors (Bakker and Reddy, 1998; Idriss et al., 2000). The defining TNFRSF feature is the presence of two to six repeats of cysteine clusters in their ECD (termed CRDs for "cysteine-rich domains"). These negatively charged repeats form an elongated structure due to intrachain disulfide bridge formation (Yan and Chao, 1991; Baldwin et al., 1992). The CRDs also provide necessary scaffold contacts for ligand binding (Banner et al., 1993) and for assembling receptors in the absence of ligand (Chan et al, 2000; Siegel et al., 2000).

Like all receptors of this family, p75NTR is a Type I transmembrane protein, and when translated, initially contains a 28-amino acid signal peptide. Removal of this signal sequence leaves a 399 amino acid receptor (human form) that undergoes a single N-linked glycosylation and several O-linked glycosylations (Figure 1.3) (Large et al., 1989; Grob et al., 1985). The p75NTR ECD contains four of the highly ordered CRD repeats. Contacts between p75NTR and neurotrophins occur mainly in the 2nd and 3rd CRDs (numbered beginning at the N terminus) (Yan and Chao, 1991; Baldwin et al., 1992; Chapman et al., 1995; Shamovsky et al., 1999). CRD1, the pre-ligand binding assembly domain (PLAD) found in TNF and Fas receptors, is also present in the p75NTR ECD. Whether CRD1 functions to pre-assemble p75NTR is currently unknown.

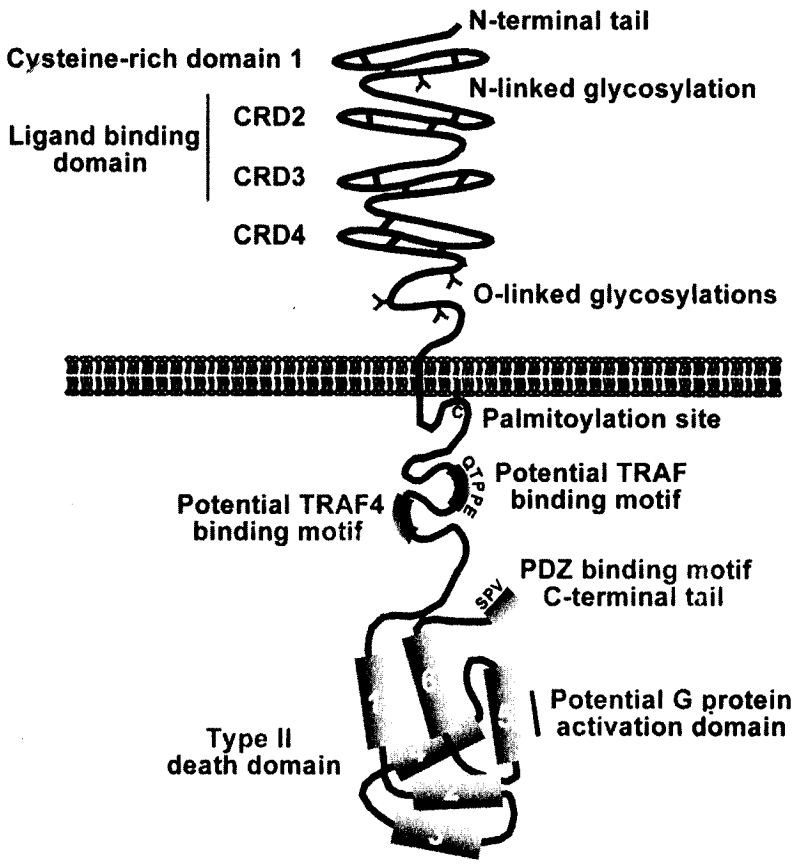


**Figure 1.3.** Structure of the p75NTR protein. p75NTR is a Type I transmembrane receptor with an extracellular domain that contains four cysteine-rich domains (CRD), and multiple O- and N -linked glycosylation sites. The intracellular domain contains a palmitoylation at cysteine 279, two potential TRAF-binding sites, a Type II death domain, a potential G protein activating domain, and a PDZ domain binding motif. (Adapted from P. Roux and P. Barker, 2002).

Like other members of the TNFRSF, p75NTR has no intrinsic enzymatic activity. Instead, signaling events are initiated by recruiting cytoplasmic adaptor proteins to several areas within the receptor tail. These adaptors typically include two classes of proteins, the TRAF (TNF receptor-associated factors) adaptors and "death domain" (DD) molecules (reviewed in Fesik, 2000). p75NTR can associate with both types of interactors (Krajewska et al., 1998; Khusigara et al., 1999; Vaillantcourt, manuscript in preparation; Yazidi-Belkoura et al., 2003; Ye et al., 1999). However, unlike other members of the TNFRSF, p75NTR interaction affinities tend to be more selective and sometimes require unconventional binding sites (Vaillantcourt, manuscript in preparation).

In addition to differences in affinity for interacting molecules, p75NTR also differs from other TNFRSF members in their oligomerization properties. For example, all TNFRSF members, with the exception of p75NTR, oligomerize into trimers. Moreover, TNFRSF ligands, which are produced as cell surface Type II transmembrane proteins, also trimerize (reviewed in Locksley et al., 2001). In contrast, p75NTR receptors form functional dimmers, and the prototypical p75NTR ligands, the neurotrophins, are dimers and are structurally unrelated to the TNFR superfamily ligands. Unlike most TNFRSFs, p75NTR also associates and functionally cooperates with a number of other receptor types. These include the tyrosine kinase Trk receptors and the NgR myelin inhibitory receptor (Bibel et al., 1999; Gargano et al., 1997; Salehi et al., 2000; Wang et al., 2002; Wong et al., 2002). Consistent with the above functional dissimilarities, p75NTR is believed to be a phylogenetically distant member of the TNFRSF. The distinct distribution of introns within the p75NTR gene compared with other TNFRSF members suggests that the divergence of p75NTR may have occurred early in evolution.

The p75NTR ICD is structurally distinct from other TNFRSF members. Within the ICD, all pro-apoptotic members of the TNFRSF contain a globular protein interaction domain, called the death domain (DD). This 80 amino acid region, consists of six conserved α-helices arranged in two compact bundles (Chapman et al., 1995; Liepinsh et al., 1997). However, two classes of DD structures, Type I and Type II, exist and are distinguished on the basis of helix homology and orientation (Feinstein et al., 1995). TNF receptor DDs

are Type I domains, which recruit adaptor proteins via homotypic DD interactions (Boldin et al., 1995; Tartaglia et al., 1993). The p75NTR DD, on the other hand, is a Type II domain with sequence homology to DDs present in MyD88, Unc5, and ankyrin.

The spatial arrangement of helix 1 in type II DDs is dramatically altered by almost 90° (Feinstein et al., 1995, Huang et al., 1996; Liepinsh et al., 1997). This difference likely has important signaling consequences since death-promoting proteins that associate with p75NTR differ considerably from those used by TNFR1 or Fas (see later, section 1.3.6.3.1.2). Accordingly, chimeric receptors that contain the extracellular portion of Fas and the intracellular portion of p75NTR do not induce apoptosis (Kong et al, 1999). Moreover, death-promoting proteins often bind to p75NTR in areas outside of the DD (Chittka et al, 1999; Coulson et al., 2000; Salehi et al., 2000; Wang et al., 2001). In fact, the fifth helix of the p75NTR DD is most similar to a 14-residue wasp venom peptide that can activate heterotrimeric G-proteins (Feinstein and Larhammer, 1990; Myers et al., 1994; Liepinsh et al., 1997). This portion of the DD is responsible for Rho-GDI/RhoA binding and may link p75NTR to growth pathways (Dostaler et al., 1996; Yamashita et al., 1999).

An additional p75NTR ICD feature includes a conserved C-terminal tripeptide (SPV) PDZ domain binding site that can interact with an atypical PDZ-containing protein tyrosine phosphatase, FAP-1 (Irie et al., 1999). The physiological significance of the PDZ motif, however, remains unclear.

As with the p75NTR ECD, the p75NTR ICD can also undergo several post-translational modifications. These modifications include palmitoylation at cysteine 279 (Barker et al., 1994b), phosphorylation at serine 304 (Higuchi et al., 2003), a number of other serine and threonine phosphorylations (Grob et al., 1985), and proteolytic processing (see below). The precise biological functions of these are unknown but may include directing the cellular localization of p75NTR, proper folding of the receptor, p75NTR oligomerization, or simply providing docking sites for potential protein-protein interactions.

### 1.3.2 p75NTR isoforms and related proteins

Alignment of p75NTR sequences from a range of vertebrate species demonstrates that p75NTR is well conserved (Hutson and Bothwell, 2001; Roux and Barker, 2002). Only one *p75ntr* gene which encodes a single, full length protein has been reported in most species, but frogs contain two nucleotide sequences, *p75ntra* and *p75ntrb*. These sequences are more closely related to each other than either is to p75NTR from any other species which suggests that the second p75NTR gene arises from genome duplication in Xenopus laevis.

Recently, a number of proteins highly homologous to p75NTR have been identified. The related gene products, xNRH1a (Xenopus Fullback), xNRH1b, and zNRH1a are the first identified members of the NRH (neurotrophin receptor homologues) protein family (Hutson and Bothwell, 2001). These homologues are distinct proteins, but they resemble p75NTR more closely than other DD-containing proteins. Frankowski and collegues (2002) also identified another Xenopus p75NTR homologue, a zebrafish p75NTR-like gene, and a rat DD homologue called PLAIDD or NRH2. NRH1 and NRH2, like p75NTR, possess DDs and a C-terminal PDZ-binding motif (SSXV). However, NRH2 lacks the extracellular CRDs that constitutes the ligand-binding domain of p75NTR. Additionally, p75NTR is present in all vertebrates, whereas NRH1 proteins are not found in mammals and NRH2 is found only in mammals. It will be interesting in future studies to determine whether deletion of these homologues together with p75NTR will identify previously unobservable, potentially redundant roles.

Truncated p75NTR isoforms are also naturally occurring and these proteins are produced by either alternative splicing or proteolysis. The p75NTR variant generated by alternative splicing, termed the short p75NTR isoform (or s-p75NTR), lacks three of the four CRDs due to complete removal of exon III (Dechant and Barde, 1997). This isoform is incapable of binding neurotrophins but does contain CRD1, the binding site for rabies virus glycoprotein (see below) and the portion necessary for TNF receptor oligomerization (above). The transmembrane and ICDs of s-p75NTR, however, are intact and identical to that of full-length p75NTR.

A constitutively active metalloproteinase is responsible for proteolytic cleavage of the p75NTR ECD. This cleavage generates a soluble p75NTR-ECD fragment capable of binding neurotrophins, and a fragment containing the transmembrane and ICDs (Zupan et al., 1989; Barker et al., 1991; DiStefano et al., 1988, 1993). High levels of these fragments are found during development and following peripheral nerve injury (DiStefano et al., 1991). Additional proteolytic processing events generate soluble p75NTR ICD fragments. Sequential cleavage of p75NTR by α- and γ-secretases, near the membrane junction of the ECD and subsequently within the TM domain, releases proteosome-sensitive, 25-30 kDa ICD fragments (Kanning et al., 2003). The smaller fragment can accumulate in the nucleus and can modulate NF-kB activation by TRAF6. Interestingly, NRH1 and NRH2 proteins are also proteolytically processed to generate soluble ICD fragments. Moreover, sometimes these products accumulate in the nucleus. The physiological functions of all of these products remains to be understood.

### 1.3.3 Expression of p75NTR

The p75ntr gene spans approximately 23 kb of human chromosome region 17 (Huebner et al., 1986, Sehgal et al., 1988b). The nucleotide sequence for the p75ntr gene is organized into six exons and transcribes a full-length 3.8 kb mRNA (Johnson et al., 1986). Regulation of the p75NTR transcript is poorly understood. No TATA or CAAT consensus sequences can be found within the p75NTR promoter (Sehgal et al., 1988a). Only conserved GC rich regions resembling Sp1 binding sites and E-box-like elements have been described (Chiaramello et al., 1995; Metsis et al., 2001). Regardless, the p75NTR promoter sequence is well conserved between species, and much is known about when and where p75NTR is expressed.

#### 1.3.3.1 Developmental Expression of p75NTR

Early in CNS development, p75NTR transcripts are abundant throughout the embryonic neural tube and within the retina (Cotrina et al., 2000; Heuer et al., 1990; Salehi et al., 2000). Once central neurons begin to differentiate, p75NTR expression becomes more limited although high expression is still found at every level of the CNS. This includes

motor and sensory neurons of the brain stem and spinal cord, neurons within the amygdala and thalamus, cortical and subcortical neurons, and several neuronal populations of the cerebellum (Allendoerfer et al., 1990; reviewed in Bothwell, 1991; Buck et al., 1987, 1988; Cotrina et al., 2000; Ernfors et al., 1988; Escandon and Chao, 1989; Heuer et al., 1990; Large et al., 1989; Salehi et al., 2000; Schatteman et al., 1988).

In the adult CNS, p75NTR expression is further reduced and restricted. High levels are found in basal forebrain cholinergic neurons (Hefti et al., 1986; Dreyfus et al., 1989; Schatteman et la., 1988; Springer et al., 1987; Yan et al., 1988) whereas low p75NTR levels are detected in motor neurons (Armstrong et al., 1991; Ernfors et al., 1989), cerebellar Purkinje cells (Cohen-Cory et al., 1991; Koh et al., 1989; Shelton et al., 1986), within the basal ganglia (Henry et al., 1994), and throughout specific brain stem nuclei (Koh et al., 1989; Schatteman et al, 1988; Sofroniew et al., 1989).

In the peripheral nervous system (PNS), p75NTR is initially expressed in neural crest cells. Levels increase during differentiation into functional ganglia, particularly within parasympathetic, enteric, autonomic cranial and sensory ganglia. In adult animals, p75NTR expression is maintained in sensory, sympathetic, enteric, and subsets of parasympathetic neurons (Carroll et al., 1992; Schatteman et al., 1993; Sutter et al., 1979; Yan et al., 1988). Other non-neuronal derivatives of the neural crest, including proliferative melanocytes and Schwann cells, also continue to express high levels of p75NTR.

Despite p75NTR's initial identification as a neuronal receptor, the highest levels of p75NTR are found outside the nervous system. Early embryos express p75NTR in tissues undergoing extensive morphogenesis and differentiation within the dermatome, sclerotome, and mesenchyme (Cotrina et al., 2000; Heuer et al., 1990; Thompson et al., 1988). As development proceeds, this expression becomes restricted to the limb buds, kidney, maxillary pad, teeth, lung, muscle, testes, pituitary, retina, skin, hair follicles, salivary glands, perivascular cells, meninges and the developing inner ear (Alpers et al., 1993; Byers et al., 1990; Russo et al., 1994; Sariola et al., 1991; Seidl et al., 1998; von

Bartheld et al., 1991; Wheeler et al., 1992; Wheeler et al., 1998). Interestingly, within these organs, p75NTR expression is highest before innervation and often at mesenchymal/epithelial boundaries.

### 1.3.3.2 Injury- and disease-induced p75NTR expression

The progressive restriction of p75NTR expression with age implies that p75NTR may be less important in the adult than during development. However, following injury or disease, p75NTR expression is often induced by within neurons, glia, and other cell populations. For example, following neurotoxic events such as seizures or ischemia, massive increases in p75NTR protein are found in dying cortical, hippocampal, and striatal neurons (Roux et al., 1999; Oh et al., 2000; Park et al., 2000; Kokaia et al., 1998; Andsberg et al., 2001). Similarly, nerve injury upregulates p75NTR within immune cells, oligodendrocytes, microglia, and Schwann cells (Chang et al., 2000; Beattie et al., 2002; Syroid et al., 2000; Lemke et al., 1988; Taniuchi et al., 1988; Nataf et al., 1998). Chronic diseases are also associated with increased p75NTR levels. The list of diseases includes multiple sclerosis (Dowling et al., 1999), diabetic neuropathy (Conti et al., 1997), Alzheimer's (Yaar et al., 1997; Mufson and Kordower, 1992) and several tumors (Krygier et al., 2001; Weis et al., 2002). With regard to the latter condition, p75NTR-expressing cancer cells typically include neural crest derivatives (melanoma, Schwannoma, pheochromocytoma) consistent with cellular reversal to a more immature, embryonic-like phenotype. The consequence of these enhanced p75NTR levels is not completely clear. Most reports show a tight link between p75NTR expression and apoptosis, and recent work demonstrates that p75NTR is required for injury-induced damage in the spinal cord and cortex (Beattie et al., 2002; Roux et al., 1999; Troy et al., 2002; Giehl et al., 2001; Oh et al., 2000). Therefore, blocking p75NTR expression and action following injury or during disease may prove effective for preventing cell death and consequently neurological disease.

### 1.3.3.3 p75NTR subcellular distribution

p75NTR can be found in most areas of a neuron including the soma, dendrites, and along axons (Tongiorgi et al., 1997, 2000; Dougherty and Milner, 1999; Kryl et al., 1999).

However, p75NTR localizes to specialized lipid compartments within cell membranes called caveolae and lipid rafts (Bilderback et al., 1997; Huang et al., 1999; Higuchi et al., 2003). This may occur through direct binding to microdomain components such as Caveolin-1 (Bilderback et al., 1999) and require phosphorylation by the β catalytic subunit of cAMP-dependent protein kinase (PKACβ) (see later) (Higuchi et al., 2003) and may be important for concentrating or separating specific signaling molecules. Recent evidence also demonstrates p75NTR presence within distinct organelle compartments including recycling endosomes in the cell body, vesicles at the growth cone, and vesicles moving along axons (see below) (Bronfman et al., 2003; Lalli et al., 2002).

### 1.3.4 Transport of p75NTR

p75NTR and Trk receptors can mediate neurotrophin transport in anterograde and retrograde directions (Johnson et al., 1987; Loy et al., 1994; Ehlers et al., 1995; DiStefano et al., 1992; Curtis et al., 1995; Grimes et al., 1996, 1997; Bhattacharyya et al., 1997, 2002; Reynolds et al., 2000; Butowt et al., 2001; von Bartheld et al., 2001; Howe et al., 2001; Riccio et al., 1997). Studies using p75NTR mutant mice or p75NTR blocking antibodies demonstrate that the receptor is necessary for retrograde transport of NT-4/5 and BDNF, but it is not necessary for NGF transport (Curtis et al., 1995). When TrkA is absent, however, p75NTR can internalize and transport NGF back to the cell body (Kahle et al., 1994).

The morphology, kinetics, and transport paths employed by p75NTR-containing vesicles may be quite unique. In PC12 cells, for example, neurotrophin-p75NTR containing vesicles are slower moving and less quickly internalized than Trk-containing vesicles. They are also internalized via clathrin-coated pits into early endosomes (Lalli et al., 2002; Bronfman et al., 2003). Eventually these endosomes accumulate in the cell body and within growth cones. Importantly, NGF can induce the endosomal association of p75NTR with its MAGE interactors, necdin and NRAGE which suggests that signaling endosomes containing activated p75NTR are important for neurotrophin signaling.

The reasons for different neurotrophin vesicles, with distinct signaling adaptors and differential trafficking of vesicles, are not known. One suggestion is that distinct targeting, sorting or recycling of specific neurotrophins may be a mechanism for regulating synaptic plasticity. Supporting this hypothesis, recent work demonstrates that neurotrophin signaling through p75NTR modulates the release of distinct neurotransmitter pools from neurotrophin signaling through Trk receptors (Yang et al., 2002). Interestingly, since lectins, pathogens and neurotoxins also bind p75NTR, receptor trafficking through p75NTR internalization may also be a mechanism for entry and spreading of toxins and virus into the nervous system (reviewed in Dechant and Barde, 2002). This mechanism is similar to the hijacking of TNFRSF members by herpes simplex virus (Montgomery et al., 1996).

### 1.3.5 p75NTR ligands

In the late 70's, NGF binding assays on neurons and PC12 cells suggested the existence of two NGF receptors (Sutter et al., 1979; Rodriguez-Tebar et al., 1992; Andres et al., 1977; Olender et al., 1980). The first NGF receptor, p75NTR, was isolated by expression cloning, and nanomolar $^{125}$I-NGF binding led to its initial naming as the low affinity NGF receptor (Chao et al., 1986; Johnson et al., 1986; Radeke et al., 1987). Years later, the second NGF receptor, TrkA, was identified and the other neurotrophins were soon discovered. Ligand binding experiments with the remaining neurotrophins demonstrated that unlike the TrkA receptor, the p75NTR ECD is not specific for NGF. Instead, the p75NTR ECD is fairly promiscuous, capable of binding all neurotrophins. Moreover, p75NTR affinities for each neurotrophin ligand are similar and in general low (Rodriguez-Tebar et al., 1990, 1992; Squinto et al., 1991). The main distinction between each neurotrophin and its ability to bind to p75NTR is probably that each neurotrophin uses slightly different contact sites (Ryden et al., 1995).

Although p75NTR binds all neurotrophins, p75NTR-mediated cellular responses to purified recombinant neurotrophins have generally been weak and unreliable. These dilemmas have long plagued the p75NTR field. Recent discoveries, however, suggest a feasible resolution. Specifically, Hempstead and colleagues have demonstrated that

unprocessed neurotrophin precursors bind p75NTR with high affinity and activate p75NTR responses at significantly lower concentrations than do mature neurotrophin (Lee et al., 2001). Additionally, while proNGF binding experiments confirm a five-fold stronger equilibrium-binding constant for p75NTR than for mature NGF, Trk affinity for proNGF is not as strong as that for mature NGF. Thus, the unprocessed pro-neurotrophins may proved to be novel p75NTR ligands with greater potencies that are specific for p75NTR.

p75NTR can bind to more than just neurotrophins. CRNF, or cysteine-rich neurotrophic factor is a 13.1 kDa p75NTR ligand isolated from the snail, Lymnaea stagnalis. CRNF binds p75NTR with similar affinity as the neurotrophins but shares no neurotrophin sequence homology (Fainzilber et al., 1996). The expression patterns for CRNF suggest that this ligand acts as a target-derived trophic factor and CRNF can regulate processes common to p75NTR action such as neurite outgrowth. It is possible, therefore, that CRNF represents the prototype of another family of p75NTR ligands.

Other non-neurotrophin p75NTR ligands include the neurotoxic prion protein fragment, PrP (26-106) (Della-Bianca et al., 2001), the Ab-peptide of the amyloid precursor protein (APP) (Yaar et al., 1997; Kuner et al., 1998; Perini et al., 2002), and the trimeric envelope glycoprotein from rabies virus (Tuffereau et al., 1998; Langevin et al., 2002). These products are believed to regulate the pathogenesis of several neurological disorders (reviewed in Yuan and Yankner, 2000) and most of these products, with the exception of rabies virus, bind p75NTR with nanomolar affinities. Some of these pathogens utilize p75NTR signaling to initiate neuronal damage. Binding of pathogens to p75NTR may therefore be important for the pathogenic mechanisms in adult neurodegenerative disorders.

### 1.3.6 p75NTR Functions

The biological roles of p75NTR are diverse but three general functions can be attributed to the receptor. First, p75NTR can positively or negatively modulate Trk receptor signaling. Second, p75NTR can autonomously activate signaling cascades that regulate

cellular apoptosis. Finally, p75NTR can regulate neurite outgrowth in response to neurotrophins and myelin-derived inhibitory proteins.

### 1.3.6.1 Lessons from the p75NTR knockout mice

Deleting endogenous genes by homologous recombination has been useful for determining the physiological functions of a variety of proteins. Therefore, to understand the physiological functions of p75NTR, two p75NTR null mice have been made. The first published mutant mouse was generated by targeted deletion of exon III of the p75ntr locus. This exon encodes CRDs 2 through 4, the areas necessary for ligand binding, and the mice with this deletion are referred to as $p75NTR^{III-/-}$ mice (Lee et al., 1992). This mutant, although lacking full-length p75NTR, still expresses the naturally occurring shorter p75NTR isoform, s-p75NTR. Thus, $p75NTR^{III-/-}$ mice are not true knockout animals. Instead they are hypomorphic p75NTR mutants (Dechant and Barde, 1997; von Schack et al., 2001). This finding prompted the generation of the second knockout mouse which lacks both the full-length and short isoforms of p75NTR by neomycin casette insertion within exon IV ($p75NTR^{IV-/-}$ mice).

Analysis of both knockout mice, largely confirm the three main p75NTR functions. For example, p75NTR can autonomously activate apoptosis, and a number of tissues from p75NTR null mice have markedly reduced apoptosis including the cells of the retina, spinal cord (Frade et al., 1999), PNS (Soilu-Hanninen et al., 1999; Syroid et al., 2000), and vasculature (Kraemer et al., 2002; Wang et al., 2000). Similarly, p75NTR function in neurite outgrowth regulation can manifest as disrupted innervation and pathfinding errors (Brann et al., 1999; Yamashita et al., 1999; Walsh et al., 1999; Anton et al., 1994; Yang et al., 2002). Progressive losses in neuron number and innervation can also be found within Trk-expressing sensory and sympathetic populations of mutant mice, as one might expect for a neurotrophin co-receptor deficit.

Interestingly, the phenotype of $p75NTR^{IV-/-}$ mice is more severe than $p75NTR^{III-/-}$ mice. $p75NTR^{IV-/-}$ mice are smaller, have hind-limb ataxia, greater peripheral nerve loss, and ruptures of aortic blood vessels resulting in perinatal lethality. Some of these phenotypes

are completely absent in the p75NTR$^{III-/-}$ mutant mice. This difference suggests that s-p75NTR may provide functional compensation for p75NTR in p75NTR$^{III-/-}$ mice. However, we have recently found aberrant expression of a 26kDa transmembrane and ICD containing p75NTR product within p75NTR$^{IV-/-}$ mice (C. Paul, unpublished results). Overexpression of the 26kDa fragment in 293T cells activates cellular responses consistent with reported p75NTR activities (C.Paul, unpublished results). These results suggest that the severe phenotype within p75NTR$^{III-/-}$ mice may not be due to functional inactivation of p75NTR, but rather reflect a gain of function phenotype due to abberantly high expression of an active p75NTR product. Future generation of complete knockout mice will be necessary to resolve these issues.

### 1.3.6.2 p75NTR modulates Trk receptor signaling

The discovery of both high and low affinity binding sites on NGF responsive cells with different dissociation rates (Vale and Shooter, 1985) and different biochemical properties (Schechter et al., 1981; Puma et al., 1983), predicted that NGF would require two separate receptors. Indeed, after many years of study, it is now well established that both p75NTR and TrkA bind independently to NGF (Rodriguez-Tebar et al., 1990, 1992; Kaplan et al., 1991a; Klein et al., 1991b; Squinto et al., 1991). However, the formation of high affinity ($K_D$ of $10^{-11}$M) sites does not require a separate molecule, but rather co-expression of both p75NTR and Trk (Hempstead et al., 1991; Mahadeo et al., 1994).

In fact, more recent work demonstrates that p75NTR can act as a Trk co-receptor and modulate Trk function by selective modulation of Trk/neurotrophin binding. p75NTR enhances the affinity of TrkA for NGF (Lee et al., 1994a; Mahadeo et al., 1994; Hempstead et al., 1991; Rodriguez-Tebar et al., 1992), while decreasing its affinity for NT-3 (Bibel et al., 1999; Mischel et al., 2001; PA. Barker, unpublished results). Similarly, p75NTR confers increased specificity toward preferred ligands for TrkB and TrkC receptors but decreased affinity toward their non-preferred ligands (see Table 1.1) (Ip et al., 1993; Bibel et al., 1999; Maisonpierre et al., 1990; Berkemeier et al., 1991; Squinto et al., 1991; Benedetti et al., 1994). Therefore, p75NTR modulates

| | Preferred | Non-preferred | Non-ligand |
|---|---|---|---|
| TrkA | NGF | NT-3, NT-4 | BDNF |
| TrkB | BDNF, NT-4 | NT-3 | NGF |
| TrkC | NT-3 | none | NGF, BDNF, NT-4 |

**Table 1.1.** Ligand binding specificities of Trk receptors. Preferred ligand show high affinity binding to Trk receptors. Non-preferred ligands show low but detectable binding affinity, whereas non-ligands do not bind or activate the corresponding Trk receptor.

Trk/neurotrophin binding to a) restrict neurotrophin specificity and to b) increase responsiveness to low neurotrophin concentrations.

These two properties are important for neurons innervating targets secreting limited amounts of neurotrophin. For example, sensory and sympathetic innervation in $p75NTR^{III-/-}$ mice is decreased and cerebellar patterning in $BDNF^{+/-}$, $p75NTR^{III-/-}$ mutant mice is disrupted, correlating with reduced neurotrophin responsiveness for these neurons (Lee et al., 1992, 1994a, b; Davies et al., 1993; Carter et al., 2003). Similarly, mice lacking one ngf allele ($NGF^{+/-}$) have reduced sympathetic neuron numbers compared with combined $NGF^{+/-}$, $p75NTR^{III-/-}$ double mutant mice. However, the increase in neuron numbers seen in the double mutant is lost in $NGF^{+/-}$, $p75NTR^{III-/-}$, $NT\text{-}3^{+/-}$ triple mutant mice (Brennan et al., 1999). In other words, in the absence of p75NTR, NT-3 can compensate for NGF to induce TrkA activation.

#### 1.3.6.2.1 Mechanisms of Trk-p75NTR interactions

The molecular mechanisms for p75NTR-dependent modulation of Trk/neurotrophin affinities are still disputed, but several models have been proposed. First, a number of reports demonstrate that binding of NGF to p75NTR is necessary to enhance TrkA responsiveness (Barker et al., 1994; Ryden et al., 1997; Hantzopoulos et al., 1994; Verdi et la., 1994; Clary et al., 1994; Lachance et al., 1997). This finding implies that p75NTR presents preferred ligand to Trk either by concentrating it locally or by presenting it in a favorable binding conformation. Recent work, however, argues against a presentation model whereby p75NTR directly binds neurotrophin. Instead, the second model proposes that p75NTR induces conformational changes upon Trk to facilitate ligand binding. More specifically, Esposito and colleagues (2001), using p75NTR ECD mutants, demonstrate that only the transmembrane and ICD portions of p75NTR are necessary to generate high affinity NGF binding sites. The discrepancy between these two models might be eliminated in the future by distinguishing between the generation of high affinity binding sites from the functional effects of p75NTR on TrkA activation.

The third model, which does not exclude models one and two, suggests that p75NTR/Trk functional collaborations may require activation of intracellular signaling mechanisms. For example, BDNF, which does not bind TrkA, can bind to p75NTR and reduce TrkA activation (MacPhee and Barker, 1997). Similarly, TrkA-activated PI3-kinase activity can block p75NTR function and this may contribute to neurotrophin/Trk affinities (Dobrowsky et al., 1994, 1995; Bilderback et al., 2001; Yoon et al., 1998). Interestingly, BDNF treatment of cells that express both p75NTR and TrkA results in increases in the phospho-serine content of the TrkA ICD (MacPhee and Barker, 1997). Hence, p75NTR-Trk interactions may be regulated by direct phosphorylation of receptor intracellular domains, reminiscent of receptor transmodulation mechanisms initiated by other TNFRSF members (Feinstein et al., 1993; Hotamisligil et al., 1994; Kanety et al., 1995).

Regardless of the mechanism for p75NTR-Trk modulation, the high degree of fine tuning and cross talk between these two classes of neurotrophin receptors suggests the two physically interact. p75NTR and Trk co-localize within plasmalemmal patches (Wolf et al., 1995; Ross et al., 1996) and several research groups have now co-immunoprecipitated p75NTR with Trk receptors. (Gargano et al., 1997; Bibel et al., 1999; Salehi et al., 2000). Whether this interaction is direct or requires stabilization by additional factors, however, remains unclear. A series of p75NTR and Trk interacting proteins have also been isolated and some can bind to both receptors. ARMS (ankyrin-rich membrane spanning), for example, is a large transmembrane protein containing ankyrin repeats, a sterile motif and a PDZ-binding motif, and can be found in association with both p75NTR and TrkA (Kong et al., 2001). Gangliosides or Caveolin, key structural proteins in lipid microdomains (Parton et al., 1996; Okamoto et al., 1998; Bilderback et al., 1997, 1999; Huang et al., 1999; Yamashita et al., 2002), can also bind to both p75NTR and TrkA and may therefore provide essential scaffold links. Conversely, other proteins can bind and interfere with Trk-p75NTR complexes, presumably to regulate signaling events. These proteins include NRAGE, Necdin, and the atypical protein kinase C (aPKC)-interacting protein p62/ZIP (Wooten et al., 2001; Mamidipudi et al., 2002; Salehi et al., 2000; Tcherpakov et al., 2002; Geetha et al., 2003).

### 1.3.6.3 p75NTR regulates cellular apoptosis

In the mid 90's several signaling pathways initiated by TNFRSF members were discovered. This spurred efforts to place p75NTR in similar signaling pathways, and so far, a number of common signaling events have been identified. These events include the generation of the lipid second messenger ceramide, the regulation of the transcription factor NF-kB, and the activation of the c-Jun N-terminal kinase, JNK. Regulation of similar signaling pathways and the presence of an intracellular DD has also motivated experiments addressing the role of p75NTR in the regulation of cellular apoptosis.

#### 1.3.6.3.1 p75NTR induces cell death

A number of cell types are sensitive to p75NTR-mediated apoptosis. To date the list includes:

- several classes of central neurons (von Bartheld et al., 1994; Frade et al., 1996, 1999, 2000a, b; Sekiya et al., 1986; Sendtner et al., 1992; Terrado et al., 2000; Ferri et al., 1998; Majdan et al., 1997; Roux et al., 1999; Park et al., 2000a, b; Yeo et al., 1997; Oh et al., 2000; Greferath et al., 2002; Friedman et al., 2000; Troy et al., 2002; Brann et al., 2002; **Chapter4**; Jover et al., 2002)
- peripheral neurons (Lee et al., 1992; Cheema et al., 1996; Majdan et al., 1997, 2001; Davey et al., 1998; Taniuchi et al., 1985; Bamji et al., 1998; Barrett et al., 1994; Coulson et al., 1999, 2000; Aloyz et al., 1998; Savitz et al., 2000; Lee et al., 2001; Freidin et al., 2001; Agerman et al., 2000; Palmada et al., 2002)
- oligodendrocytes (Casha et al., 2001; Casaccia-Bonnefil et al., 1996; Yoon et al., 1998; Gu et al., 1999; Mukai et al., 2000; Kimura et al., 2001; Beattie et al., 2002; Harrington et al., 2002)
- Schwann cells (Ferri et al., 1999; Syroid et al., 2000; Soilu-Hanninen et al., 1999; Petratos et al., 2003)
- a number of non-nervous system cell types including vascular cells (Wang et al., 2000; Kraemer et al., 2002) and keratinocytes (Botchkarev et al., 2000)
- several immortalized cell lines (Bunone et al., 1997; Lievremont et al., 1999; Perini et al., 2002; Gentry et al., 2000; Salehi et al., 2000; Irie et al., 1999; Ye et al., 1999a, b;

Mukai et al., 2000; Wang et al., 2000; Kimura et al., 2001; Bono et al., 1999; **Chapter 4**).

Although this list is long, p75NTR apoptotic responses are cell-type specific and are often restricted to distinct developmental stages or pathological states. For example, retina and spinal cord cells undergo p75NTR-dependent death during embryonic development and sympathetic neurons require p75NTR for death later in postnatal life (Frade et al., 1996, 1999; Bamji et al., 1998). In the adult, p75NTR levels are increased after nervous system injury and disease (Ernfors et al., 1989; Armstrong et al., 1999; 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; Beattie et al., 2002; Troy et al., 2002; Greferath et al., 2002; Park et al., 2000), and more often than not, these p75NTR-expressing cells undergo apoptosis (Roux et al., 1999; Greferath et al., 2002; Troy et al., 2002; Beattie et al., 2002; Park et al., 2000). In fact, the induction of apoptosis within cortical and hippocampal neurons after pilocarpine-induced seizure, within oligodendrocytes after spinal cord injury, or in axotomized sensory and motor neurons, is almost completely blocked when p75NTR is removed (Troy et al., 2002; Beattie et al., 2002; Cheema et al., 1996; Ferri et al., 1998, 1999).

p75NTR can induce apoptosis in cells where Trk activation is reduced or absent (eg. developing oligodendrocytes) (Casha et al., 2001; Casaccia-Bonnefil et al., 1996; Yoon et al., 1998; Gu et al., 1999; Mukai et al., 2000) and in Trk-expressing cells (eg. sympathetic neurons) (Bamji et al., 1998; Palmada et al., 2002; Lee et al., 2001). It is therefore difficult to interpret whether p75NTR facilitates cell death through activation of autonomous pro-apoptotic pathways or by inhibiting Trk activity. Of course, nothing prevents p75NTR from using both mechanisms, but recent results demonstrate that removal of p75NTR in TrkA-/- knockout mice (TrkA-/-, p75NTRIII-/- double knockout mice), substantially rescues TrkA-/- sympathetic neurons from developmental death (Majdan et al., 2001). Furthermore, in Trk-expressing cells, p75NTR often requires non-preferred Trk ligands such as BDNF and proNGF to induce apoptosis (Bamji et al., 1998;

Palmada et al., 2002; Lee et al., 2001). Together, these data suggest that p75NTR autonomous signaling plays a significant role.

The role of ligand binding in p75NTR-induced death is also controversial. Overexpression of p75NTR alone can cause cell death in vitro (Rabizadeh et al., 1993; Bunone et al., 1997; Lievremont et al., 1999; Roux et al., 2000; Ye et al., 1999a; Bhakar submitted; Chapter 4) and in vivo (Majdan et al., 1997), presumably by oligomerization-induced activation of the receptor. However, a number of cellular systems demonstrate a neurotrophin requirement (Casaccia-Bonnefil et al., 1996; Yoon et al., 1998; Gu et al., 1999; Soilu-Hanninen et al., 1999; Trim et al., 2000; Salehi et al., 2000; Cotrina et al., 2000; von Bartheld et al., 1994; Davey et al., 1998; Frade et al., 2000b; Bamji et al., 1998), or a requirement for binding by the unprocessed proneurotrophins (Beattie et al., 2002; Lee et al., 2001) and the neurotoxic peptide ligands (Della-Bianca et al., 2001; Yaar et al., 1997; Perini et al., 2002). This variance in findings might reflect cell-type specific differences, including autocrine signaling mechanisms in ligand-independent experimental systems. However, the variance might just as well reflect the supra-physiological limits attainable by current biochemical approaches.

Alternatively, both ligand-dependent and independent processes may be relevant. Neuronal death resulting from a lack of neurotrophin support may engage death-promoting molecules like p75NTR that signal when unoccupied. Indeed, the two causes of death may be inextricably linked. In support of this interpretation, NGF-deprivation induced apoptosis is decreased in differentiated PC12 cells, sensory neurons, and sympathetic neurons when p75NTR expression is reduced (Rabizadeh et al., 1993; Barrett et al., 1996; Barrett et al., 1994; Bamji et al., 1998). Also, when p75NTR expression is increased, death by neurotrophin-deprivation is accelerated (Barrett et al., 2000). Interestingly, site-directed mutagenesis studies suggest that a juxtamembrane intracellular region dubbed Chopper (Coulson et al., 2000) or a 30-amino acid region that lies in the fourth and fifth helices of the DD called the dependence domain, are crucial for these ligand-independent apoptotic effects (Rabizadeh et al., 2000). Presumably, the newly

discovered higher affinity proneurotrophin ligands will provide a reliable means to compare p75NTR action in a variety of cellular models and will resolve these issues.

#### 1.3.6.3.1.1 Mechanisms of p75NTR-induced apoptosis

Identifying the cellular machinery necessary for p75NTR to kill has also been the focus of intensive research in the past few years. In general, apoptosis can be initiated by two main pathways (Figure 1.4). The first pathway, called the extrinsic death pathway, begins with activation of cell surface death receptor (DR) members of the TNFR superfamily. In response to ligand binding, death receptors recruit TRADD and FADD adaptor proteins, along with initiator Caspase 8, into a 'death-inducing signaling complex' (DISC). The DISC is directly associated with receptor DD tails, and the aggregation of these components results in the activation and release of Caspase 8. Subsequent to these events, active Caspase 8 cleaves and activates effector Caspases, and active effector Caspases go on to cleave substrates for the execution of cell death (reviewed in Aggarwal, 2000; Baud and Karin, 2001; Joza et al., 2002; Opferman and Korsmeyer, 2003).

The second pathway, called the intrinsic death pathway, is initiated by cellular responses that signal to mitochondrial regulators of the Bcl-2 protein family (reviewed in Matsuyama and Reed, 2000; Shi, 2002; Harris and Johnson, 2001; Opferman and Korsmeyer, 2003). Typically, the pro-apoptotic "BH3-domain only" members are the first to accumulate and activate. Activated BH3-domain only proteins then either block prosurvival Bcl-2 members or directly activate pro-apoptotic multidomain members such as Bax and Bak (Letai et al., 2002). In either case, Bax and Bak oligomerize allowing for release of pro-apoptotic mitochondrial proteins into the cytosol and disruption of mitochondrial integrity. Released mitochondrial proteins such as Cytochrome c bind to substrates that facilitate initiator Caspase 9 activation. Thereafter, Caspase 9 activates the effector Caspases for initiation of the final stages of cell death.

Cultured sympathetic neurons and cerebellar granule neurons have been useful model systems to delineate the components of neuronal death. Both of these neuron types express p75NTR. Moreover, when trophic support is removed, NGF for sympathetic

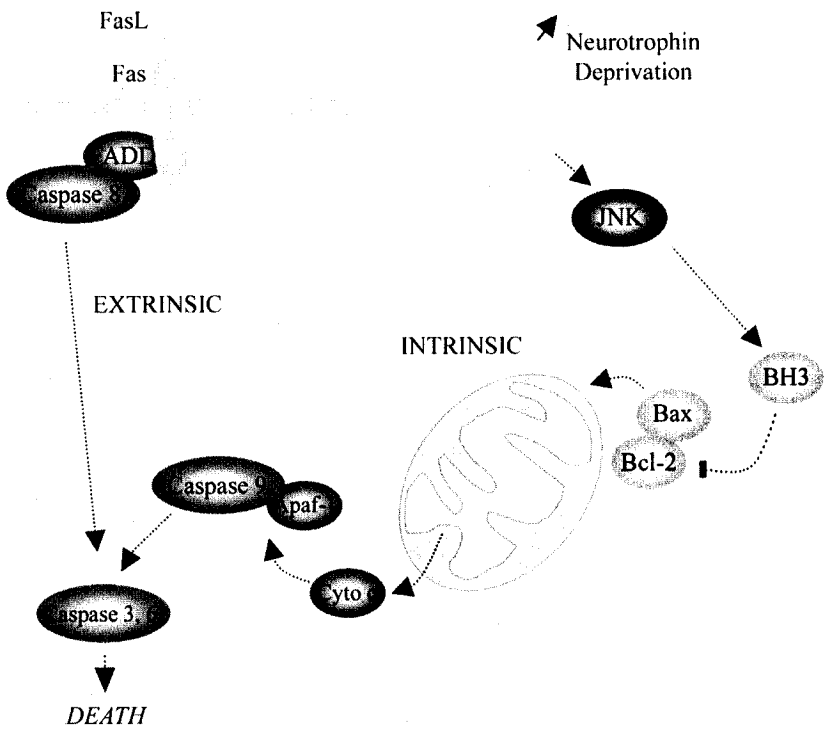


**Figure 1.4.** The extrinsic and intrinsic cell death pathways. The extrinsic pathway begins with death receptor (Fas) recruitment of FADD for Caspase 8-dependent death. The intrinsic pathway, which can be initiated by neurotrophin withdrawal, relies on mitochondrial regulators of the Bcl-2 protein family for Cytochrome c release and activation of Caspase 9-dependent death.

neurons and potassium chloride (KCl) for cerebellar granule neurons, these cells undergo Caspase activation and classical apoptotic changes dependent upon the loss of mitochondrial integrity. These events are consistent with activation of the intrinsic death pathway. In sympathetic neurons, the loss of survival signals also promotes the activation of JNK and the mitochondrial accumulation and activation of Bax. These two events are also absolutely required for sympathetic neurons to die (Martin et al., 1988; Deshmukh et al., 1996, 1998; Martinou et al., 1999; Putcha et al., 1999; Eilers et al., 2001; Bruckner et al., 2001; Harding et al., 2001; Deckwerth et al., 1996).

The membrane proximal events linking NGF withdrawal to activation of the JNK pathway, in sympathetic neurons, are not yet known. Experiments using dominant negative mutants to block NGF-deprivation induced death, however, have implicated several downstream events. These include activation of the small GTPases Cdc42 and Rac1 (Bazenet et al., 1998), activation of the MAPKKK components including members of the MEKK and MLK protein families (Fan et al., 1996; Hirai et al., 1996; Rana et al., 1996; Tibbles et al., 1996; Sakuma et al., 1997), and subsequently, the MAPKKK substrates, MKK4 and 7 (Xu et al., 2001). Active MKK4 and 7 can then directly phosphorylate and activate JNK (Bruckner et al., 2001).

The link between JNK activation and the activation and accumulation of Bax in neuronal death is also not completely clear. The best characterized event is JNK-dependent phosphorylation of the transcription factor, c-Jun. Activated c-Jun is then believed to propagate pro-apoptotic signals by increasing the transcription of BH3-domain only protein products, Bim and Dp5 (Harris et al., 2001; Whitfield et al., 2001; Putcha et al., 2001). Recent work, however, suggests that neuronal apoptosis may also require post-translational modifications of BH3-domain only proteins. For example, activation of the Cdc2 and JNK kinases can lead to pro-apoptotic phosphorylation and activation of Bad, Bim, and Bmf (Donovan et al., 2002; Konishi et al., 2002; Putcha2003; Lei and Davis, 2003). Pro-apoptotic phosphorylations of Bad and Bim are found in cerebellar granule and sympathetic neuronal death, respectively (Donovan et al., 2002; Konishi et al., 2002; Putcha2003). Moreover, expression of a phospho-specific dominant inhibitory Bad

mutant can significantly reduce trophic-deprivation induced death of cerebellar granule neurons (Konishi et al., 2002).

The mechanisms of p75NTR-mediated apoptosis are beginning to emerge. In cells undergoing p75NTR-activated death, p75NTR can activate Rac (Harrington et al., 2001) and JNK (Casaccia-Bonnefil et al., 1996; Yoon et al., 1998; Aloyz et al., 1998; Bamji et al., 1998; Friedman et al., 2000; Roux et al., 2001; Chapter 4). One study suggests that Rac is necessary for p75NTR-induced death, and we and others have recently shown that blocking JNK activity with chemical inhibitors or with dominant-inhibitory mutants, completely attenuates p75NTR-induced death (Chapter 4; Harrington et al., 2001; Friedman et al., 2000). p75NTR-induced death also correlates with the release of Cytochrome c from mitochondria and selective activation of Caspases 9, 6, and 3 (Chapter 4; Gu et al., 1999; Wang et al., 2001; Troy et al., 2002; Harrington et al., 2001; Jover et al., 2002). Additionally, under conditions in which p75NTR induces JNK phosphorylation and death, we have shown that p75NTR specifically increases the phosphorylation and oligomerization of the BH3-only protein Bad (Chapter 4). Furthermore, functional deletion of Bad, using RNAi or the dominant inhibitory phosphorylation mutant, demonstrates that Bad is necessary for p75NTR induced death. Therefore, although homology to the TNFRSF initially suggested that p75NTR would activate an extrinsic death pathway common to death receptors (DR), most data suggest that p75NTR-induced death is more comparable to intrinsic neuronal death mechanisms.

### 1.3.6.3.1.2 p75NTR death promoting interactors

The p75NTR-interacting proteins that link the receptor to the JNK pathway are unknown. No GEFs or GTPase-activating proteins (GAPs), which regulate Rac, Rho or Cdc42 activity, have been found in association with p75NTR or with p75NTR interactors. The small GTPase RhoA, in complex with its guanine dissociation inhibitor RhoA-GDI, can bind to the fifth helix of the p75NTR DD. However, this interaction has not been linked to JNK activation or apoptotic signaling (Yamashita et al., 1999, 2003).

One interactor, NADE (p75NTR-associated cell death executor), interacts with the DD of p75NTR and mediates caspase-dependent apoptosis in response to NGF in 293T, PC12, nnr5, cortical neurons, and oligodendrocytes. NADE might also be particularly relevant for ischemia-induced apoptosis to the hippocampus (Mukai et al., 2000; Park et al., 2000). NADE is 22 kilodaltons with a central region (amino acids 41-71) sufficient to induce apoptosis. The C-terminus of NADE (amino acids 72-112), however, contains a leucine-rich nuclear export signal (NES) sequence and two ubiquitination boxes that are essential for endogenous NADE effects. These effects include nuclear export of NADE, NADE self-association, NADE interaction with p75NTR and the 14-3-3e protein, and NADE-dependent NGF-initiated apoptosis (Mukai et al., 2002; Kimura et al., 2001). No obvious connections to the JNK pathway, however, can be attributed to NADE-induced apoptosis.

Another p75NTR interactor, NRAGE (Neurotrophin receptor-interacting MAGE), can regulate p75NTR-dependent apoptosis, and when overexpressed, induces apoptosis in a JNK-dependent manner (Salehi et al., 2000, 2002). NRAGE is an 86 kDa MAGE family member that interacts with the juxtamembrane region of the p75NTR ICD. The MAGE homology domain within NRAGE is approximately 200 amino acids long and is necessary for its binding to p75NTR. NRAGE expression is highest in proliferating neural populations from the developing brain and spinal cord, but can also be found in adult basal forebrain cholinergic neurons, within the hippocampus, and in cell types where p75NTR expression is induced following injury (Frade and Barde, 1999; Kendall et al., 2002). NRAGE facilitates p75NTR-mediated death of sympathetic precursor cells but this process can be blocked by TrkA expression, presumably by disrupting a p75NTR-NRAGE complex (Salehi et al., 2000).

Work from our lab (Salehi et al., 2002) demonstrates that NRAGE is a potent regulator of JNK activity, mitochondrial release of Cytochrome c, activation of Caspase 9, and Caspase-dependent cell death. Blocking JNK activity ablates NRAGE-mediated Caspase activation and cell death. These findings identify NRAGE as a p75NTR interactor capable of inducing Caspase activation and cell death through a JNK-dependent mitochondrial pathway (Salehi et al., 2002). Additional mechanisms of NRAGE-induced

death, however, may be possible. NRAGE can interact with the RING domains of anti-apoptotic IAP (inhibitor of apoptosis protein) homologues ITA and XIAP to facilitate interleukin-3 withdrawal induced apoptosis (Jordan et al., 2001). NRAGE can also induce cell cycle arrest when overexpressed and may, therefore, facilitate p75NTR-dependent apoptosis through an up-regulation of cell cycle proteins (Salehi et al., 2000; Kendall et al., 2002).

Traditionally, little attention has been paid to the interplay between neurotrophins and the cell cycle. However, a role for cell cycle proteins in p75NTR-induced cell death, has previously been demonstrated. NGF treatment of p75NTR-expressing retinal neurons increases cyclin B2 levels, cell cycle entry, and apoptosis (Frade, 2000b). Similarly, p75NTR-dependent apoptosis can be blocked by cyclin-dependent kinase (CDK) inhibitors. Additionally, the inappropriate activation of cell cycle regulatory molecules commonly activates apoptosis requiring the tumour suppressor p53, and p53 has previously been implicated in p75NTR-induced apoptosis (Bamji et al., 1998; Frade, 2000). These results raise the intriguing possibility that the apoptotic effects of p75NTR involve conflicting signals for cell division and growth arrest. Indeed, p75NTR is expressed in many cells at the time they become post-mitotic (Farinas et al., 1998). As well, p75NTR can retard cell-cycle progression in tumor cells (Kwaja et al., 2003) and nestin-positive proliferating cells in vivo (Hosomi et al., 2003). Furthermore, ceramide, a lipid second messenger activated by p75NTR (Dobrowsky et al., 1994) can mimic NGF-mediated growth arrest and promote the differentiation of embryonic hippocampal neurons when applied exogenously (Brann et al., 1999).

A number of p75NTR-binding proteins also regulate cell cycle events. p75NTR can interact with the MAGE family members, Necdin and MAGE-H1, to accelerate NGF-mediated differentiation (Tcherpakov et al., 2002). Interestingly, Necdin is predominantly expressed in postmitotic neurons and can induce growth arrest of proliferative cells, possibly by interacting with the transcription factors E2F1 and p53. p75NTR can also bind the zinc finger proteins SC-1 (Schwann cell factor-1) and NRIF1/2 (Neurotrophin receptor-interacting factor) to disrupt the cell cycle and sometimes promote apoptosis

(Chittka et al., 1999; Casademunt et al., 1999; Benzel et al., 2001). These functions are supported in vivo, since NRIF1 null mice show reduced developmental death in the retina, similar to that observed in mice lacking p75NTR (Casademunt et al., 1999; Frade and Barde, 1999). Moreover, NRIF2 and p75NTR$^{IV-/-}$ null mice are small. However, little is known regarding the signal transduction mechanisms of SC-1 or NRIF proteins in cell cycle or apoptotic regulation.

### 1.3.6.3.2 p75NTR promotes survival

Many members of the TNFR superfamily are bifunctional in that they activate both apoptotic and prosurvival signals (reviewed in Baker and Reddy, 1998). Similarly, recent work demonstrates that p75NTR can, in some circumstances, facilitate cell survival. For example, activation of p75NTR can promote the survival of Schwann cells (Khursigara et al., 2001), cancer cells (Roux et al., 2001; Gentry et al., 2000; Descamps et al., 2001; Mamidipudi et al., 2002; Wooten et al., 2001; Yazidi-Belkoura et al., 2003; Foehr et al., 2000; Hughes et al., 2001; Lachyankar et al., 2002) and specific developing cortical, retinal, hippocampal, cerebellar and sensory neuron sub-populations (Bhakar unpublished results; Brann et al., 1999; De Freitas et al., 2001; Hamanoue et al., 1999; Hutson et al., 2001; Culmsee et al., 2002; Courtney et al., 1997; Bui et al., 2002). After neurotoxic stress, neurotrophin treatment of cortical and hippocampal neurons, which express p75NTR but not TrkA, protects these cells (Shimohama et al., 1993; Kume et al., 2000; Cheng and Mattson, 1991; Cheng et al., 1993; Culmsee et al., 2002; Bui et al., 2002). Similarly, when overexpressed, p75NTR can protect PC12 cells that have been deprived of serum (Roux et al., 2001). Thus, significant evidence supports a role for p75NTR in the facilitation of survival.

#### 1.3.6.3.2.1 Mechanisms of p75NTR-mediated survival

The mechanisms by which p75NTR activates prosurvival signals are not known, but many TNFRSF members promote survival by activating NF-kB (Pahl et al., 1999; Denk et al., 2000). The transcription complex NF-kB (nuclear factor-kappa B) is made of two subunits taken from the Rel Family. Rel members include RelA [p65], NFkB2 [p52/p100], NFkB1 [p50/p105], RelB, and c-Rel, and each member has a 300-amino acid

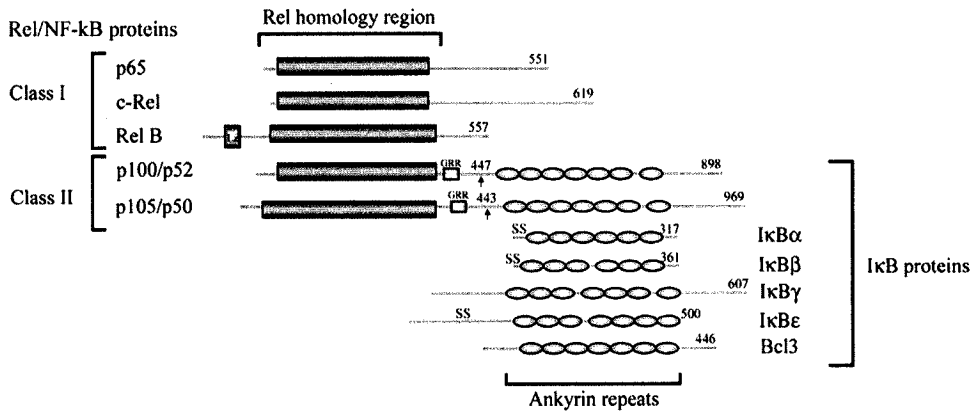


**Figure 1.5.** Mammalian Rel/NF-κB and IκB proteins. The five mammalian Rel family members are divided into two classes: Class I proteins do not require proteolytic processing, Class II proteins do require proteolytic processing. All members dimerize, the most commonly detected NF-κB dimer is RelA/p50. All NF-κB proteins contain a Rel homology domain (RHD) which mediates their dimerization and binding to DNA. The RHD also contains at its C-terminus, a nuclear localization sequence (NLS) which is recognized and masked by the IκB proteins. All IκB proteins contain 6-7 ankyrin repeats, which mediate their binding to RHDs. IκBα, IκBβ, and IκBε contain an N-terminal regulatory domain, within which there are two conserved serines (SS). Phosphorylation at these sites targets the IκBs to ubiquitin-dependent degradation. GRR, glycine-rich region; Lz, leucine zipper; arrows indicate C-terminal residues of p50 and p52 following p105 and p100 processing, respectively. Adapted from Karin et al. 2002, Nature Cancer Reviews (2):301.

region of homology (the Rel homology domain) containing motifs for dimerization, DNA binding, and nuclear localization (Figure 1.5) (reviewed in Verma et al., 1995; Karin et al., 2002). The most usual form of NF-kB is a heterodimer of p65 and p50, which normally exists dormant in the cytoplasm by virtue of its interaction with a member of an inhibitory protein family called IkB. IkB proteins include IkBα, IkBβ, IkBε, p105, p100, and Bcl-3 (Figure 1.5).

p75NTR-dependent NF-kB activation has been reported following neurotrophin treatment of Schwann cells (Carter et al., 1996; Khursigara et al., 1999), oligodendrocytes (Yoon et al., 1998; Ladiwala et al., 1998) sensory (Hamanoue et al., 1999) and sympathetic neurons (Maggirwar et al., 1998), PC12 cells (Foehr et al., 2000; Mamidipudi et al., 2002; Wooten et al., 2000; Bui et al., 2001), PCNA cells (Carter et al., 1996; Bhakar et al., 1999), Schwannomas (Gentry et al., 2000), P19 neuronal cells (Burke et al., 2003), hippocampal neurons (Bui et al., 2001), neuroblastomas (Bui et al., 2001) and 293T cells (Ye et al., 1999). Unfortunately, the level of NF-kB activation is modest compared to activities induced by other TNFRSF members, and more often than not, this modest effect can only be seen after cells have been subjected to severe stress, including temperature changes, nutrient withdrawal, and hypoxia (Bhakar et al., 1999; Cosygaya et al., 2001; Ladiwala et al., 1998; Carter et al., 1996; Hughes et al., 2001; Khursigara et al., 2001). In fact, it is plausible that p75NTR does not directly activate NF-kB in most systems, but instead p75NTR functions to enhance NF-kB activation by other receptors (Chapter 2; Bhakar et al., 1999; Cosygaya et al., 2001; Recio et al., 1997). In support of this interpretation, many TNFRSF members can functionally cooperate to enhance intracellular signals, especially in response to stress as part of the pro-inflammatory response (MacEwan, 1996; Haxhinasto et al., 2002; Cheng et al., 2003). Moreover, p75NTR has been previously reported to functionally cooperate with several receptor types (MacEwan, 1996; Foehr et al., 2000; Savitz et al., 2000; Kuner et al., 1998; Perini et al., 2002; see section 1.3.6.2).

The mechanisms by which p75NTR may regulate NF-kB are not completely clear. In non-neuronal cells, activation of NF-kB requires activation of kinase cascades that

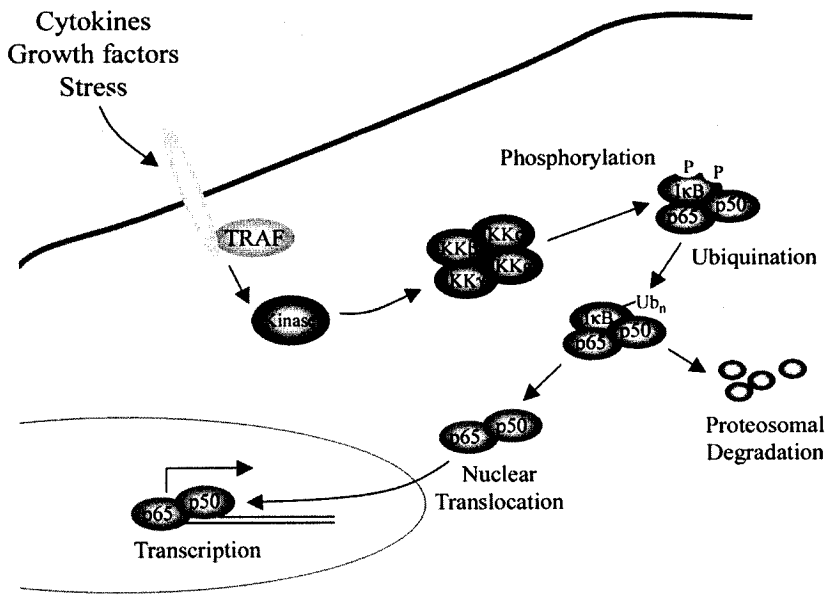


**Figure 1.6.** Schematic diagram of the canonical pathway for NF-kB activation. In response to ligand binding, receptors like TNFR, recruit and bind adaptor TRAF proteins to activate kinases that culminate in the activation of the IKK kinase complex. Active IKK phosphorylates IkB proteins on two serine residues to initiate ubiquitin-dependent IkB degradation. This event releases NF-kB dimers (p65/p50) to translocate to the nucleus and bind to consensus kB DNA sequences for the initiation of gene transcription.

converge on IKK1 and IKK2, related regulatory kinases within a kinase complex called IKK (IkB Kinase). Once active, IKKs phosphorylate IkB family members on specific serine residues to initiate Ubiquitin-dependent IkB degradation. This event releases NF-kB dimers to translocate to the nucleus and bind to consensus kB DNA sequences for the initiation gene transcription (Figure 1.6).

p75NTR activation can result in slight increases in IKK activity (Foehr et al., 2000), IkB phosphorylation on serine residues, IkB degradation (Bhakar et al., 1999; Cosygaya et al., 2001; Foehr et al., 2000; Mamidipudi et al., 2002), and sometimes unusual tyrosine phosphorylation of IkBα (Bui et al., 2001, 2002). p75NTR activation has also been linked to increased production of the neuroprotective NF-kB-regulated genes including Bcl-2, Bcl-xL, iNOS and COX-2 (Bui et al., 2001; Culmsee et al., 2002; Foehr et al., 2000; Burke et al., 2003). Most of these events, however, have not been linked to p75NTR-mediated survival outcomes.

Recent work has demonstrated that, similar to Trk receptors, p75NTR can promote survival by activating the serine/threonine kinase, Akt (Roux et al., 2001; Bui et al., 2002). Activation of Akt by p75NTR requires PI3-K activity and this event is associated with increased tyrosine phosphorylation of p85 and Shc, and reduced cytosolic tyrosine phosphatase activity. There are several Akt substrates that could mediate a p75NTR-initiated survival response (see Trk section). One intriguing possibility includes direct regulation of the NF-kB pathway by phosphorylation of IKK1 (Kane et al., 1999; Ozes et al., 1999; Romashkova et al., 1999). In fact, experiments performed by Bui and colleagues (2002) demonstrate that inhibition of PI3-kinase/Akt activity prevents NGF-dependent activation of NF-kB in PC12 cells and hippocampal neurons (Bui et al., 2002).

### 1.3.6.3.2.2 p75NTR survival promoting interactors

The cellular components linking p75NTR activation to the Akt or NF-kB pathways are also unknown. In non-neuronal cells, receptor-mediated activation of NF-kB occurs via two main pathways. The classical pathway begins by recruiting intracellular TRAF (TNF receptor associated factors) adaptor proteins to receptor ICDs. In the case of TNFRSF

members, this process can occur through direct binding or through adaptor proteins such as TRADD and RIP2. In the case of IL-1 (Interleukin-1)/Toll Receptor family members, the process can occur through MyD88 and IRAK (reviewed in Cao et al., 1999). Recruited TRAF proteins have E3 ubiquitin (Ub) ligase activity. Then, through the assembly of novel K63-linked poly-Ub chains on downstream targets, kinase activity is initiated, which is responsible for the phosphorylation and activation of IKK2 (Deng et al., 2000; Wang et al., 2001; Yang et al., 2001). IKK2 subsequently phosphorylates IkB inhibitors allowing for release and activation of NF-kB. The non-cannonical pathway requires activation of cellular signals that converge on the MAPKKK, NIK (NF-kB inducing kinase). NIK subsequently activates IKK1, and this allows for processing of p100 to generate an active NF-kB dimer (Yin et al., 2001; Xiao et al., 2001).

Several studies show that components of the classical NF-kB pathway can interact with p75NTR. The TRAF proteins, for example, bind to different regions of the p75NTR intracellular domain through conserved C-terminal coiled-coil TRAF domains (Ye et al., 1999b; Khursigara et al., 1999, 2001; Krajewska et al., 1998; Zapata et al., 2000; Vaillantcourt, manuscript in preparation). Each of the six mammalian TRAF members contain this C-terminal TRAF domain, responsible for TRAF homo- and hetero-dimerization. Most members, with the exception of TRAF1, also have an N-terminal RING finger domain, followed by clusters of five to seven zinc finger domains. These domains are important for propagation of downstream signaling events (reviewed in Bradley and Pober, 2001).

Initially, p75NTR was thought to bind all six mammalian TRAF proteins, and its interaction with TRAF2 and TRAF6 was thought to facilitate p75NTR-dependent activation of NF-kB (Khursigara et al., 1999; Ye et al., 1999b). Recent analysis, however, has found that neither TRAF2, 3 nor 5 bind to p75NTR (Zapata et al., 2000). In addition, TRAF6 association with p75NTR may be more relevant for activating the transcription factor ATF-2 than for activating NF-kB (Khursigara et al., 2001). To resolve these contradictions, we recently examined the relative affinity of each TRAF member for p75NTR and found the highest affinity was for TRAF4. Specifically, TRAF4 binds to the

p75NTR conserved JXM region between amino acids 303-329, and this interaction has little effect on NF-kB signaling. Instead, TRAF4 has dramatic effects on p75NTR cellular trafficking such that TRAF4 expression causes the retention of p75NTR in the endoplasmic reticulum (Vaillancourt, manuscript in preparation).

We and others have confirmed that TRAF6 can also transiently bind to p75NTR (Khursigara et al., 1999; Wooten et al., 2001; Mamidipudi et al., 2002; Vaillancourt, manuscript in preparation). This interaction is ligand-dependent, requires the p75NTR JXM domain, and likely occurs via a complex of additional co-factors. One co-factor, IRAK (IL-1 receptor-associated kinase), is a member of a family of cytoplasmic serine-, threonine-specific innate-immunity kinases (SIIK). IRAK shares some homology with human mixed-lineage kinase (hMLK) proteins and the Drosophila kinase Pelle, which activates Dorsal, the Drosophila equivalent of NF-kB. IRAK has an N-terminal DD of 120 amino acids and a central 300 amino acid kinase domain. When cells are stimulated with IL-1, IRAK associates transiently with the IL-1 receptor through the adaptor MyD88. Following this, IRAK becomes highly phosphorylated and then dissociates to bind TRAF6 and activate the NF-kB pathway. Recent work by Wooten and colleagues (Wooten et al., 2001; Mamidioudi et al., 2002) demonstrates that in PC12 cells, p75NTR can recruit IRAK together with MyD88, the atypical PKC interacting protein p62 and TRAF6. This complex then recruits and activates IKK2 for NF-kB activation.

Other co-factors supporting TRAF association and/or NF-kB activation may include the adaptor protein TRADD, the RIP2 kinase, and the Fas-associated phosphatase-1, FAP-1. Neurotrophin treatment of MCF-7 breast cancer cells leads to association of TRADD with p75NTR. This association requires the TRADD DD and has been implicated in p75NTR-activated pro-survival signals (Yazidi-Belkoura et al., 2003). RIP2, also known as RICK or CARDIAK, is a serine/threonine kinase that contains a caspase recruitment domain (CARD) and is capable of associating with the TNF receptor complex (Inohara et al., 1998; McCarthy et al., 1998; Thome et al., 1998). RIP2 is highly expressed in newly isolated Schwann cells and the CARD domain of RIP2 can interact with the 5th helix of the p75NTR DD in an NGF-dependent manner. This interaction results in enhanced

p75NTR-activated NF-kB activity and functions to reduce NGF-induced apoptosis of Schwann cells (Khursigara et al., 2001). The Fas interactor, FAP-1, contains a putative ezrin membrane binding domain and six PDZ domains. FAP-1 can bind the conserved C-terminal Ser-Pro-Val residues of p75NTR through its third PDZ domain when highly overexpressed (Irie et al., 1999; Sato et al., 1995). This binding correlates with modest increases in p75NTR-mediated NF-kB activation. However, the precise role of FAP-1 in p75NTR function is not known.

### 1.3.6.4 Regulation of neurite outgrowth by p75NTR

New evidence demonstrates that the functions of p75NTR extend beyond modulation of Trk signals and the regulation of cell survival and death. In particular, p75NTR is a key regulator of axon elongation. For example, in response to NGF, hippocampal and ciliary neurons, which express p75NTR and not TrkA, extend neurites (Brann et al., 1999; Yamashita et al., 1999). Accordingly, axon outgrowth deficits can also be found in spinal cord and forelimb motor neurons of $p75NTR^{III-/-}$ embryos (Yamashita et al., 1999; Bentley et al., 2000). Interestingly, the p75NTR ligand, CRNF, has also been shown to stimulate neurite outgrowth of pedal A motor neurons (Fainzilber et al., 1996).

Whereas p75NTR binding to neurotrophins or CRNF leads to axonal elongation, p75NTR seems to be a major transducing component of neurite outgrowth inhibitory signals in response to myelin-derived inhibitors (Yamashita et al., 2002; Wang et al., 2002; Wong et al., 2002). For example, p75NTR can directly interact with the brain ganglioside GT1b and the lipid-anchored Nogo receptor (NgR). Both are established binding partners for the major growth-inhibiting components of myelin which include MAG (myelin-associated glycoprotein), Nogo, and OMgp (oligodendrocyte-myelin glycoprotein) (reviewed in Vyas and Schnaar, 2001; Fournier et al., 2002). When primary cerebellar and sensory neurons are treated with these inhibitory proteins, neurite outgrowth is inhibited. However, MAG or Nogo-mediated growth inhibition does not occur in cerebellar or sensory neurons derived from p75NTR knockout animals (Yamashita et al., 2002, 2003; Wang et al., 2002; Wong et al., 2002). In fact, growth inhibition through p75NTR might account for the cholinergic hyperinnervation seen in the hippocampus of $p75NTR^{III-/-}$

mutant mice (Yeo et al., 1997) and the increased NGF-outgrowth responsiveness of sympathetic neurons taken from these mice (Kohn et al., 1999). Similarly, overexpression of NGF within p75NTR$^{III-/-}$ mutant mice leads to aberrant sympathetic axon growth in myelin-rich areas where these axons would not normally grow (Walsh et al., 1999), suggesting that p75NTR is necessary for proper growth inhibition.

Neurotrophin or myelin-inhibitor interaction with p75NTR may also provide necessary cues to properly guide axon growth. Sympathetic and cortical subplate axons, for example, innervate inappropriate targets when p75NTR is removed (Lee et al., 1994; McQuillen et al., 2000). Moreover, MAG-induced growth cone turning is disrupted in Xenopus axons treated with p75NTR blocking antibodies (Wong et al., 2002). Interestingly, the p75NTR interacting protein NRAGE, associates with the DD-containing axon guidance receptor UNC5H1 (Williams et al., 2003). Together, these data suggest that p75NTR plays a crucial role in the growth and guidance of axons.

#### 1.3.6.4.1 Mechanisms of p75NTR-mediated growth regulation

Exciting new possibilities for how p75NTR might regulate outgrowth have recently been reported, and a key regulatory event is modulation of RhoA activity. When p75NTR-expressing neurons are treated with neurotrophin, for example, RhoA activity is attenuated and outgrowth is favored (Yamashita et al., 1999, Higuchi et al., 2003). On the other hand, when myelin-inhibitory proteins are applied, RhoA activity increases, and this directs myelin outgrowth inhibition by causing the actin cytoskeleton to become more rigid (Yamashita et al., 2002, 2003; Wang et al., 2002; Wong et al., 2002).

The precise binding partners and associated GEFs required for p75NTR regulation of RhoA are not completely clear. Recent work demonstrates that the association of myelin-inhibitory proteins with p75NTR leads to direct interactions between p75NTR and the Rho nucleotide dissociation inhibitor, Rho-GDI (Yamashita et al., 2003). This interaction uses the fifth helix of the p75NTR DD and functions to inhibit Rho-GDI activity. Displacing Rho-GDI from RhoA facilitates GEF-mediated activation of RhoA and

suggests, under these conditions, p75NTR inhibits neurite growth by functioning as a displacement factor.

For neurotrophin-dependent regulation of RhoA, new data suggests that a variant of the b catalytic subunit of cAMP-dependent protein kinase, PKACb is required. Neurotrophin binding to p75NTR within cerebellar neurons increases intracellular cAMP levels, PKA activity, and causes the association of PKACb with p75NTR. Activated PKA results in phosphorylation of p75NTR at Ser304, and this event is necessary for p75NTR to translocate to lipid rafts. Therefore, the translocation of p75NTR may be a prerequisite for RhoA inactivation and neurite outgrowth. This relationship, however, remains to be shown (Higuchiet al., 2003). Interestingly, MAGE family members that interact with p75NTR also support neurite extension (Tcherpakov et al., 2002). Whether MAGE proteins modulate Rho activity for growth is currently unknown.

# RATIONALE AND OBJECTIVES

## Rationale

During brain development and neurodegenerative disease, large numbers of neurons die through a genetically defined suicide program called apoptosis (reviewed in Roth and D'Sa 2001). The cellular machinery that controls this neuronal apoptosis relies on the availability of neurotrophic factors such that insufficient or inappropriate trophic support rapidly activates death. Linking these trophic conditions to the death machinery, however, remains a significant challenge.

One possible link is the p75 neurotrophin receptor which binds and responds to the neurotrophin trophic factors and significantly regulates apoptosis in a number of neuronal populations (reviewed in Roux2002). The signal transduction pathways employed by p75NTR are beginning to be understood but are still largely incomplete. So far, it is clear that p75NTR can regulate RhoA, Akt, and JNK activities and that p75NTR can interact with several adaptor proteins. These findings suggest that careful analysis of p75NTR signaling mechanisms will provide critical information for understanding neuronal apoptosis. Therefore, for my doctoral thesis, I have attempted to elucidate molecular mechanisms underlying neuronal apoptosis by characterizing cellular signals regulated by p75NTR.

## Objectives

The three main objectives of this thesis are:

1. To determine whether p75NTR activates NF-kB, given that related TNFRSF members activate the NF-kB transcription factor to regulate cellular apoptosis.

2. To clarify the role and pattern of transcriptionally active NF-kB within the nervous system, since little is known regarding neuronal NF-kB function and location.

3. To identify the signaling pathways required for p75NTR-induced death, as p75NTR can regulate apoptotic events and has structural homologies to known death receptors.

# PREFACE TO CHAPTER 2

The discovery of the TNF receptor superfamily (TNFRSF) has been important for understanding how receptors lacking enzymatic activity function. At the beginning of our investigation, several members of this superfamily (including TNFR1) were shown to regulate cell death, and the signal transducing pathways for regulating cell death were just being described. Evidence that p75NTR could also regulate apoptosis suggested that p75NTR would activate cellular responses in a manner similar to its TNF receptor homologs. One TNFRSF-mediated cellular response that regulates apoptosis is the activation of the transcriptional complex, NF-kB. Therefore, Chapter Two considers whether p75NTR activates the NF-kB pathway in a variety of cell types and, if so, to what degree. Chapter 2 also investigates whether p75NTR contributes to NF-kB activation by other stimuli because: 1) TNFRSF members share common downstream TRAF effectors and 2) convergent signals between different receptor types can amplify NF-kB activation.

# CHAPTER 2

# THE P75 NEUROTROPHIN RECEPTOR (P75NTR) ALTERS TUMOR NECROSIS FACTOR-MEDIATED NF-KB ACTIVITY UNDER PHYSIOLOGICAL CONDITIONS, BUT DIRECT P75NTR-MEDIATED NF-KB ACTIVATION REQUIRES CELL STRESS

# p75NTR alters TNF-mediated NF-kB activity under physiological conditions but direct p75NTR-mediated NF-kB activation requires cell stress

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## ABSTRACT

The p75 neurotrophin receptor (p75NTR) has been linked to activation of the NF-kB transcriptional complex in oligodendrocytes, Schwann cells and PCNA cells. In this report, tumor necrosis factor- and neurotrophin-mediated NF-kB activation were compared in several cell lines. All cell types showed TNF-mediated activation of NF-kB but direct neurotrophin-dependent activation of NF-kB was never observed under normal growth conditions. In PCNA cells, a modest NGF-dependent induction of NF-kB was detected but only after cells were subjected to severe stress. Although NGF binding did not directly activate NF-kB under normal conditions, NGF consistently altered TNF-dependent NF-kB activation in each cell type examined; extended exposure to NGF and TNF always increased NF-kB activation over that achieved with TNF alone. The increase in NF-kB activity mediated by NGF correlated with reduced levels of IkBα NGF added alone had no effect on IkBα levels but when added with TNF, NGF treatment significantly reduced IkBα levels. We propose that modulation of cytokine receptor signaling is a significant physiological function of the p75 neurotrophin receptor and that previous reports of direct NF-kB activation through p75NTR reflect this modulatory activity.

# INTRODUCTION

The neurotrophins are a well conserved family of proteins which play critical roles in the maintenance and development of the nervous system (Cowley et al., 1994; Ernfors et al., 1994a, b; Jones et al., 1994; Klein et al., 1993, 1994; Smeyne et al., 1994). Their cellular effects are mediated by two distinct classes of cell surface receptors. The trk receptors, a highly related family of receptor tyrosine kinases, recognize the neurotrophins with a relatively high degree of binding specificity: TrkA preferentially binds NGF[4], TrkB prefers BDNF[5] and NT-4/5[6], and TrkC interacts only with NT-3[7] (Kaplan and Miller, 1997). The other class of neurotrophin receptor contains only the p75NTR[8]. This receptor is a member of the TNF[9] receptor superfamily that includes CD27, CD30, CD40, 4-1BB, OX40, the fas antigen and the tumor necrosis factor receptors TNFR1 and TNFR2 (Baker and Reddy, 1996). Unlike the trk receptors, defining the precise physiological role of the p75NTR has proven difficult (Barker, 1998). Several studies indicate that p75NTR can functionally interact with trk receptors to enhance or dampen intracellular signals. For example, when p75NTR is co-expressed with trkA, it tends to dampen basal levels of TrkA activation and reduce responses of trkA to nonpreferred ligands (Barker, 1998; MacPhee and Barker, 1997; Benedetti et al., 1994; Ip et al., 1993; Bibel et al., 1999). However, p75NTR also facilitates NGF binding to trkA and thus increases trkA responses to preferred ligands (Mahadeo et al., 1994; Barker et al., 1994; Hantzopoulous et al., 1994; Verdi et al., 1994).

p75NTR also has an autonomous signaling role that in some respects is similar to other members of the TNF receptor superfamily. Binding of each of the neurotrophins to p75NTR evokes activation of cellular sphingomyelinase which results in increased ceramide production (Dobrowsky et al., 1994, 1995) and recent studies suggest that p75NTR may behave as a ligand-activated apoptotic receptor during development (Cassacia-Bonnefil et al., 1996; Frade et al., 1996, Majdan et al.,1997; Frade and Barde,

[4] nerve growth factor
[5] brain-derived neurotrophic factor
[6] neurotrophin 4/5
[7] neurotrophin-3
[8] p75 neurotrophin receptor
[9] tumor necrosis factor

1999). Specific p75NTR interacting proteins have proven difficult to identify but the receptor's apoptotic function may be subserved by a region of intracellular homology shared between p75NTR and other apoptotic receptors of the TNF receptor superfamily. This 80 amino acid region, termed the death domain, is required to mediate interactions of other TNF receptor superfamily members with downstream apoptotic effectors (Schulze-Osthoff et al., 1998).

One well studied effect of TNF receptor superfamily members is activation of the transcription complex NF-kB (Baldwin, 1996). In response to ligand binding, receptors of this class bind TRAF proteins through their intracellular domains and activate a kinase cascade that culminates in activation of IKKα and IKKβ and subsequent phosphorylation of IkB subunits, which targets them for ubiquitination and proteosomal degradation (Karin and Delhase, 1998). IkB degradation releases NF-kB subunits from their latent cytoplasmic state and allows them to translocate to the nucleus where they regulate specific gene regulatory events. There are preferential interactions of the six TRAF proteins with various members of the TNF receptor superfamily (Aizawa et al., 1997; Cao et al., 1996; Ishida et al., 1996; Sandberg et al., 1997) and it is likely that differences in these TRAF protein associations play a crucial role in determining levels of NF-kB activation which occurs in response to a particular stimulus. To date, only TRAF6 has been reported to interact with p75NTR (Khursigara et al., 1999).

NGF binding to p75NTR activates NF-kB in fibroblasts overexpressing p75NTR, in primary mouse Schwann cells (Carter et al., 1996) and in primary rat oligodendrocytes (Yoon et al., 1998; Ladiwala et al., 1998). To extend these results, we examined p75NTR-mediated NF-kB activation in PCNA, 293HEK, 3T3, and A875 melanoma cells. Here we show that neurotrophin binding to p75NTR does not directly activate NF-kB under normal physiological conditions but instead modulates NF-kB activation induced by other stimuli.

## EXPERIMENTAL PROCEDURES

**Materials.** NGF was purchased from Collaborative Research and TNF was purchased from R & D Systems. BDNF was provided by Regeneron Pharmaceuticals (Tarrytown, New York), NT-3 and NT-4 were purchased from Peprotec and the MC192 antibody (Chandler et al., 1984) was prepared from ascites fluid as described (Barker and Shooter, 1994). Antibodies against IkBα and IkBβ were purchased from Santa Cruz Biotechnology. Other reagents were purchased from either Sigma or ICN.

**Cell Culture and Transfections.** HeLa, 293HEK, 293T-HEK, A875, MG87-3T3 and PCNA cells were all maintained in Dulbecco's modified Eagle's medium containing 10% bovine calf serum, 2 mM L-glutamine, and 100 μg/ml penicillin/streptomycin in 5% $CO_2$ at 37° C. For transient transfections, 5 μg of CMV-driven rat p75NTR expression plasmid was introduced into cells on 100 mm plates using the calcium phosphate precipitation method. For transient transfections, 100 ng of an expression plasmid driving expression of enhanced green fluorescent protein (pEGFP-N1 - Clontech) was included to monitor transfection efficiency. To produce cell lines in which p75NTR levels could be induced with doxycycline, MG87-3T3 fibroblasts were stably transfected with a plasmid driving expression of the rtTA chimeric transcription factor (Gossen et al., 1995). Individual clones were screened for doxycycline inducible expression in transient transfection assays (data not shown) and lines showing lowest basal expression and strong doxycycline-induced expression (termed TIM, for tet-inducible MG87-3T3) were stably transfected with an expression plasmid containing rat p75NTR under control of a doxycycline inducible promoter. A total of 30 of these clones were analyzed and two lines (termed TIMP75-3 and TIMP75-12) which showed undetectable basal expression and strong doxycycline-inducible expression of P75NTR (data not shown) were used for detailed analyses.

**Electrophoretic Mobility Shift Assays.** Cultured cells were plated on 60- or 100-mm dishes, washed twice in DMEB[10] and then incubated in 2 or 5 ml (respectively) of DMEB supplemented as described in the figure legends. For pre-treatment experiments, cells

were washed twice in DMEB, preincubated in DMEB at room temperature for times indicated in the figure legends then incubated in 5 ml of DMEB or DMEB supplemented with NGF for 1 hour at 37°C followed by induction with TNF for 2 hours at 37°C. After the induction period, medium was removed, plates were placed on ice, rinsed with ice-cold Tris-buffered saline (20 mM Tris (pH 8.0), 137 mM NaCl) and then lysed in 10 mM HEPES (pH 7.9), 0.1% NP-40, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. Whole cell extractions were prepared by washing cells in PBS with 50 nM pyrrolidine dithiocarbamate and extracted in buffer consisting of 20 mM HEPES (pH 7.9), 0.35 M NaCl, 20% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, and 1% NP-40. Nuclear extractions were prepared in 20 mM HEPES (pH 7.9), 0.42 M NaCl, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol (DTT), 5 μg/μl leupeptin, 5 μg/μl pepstatin, 5 μg/μl aprotinin, 0.5 mM spermidine, 0.15 mM spermine, 100 μM sodium vanadate, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). Extracted lysates were analyzed for total protein content by BCA assay (Pierce), performed in triplicate. EMSA[11] were performed essentially as described (Singh and Aggarwal, 1995) on nuclear and whole cell lysates using an NF-kB binding element from the HIV-LTR as a probe. Gels were exposed to XRP film (Kodak) and scanned using an EPSON 1210 scanner. For quantitation, scanned images were analyzed by densitometry of bands using Image software. Statistical comparisions of TNF and TNF + NGF conditions were performed using paired T tests.

**Survival and apoptosis assays**. MTT assays were used for quantitation of mitochondrial activity as per manufacturer's instructions (Promega), with optical density quantified on an Titertek ELISA plate reader and expressed as the difference between OD540 and OD690. To quantitate the ratio of MTT-positive cells within stressed cell populations, at least four fields of 100 cells each were counted under phase contrast illumination (total cell number) and under bright field (MTT-positive cells). Data was normalized for total cells counted per field and three separate experiments were compared. To quantify apoptosis of PCNA cells, cells were stained with propidium iodide (100 ng/ml) for 30

[10] Dulbecco's modified Eagle's medium containing 0.1% bovine serum albumin
[11] electrophoretic mobility shift assays

minutes prior to scoring for an apoptotic morphology. A substantial proportion of apoptotic cells were non-adherent at the time of assay and therefore both adherent and non-adherent cells were quantified. The apoptotic index is the sum of adherent and non-adherent apoptotic figures, corrected for counting volumes.

**Transcriptional assays.** NF-kB transcription was monitored using a pUC19-derived NF-kB reporter gene which contains a tandem array of three functional kB sites derived from the HIV-LTR. These kB elements are just proximal to an SV40 minimal promoter driving expression of a LacZ open reading frame modified to include a nuclear localization signal at the amino terminus and an SV40 polyA sequence (plasmid pBA429) (Mercer et al., 1991). For assays of NF-kB transcriptional activity, calcium phosphate precipitates were used to transfect plasmid p429 together with plasmid p412, a GFP expression plasmid used to monitor expression levels and with either p288, a p75NTR expression plasmid, or with parental vector. β-galactosidase activity was monitored by ONPG conversion using a Promega kit. Each data point was performed in quadruplicate and experimental results were analyzed by multiple analysis of variance (ANOVA), with statistical probabilities assigned using the Tukey test for multiple comparisons.

**Immunoblotting.** Cytoplasmic or whole cell lysates were normalized for protein content using the BCA assay (Pierce), diluted in Laemmli sample buffer, boiled 5 minutes, separated on 10% or 12% SDS-polyacrylamide gels and transferred to nitrocellulose. Immunoblots were first blocked in 10 mM Tris (pH 7.4), 150 mM NaCl, 2% bovine serum albumin, 0.2% Tween 20 and then incubated with antibodies directed against IkBa or against the p75NTR intracellular domain (Majdan et al., 1997). Blocking, primary and secondary incubations for p75NTR immunoblots were performed in 10 mM Tris (pH 7.4), 150 mM NaCl, and 0.2% Tween 20 with 5% (w/v) dry skim milk. Immunoreactive bands were detected using enhanced chemiluminesence (ECL) according to the manufacturer's instructions (Dupont) and scanned images were quantified using NIH Image.

## RESULTS

Previous results indicate that p75NTR activates NF-kB in fibroblasts, Schwann cells and oligodendrocytes (Carter et al., 1996; Yoon et al., 1998). To test whether p75NTR-mediated activation of NF-kB is a general phenomenon, the activation of NF-kB by p75NTR was examined in cells which do not express endogenous p75NTR or trk receptors. 293HEK cells were transiently transfected with a p75NTR expression vector or with the parental control vector and then treated for 2 hours with either neurotrophin, TNF, or MC192, a rat p75NTR-specific monoclonal antibody. EMSA of extracted nuclear proteins revealed that TNF elicited a robust NF-kB response yet neither the p75NTR-specific antibody nor any of the neurotrophins activated NF-kB (Figure 2.1A). Various induction times (up to 12 hours) and NGF doses (5 to 500 ng/ml) were examined but an NGF-mediated NF-kB activation was never observed in 293HEK. Similar experiments were performed in p75NTR transfected HeLa cells and 3T3 fibroblasts, which are commonly used cellular models for TNF signaling, but neither of these transfected cell types showed any evidence of NF-kB activation in response to NGF at any concentration or time point; however, TNF treatment consistently produced robust NF-kB activation in these same cell lines (data not shown). To test whether neurotrophin-mediated NF-kB activation may be a feature of cell lines that express high endogenous levels of p75NTR, the A875 melanoma cell line was also examined. As with the other cell lines, EMSA revealed that TNF treatment resulted in robust NF-kB activation but NGF, BDNF and NT3 had no effect on NF-kB activation under these conditions (Figure 2.1B).

Previous reports showing that NGF binding to p75NTR increases NF-kB activity in EMSA in PCNA cells (Carter et al., 1996) and in primary rat oligodendrocytes (Yoon et al., 1998; Ladiwala et al., 1998) suggest that p75NTR can activate NF-kB in primary cells and in some cell lines. We therefore examined p75NTR-mediated activation of NF-kB in PCNA cells, to determine if our failure to detect NF-kB activation was due to cell type-specific differences in p75NTR signaling. Surprisingly, p75NTR-mediated NF-kB activation was not observed in PCNA cells in response to any of the four mammalian neurotrophins (Figure 2.2A and data not shown). One possibility for this was that PCNA

cells might produce endogenous neurotrophins which dampen an NF-kB response to exogenous ligand. However, cells plated at low density and washed extensively to remove endogenous neurotrophin still showed no evidence of NGF-mediated activation of NF-kB. A second possibility is that p75NTR-dependnet NF-kB activation depends on culture conditions; Notably, NF-kB responses can be altered under conditions of cellular stress (Guzhova et al., 1997) and in the first report of p75NTR-mediated NF-kB activation (Carter et al., 1996), cells were subjected to two stressful conditions; a temperature shock and a period of serum starvation (Dr. Bruce Carter – personal communication). We therefore tested whether these conditions might increase the ability of PCNA cells to respond to NGF. For these experiments, PCNA cells were left in room air at 20°C for several hours in serum-free media; this procedure reduced cellular mitochondrial activity (Figure 2.2B) and significantly increased the incidence of apoptotic nuclei (Figure 2.2D). Scoring of individual MTT-treated cells showed that after only 7 hours of this treatment (Figure 2.2C), cells showed strongly reduced mitochondrial activity yet the majority still remained adherent. Virtually no cells had detectable mitochondial activity after 21 hours. EMSA from PCNA cells pretreated in this manner for 7 hours are shown in Figure 2.2A. The stress treatment reduced basal NF-kB activity and strongly attenuated NF-kB activation induced by TNF. The stressed cells also revealed an NGF-dependent induction of NF-kB, an activation not observed in the unstressed cultures. Moreover, the shifted complex induced by both TNF and NGF in the stressed cells migrates more slowly than the predominant complex which is activated by TNF in PCNA cells under physiological conditions. This presumably reflects activation of a different NF-kB dimer combination. Therefore, NGF binding to p75NTR does not directly activate NF-kB under physiological conditions but does activate an NF-kB complex in severely stressed PCNA cells.

TNF receptor superfamily members share common downstream effectors, such as the TRAF proteins, and convergent signals between various receptor types have been reported to amplify ligand-mediated NF-kB activation (McKean et al., 1995). Therefore, even though p75NTR may not directly activate NF-kB under physiological conditions, it is possible it may modulate NF-kB activation induced by other stimuli. To test this, 293HEK cells transiently transfected with a p75NTR expression construct were exposed

to TNF, NGF, or combinations of both for either 2, 6 or 10 hours and then examined for NF-kB activation by EMSA. Figure 2.3A shows that two hours of TNF treatment causes a robust increase in NF-kB activity. This activation is attenuated when NGF is present, indicating that ligand-signaling through p75NTR can affect signaling of other related cytokine receptors. Intriguingly, the modulatory effect of p75NTR changes with increasing time; after 6 hours treatment, NF-kB activation produced by TNF is equivalent to that mediated by TNF and NGF together and by 10 hours, TNF-mediated NF-kB activation is increased in the presence of NGF. Under these physiological conditions, neither increasing time nor concentration of NGF altered the mobility of the primary NF-kB complex induced by TNF. To confirm these results, we also examined a 293 subline (293T-HEK) using a different nuclear protein extraction protocol (Carter et al., 1996). Figure 2.3B shows that 293T-HEK cells transfected with p75NTR are strongly modulated by NGF binding to p75NTR, with NGF initially reducing TNF-induced NF-kB activation but then increasing NF-kB activation with time, qualitatively identical to that shown in Figure 2.3A. Our transfection efficiency in 293HEK cells is about 70% and thus the magnitude of the NGF-induced modulation is likely an underestimate of the true magnitude of the modulatory effect of p75NTR. Densitometric quantification of scanned films revealed that the combination of NGF and TNF produced a significant average reduction of 26% at two hours ($p < 0.01$), a 21% increase at 10 hours ($p < 0.03$) but no change at six hours ($p < 0.47$).

To determine if p75NTR can exert modulatory effects on TNF signaling in cell lines expressing endogenous p75NTR, we turned to the A875 melanoma cell line. Primary melanocytes, originating from the neural crest, and melanoma cell lines normally co-express p75NTR and TNF receptors but have little or no trkA expression (Marano et al., 1987; Barker et al., 1993). Figure 2.4A shows that NGF does not directly activate NF-kB in this cell type but when examined two hours after cytokine addition, NGF clearly increases TNF-mediated NF-kB activation, with maximal increases (60%) at 25 ng/ml and more moderate increases (30-40%) at higher NGF concentrations. In A875 cells, NGF increased TNF-mediated NF-kB activation at all time points examined (Figure 2.4B); thus, in each cells line examined, NGF ultimately results in increased TNF

mediated activation of NF-kB by 10 hours. To determine if the synergistic effects of NGF and TNF are observed if cells are pretreated with NGF, A875 cells were pretreated with NGF for one hour and then induced with TNF for an additional two hours. Figure 2.4C shows that the modulatory effect of NGF on TNF signaling is maintained under these pretreatment conditions.

To begin to determine the mechanism by which p75NTR modulates TNF-mediated NF-kB activation, levels of IkBα were examined in cells treated with TNF, NGF or the two together. For these experiments, we used a 3T3-derived cell line (TIMP75-3 cells) in which p75NTR levels can be regulated by the addition of doxycycline. Figure 2.5A (lower panel) shows that p75NTR is undetectable in the absence of doxycycline but receptor expression increases dramatically in response to 18 hour doxycycline treatment. In the absence of detectable p75NTR expression, long-term TNF treatment led to a moderate reduction in IκBα steady-state levels which were not affected by the addition of NGF (lanes 3 and 4). When p75NTR was inducibly expressed, however, the reduction in IkBα protein induced by the combination of NGF and TNF was significantly greater than by TNF alone (Figure 2.5A – upper panel, lanes 7 and 8; $p < 0.02$).

Following TNF treatment, IkBα proteins are rapidly degraded then resynthesized and measurement of steady-state levels of IkBα represents the balance between these two processes. To directly test if NGF facilitates TNF-mediated IkBα degradation, the effects of NGF and TNF on IkBα levels were determined in the presence and absence of cycloheximide, a protein synthesis inhibitor. If NGF acts to facilitate IkBα degradation, its effect on IkBα levels should still be observed in the presence of translation inhibitors. Figure 2.5B shows, the combination of NGF and TNF produced a greater reduction in IkBa steady-state levels in A875 cells than did TNF alone (average decrease of 30%), consistent with the findings in the TIMP75-3 line. In the presence of cycloheximide, the effect of combining NGF and TNF was retained, with considerably more IkBα degradation observed compared to TNF alone. This argues that the mechanism by which NGF acts involves at least in part the facilitation of TNF-mediated IkBα degradation.

EMSA revealed that the maximal increase in NF-kB activity induced by NGF is about 3-4 fold in both transfected 293HEK cells and in A875 cells. To determine if this moderate increase in activated NF-kB complexes results in increased NF-kB-dependent transcription, a NF-kB-responsive LacZ reporter construct was transfected into 293HEK cells together with a p75NTR expression plasmid or with a parental control vector. Figure 2.6 shows that NGF added alone did not activate transcription from the LacZ reporter construct in 293HEK cells transfected with either control vector or p75NTR expression plasmid. NGF also had no effect on TNF-mediated NF-kB transcription in cells transfected with the parental expression vector, indicating that NGF does not exert p75NTR-independent effects on NF-kB. In cells expressing p75NTR, however, the combination of NGF and TNF significantly increases NF-kB activation compared to cells treated with TNF alone ($p < 0.0001$) and therefore suggests that the moderate increases in NF-kB binding activity result in significant increases in the NF-kB transcriptional response.

## DISCUSSION

The signaling properties of the p75NTR are not well defined. p75NTR-dependent sphingomyelinase activation and ceramide generation have been observed in a number of cell types under differing conditions, suggesting that activation of this signaling cascade may be a general property of p75NTR activation (Dobrowsky et al., 1994, 1995; Blochl et al., 1996). We have previously shown that a signaling cascade involving ceramide may be the mechanism through which p75NTR regulates trkA activity (MacPhee and Barker, 1997). In addition, binding of neurotrophin to p75NTR leads to phosphorylation of c-jun (Cassacia-Bonnefil et al., 1996; Yoon et al., 1998; Bamji et al., 1998) and p75NTR can facilitate apoptosis both in trkA-expressing and trkA-lacking cells (Cassacia-Bonnefil et al., 1996; Frade et al., 1996; Majdan et al., 1997; Yoon et al., 1998; Bamji et al., 1998; Barrett and Georgiou, 1996). Finally, p75NTR has been reported to activate NF-kB in oligodendrocytes, Schwann cells and in PCNA cells (Carter et al., 1996; Yoon et al., 1998; Ladiwala et al., 1998). In this study, we have examined the capacity of p75NTR to activate NF-kB in a variety of cell types and asked whether p75NTR may influence activation of NF-kB mediated by TNF. Our results indicate that neurotrophin binding to p75NTR does not activate NF-kB under physiological conditions but instead show that p75NTR modulates NF-kB signaling mediated by other cytokine receptors.

Our inability to detect direct p75NTR-mediated NF-kB signaling contrasts with earlier findings (Carter et al., 1996; Yoon et al., 1998; Ladiwala et al., 1998). There are at least two explanations for this discrepancy. One is simply that signaling elements required for direct p75NTR-mediated NF-kB activation are absent from the cell types we have examined, leading to the somewhat pedantic conclusion that p75NTR acts in a cell-type specific manner. Indeed, our results do not rule out the possibility that some cell types may support direct p75NTR-mediated activation of NF-kB under physiological conditions. However, our observation of NGF-mediated NF-kB activation only within cells which were severely stressed prior to the NGF exposure suggests an alternative explanation. Results of our work and others have shown that NGF binding to p75NTR expressed on cultured oligodendrocytes results in nuclear translocation of the p65 NF-kB subunit and activation of NF-kB (Yoon et al., 1998; Ladiwala et al., 1998) and in both of

these studies, the oligodendrocytes analyzed were maintained in serum-free media in which death occurs continually at a low rate (Cassacia-Bonnefil et al., 1996). Also, serum-starvation of cultured Schwann cells is apparently a prerequisite for NGF-dependent nuclear translocation of the p65 subunit of NF-kB (Khursigara et al., 1999). These conditions may be analogous to the stress paradigm used in our studies and together, these results are consistent with the possibility that cellular stress is necessary to observe the NGF-induced NF-kB activation reported previously by ourselves and others (Khursigara et al., 1999; Carter et al., 1996; Yoon et al., 1998; Ladiwala et al., 1998). The mechanism by which cellular stress may increase responsiveness to NF-kB is uncertain but one possibility is that stress induces increases in the production of TNF or cytosolic signaling elements to "prime" the NF-kB pathway in an autocrine manner. In this scenario, NGF acting through p75NTR is not a primary inducer of the pathway but rather synergizes with a stress-induced signal to increase NF-kB activity.

Our results show that although neurotrophin binding to p75NTR does not directly activate NF-kB signaling in either A875 melanoma cells, in transfected 293HEK cells or in stably transfected 3T3 cell lines, NGF binding to p75NTR had a clear effect on levels of NF-kB activation mediated by TNF - NGF binding to p75NTR ultimately increased levels of TNF-induced NF-kB activation in each cell line analyzed. In A875 cells, NGF potentiates TNF-mediated NF-kB signaling at every time point examined whereas 293 cells showed a more complex biphasic response to NGF. This biphasic response could be due to slight differences in template concentration or p75NTR expression between conditions, however, given our extensive protein concentration analysis, this effect probably reflects the fact that A875 cells are neural crest derivatives which normally express p75NTR and are therefore a more appropriate intracellular signaling milieu for p75NTR than 293 cells. Consistent with this, the concentration of NGF required for activation of the modulatory effect was considerably lower in A875 cells than in 293 cells (Figure 2.4A and data not shown). This modulatory effect of p75NTR on NF-kB activation likely reflects a *bona fide* physiological action of the p75NTR since the NGF-mediated increases in active NF-kB complexes occur in a variety of cells grown under normal conditions using relatively low concentrations of NGF. More importantly, these NGF-mediated increases are

reflected in significant changes in NF-kB driven transcription. Together, these results suggest that a major effect of p75NTR on NF-kB signaling in many cells may be to modulate NF-kB activation mediated by other stimuli. Our preliminary results indicate that this modulatory effect is not NGF-specific but also is observed with other neurotrophins (data not shown).

Alternative explanations might also account for this increased NF-kB activity. One possibility is that TNF increases p75NTR levels sufficiently to allow NGF to induce NF-kB. However, we show abundant p75NTR expression in many cells that demonstrate a complete lack of NGF induced NF-kB activation. This is perhaps most clearly shown in the A875 cells, which express abundant p75NTR. Since cells which express p75NTR in abundance show no direct NF-kB activation in response to NGF, it is reasonable to conclude that NGF affects TNF signaling, not the reverse. Also, although TNF can regulate expression through NF-kB elements within the CMV promoter present in expression constructs, in A875 cells which show the same qualitative effect, levels of p75 remain unchanged in the 10 hour time course of our experiments (see Figure 2.4B).

Analysis of IkBα levels further supports a p75NTR-regulated modulatory ability. Using either transiently transfected cells or cells which express p75NTR endogenously, we found that NGF markedly reduces steady-state levels of IkBα and does so by enhancing TNF-mediated degradation of the protein. The effect of NGF on IkBα degradation is readily apparent and suggests that p75NTR activation is likely to impinge on the activity of IKKα or IKKβ kinases which phosphorylate IkBα and thus target it for degradation (Ling et al., 1998; Mercurio et al., 1997). The p75NTR signaling cascade that may contribute to this effect remains unknown but the recent discovery of an interaction between p75NTR and TRAF6 raises the possibility that TRAF family members may play some role. The reduction in IkBα levels that resulted from NGF treatment was quite dramatic and the magnitude was clearly greater than the NGF-dependent changes observed by EMSA. It is possible that NGF may selectively affect IkBα degradation yet spare IkBβ or other IkB family members – we tested if IkBβ family members were

affected by NGF treatment but the poor quality of the commercially available IkBβ reagents precluded definitive results.

p75NTR potentiates TNF-mediated IkBα degradation and a key goal of future studies will be to define the precise convergence point of the NGF and TNF pathways. The precise mechanism(s) underlying the effect of p75NTR on TNF-mediated NF-kB activation remains unclear but could reflect a competition for common signaling elements which converge at or above the level of IkBα subunit phosphorylation. No reports have demonstrated a direct interaction between these receptors, however, DD-containing and TRAF adaptor proteins can interact with both receptor types.

A similar type of transreceptor effect on NF-kB activation has been described in other systems. For example, although TCR[12] activation normally produces a very small NF-kB response, TCR activation dramatically increases NF-kB activity mediated by the IL-1 receptor (McKean et al., 1995) and this TCR-mediated increase in IL-1 dependent NF-kB activity has recently been shown to result from increased TCR-dependent IkB degradation (Kalli et al., 1998). Together with our results, this suggests that transmodulatory mechanisms may be an important means for regulating cellular NF-kB activity. Therefore, p75NTR may function not only to regulate activity of receptors with which it shares ligands, such as the Trks, but may also act to modulate signaling activity of receptors with which it shares functional or structural homology, such as the TNF receptors.

[12] T cell receptor

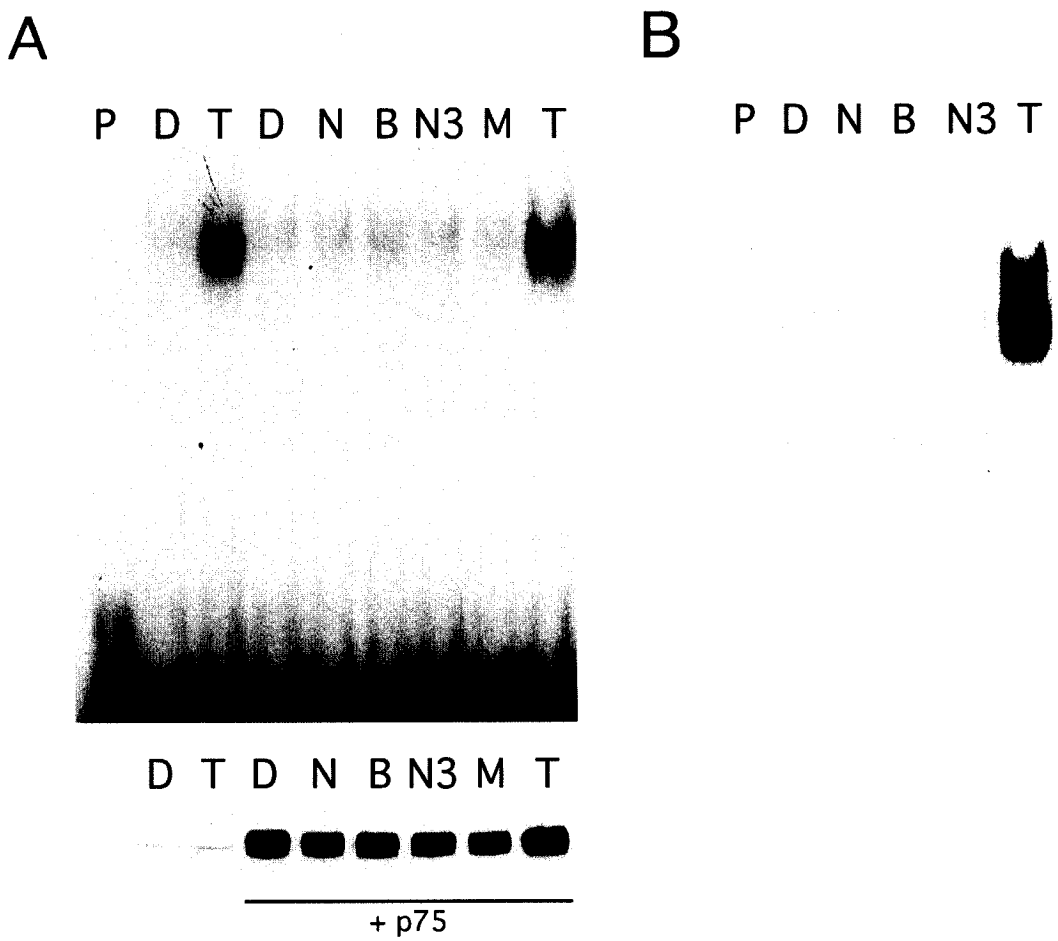


**Figure 2.1.** Neurotrophins do not directly activate NF-kB in 293HEK or A875 cells. (A). 293HEK cells were transiently transfected either with a control vector or with p75NTR expression vector and two days later were incubated for 2 hours in either DMEB alone, or DMEB containing TNF (5ng/ml), NGF (250ng/ml), BDNF (250ng/ml), NT3 (250ng/ml), or MC192 (1μg/ml). Nuclear extracts were analyzed by EMSA (upper panel) using a labeled NF-kB probe. To confirm p75NTR expression, cell lysates were analyzed for p75NTR content by immunoblotting (lower panel). (B). A875 cells were treated with neurotrophin or TNF for 2 hours and nuclear extracts were then analyzed by EMSA as described in Materials and M ethods. These experiments were repeated three times. In addition, concentrations of 5-500 ng/ml neurotrophins were tested for periods of up to 10 hours in both cell lines but direct neurotrophin-mediated activation of NF-kB was never observed (data not shown). Abbreviations are P for probe alone (no cellular extract added), D for DMEB, T for TNF, N for NGF, B for BDNF, N3 for NT-3, and M for MC192.

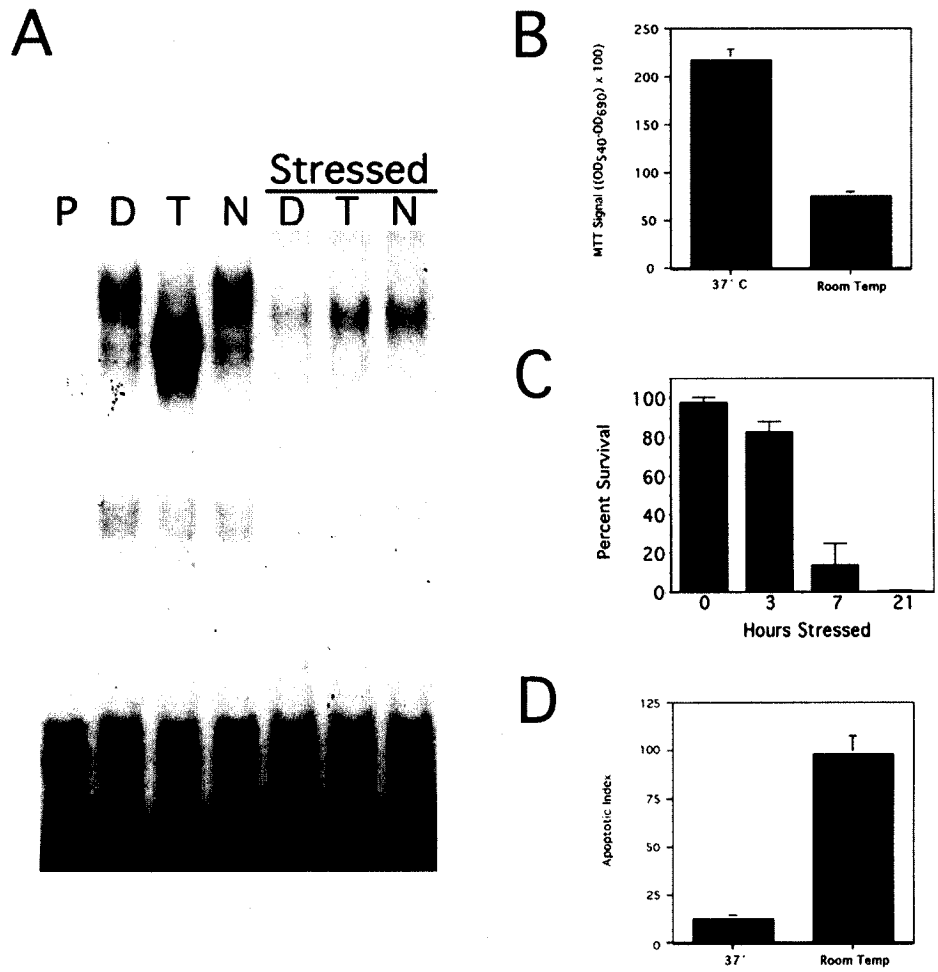


**Figure 2.2.** NGF mediates activation of NF-kB in PCNA cells only after severe cellular stress. PCNA cells were maintained either in serum-containing media at 37°C in a 5% CO2 atmosphere (control) or in serum-free media in room air (20°C) for several hours (stressed) as described in "Results". Parallel cultures were analyzed in four ways. For NF-kB activity, unstressed (lanes 2-4) and cells stressed for 7 hours (lanes 5-7) cells were left untreated (DMEB) or were treated with either NGF (100 ng/ml) or TNF (20 ng/ml) for an additional 2 hours at 37°C in a 5 % CO2 atmosphere after which nuclear proteins were extracted and analyzed by EMSA using a labeled NF-kB probe (A). To measure mitochondrial activity, total MTT activity was quantified after 18 hours of stress (B) and scoring of cellular MTT production was compared after 0, 3, 7, and 21 hours of stress (C). For cell death analysis, apoptotic bodies were determined by propidium iodide staining after 18 hours of stress (D). Each experiment was repeated three times. Abbreviations are D for DMEB, T for TNF and N for NGF.

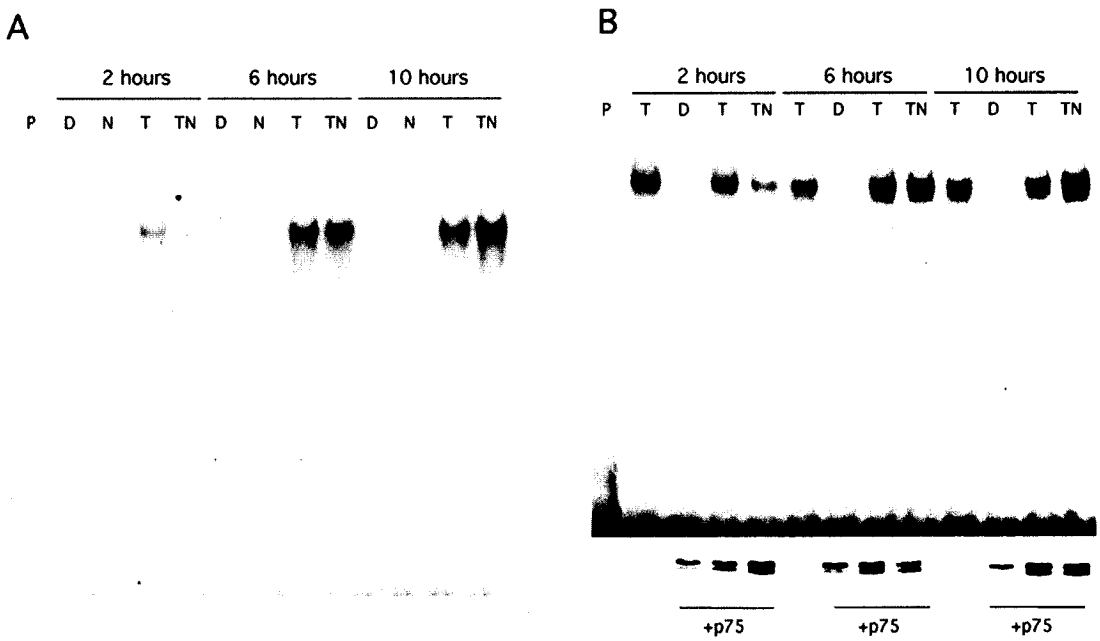


**Figure 2.3.** NGF modulates TNF-induced NF-kB activation in 293HEK cells expressing the p75NTR cell surface receptor in a time-dependent manner. (A) 293HEK cells transiently transfected with a p75NTR expression vector were incubated with TNF (5 ng/ml) with or without NGF (250 ng/ml) for 2, 6 or 10 hours. Nuclear extractions were performed and analyzed for NF-kB binding activity by EMSA using a labeled NF-kB probe. Experiments were repeated four times with similar results. (B) Similar experiments were performed on a 293T-HEK subline transfected with a control vector or with a p75NTR expression vector and cellular proteins were isolated using a whole cell extraction protocol (see Materials and Methods). p75NTR expression levels determined by immunoblotting of cellular lysates are shown in lower panel. Experiments were repeated three times with similar results. Abbreviations are P for probe alone (no cellular extract added), D for DMEB, T for TNF, N for NGF and TN for TNF + NGF.

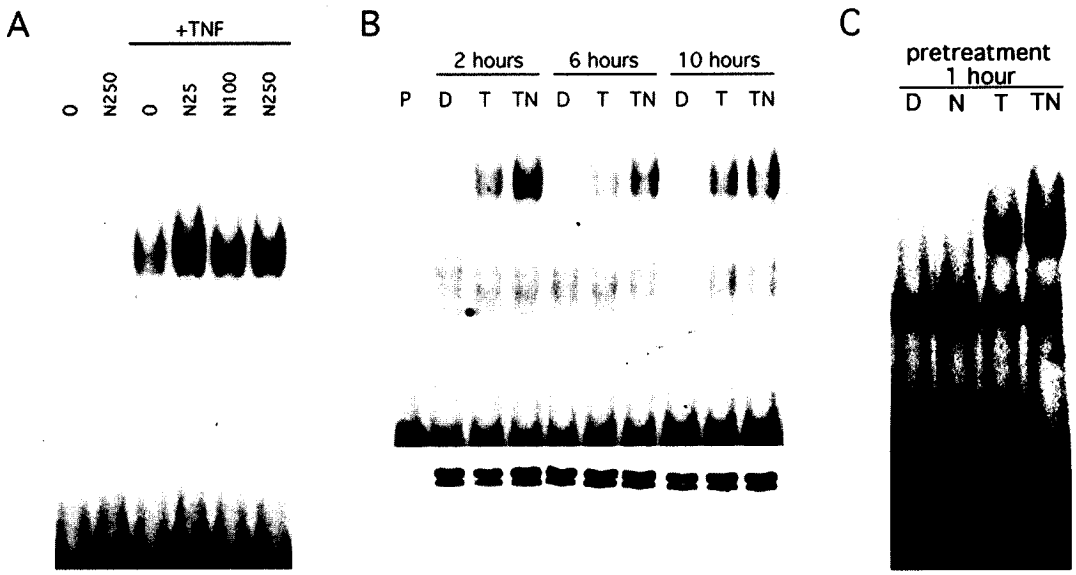


**Figure 2.4.** Neurotrophins do not directly activate NF-kB in A875 cells but NGF increases TNF-induced NF-kB activation in a time- and dose-dependent manner. (A) A875 cells were incubated for 2 hours in DMEB supplemented with either NGF (250 ng/ml) or TNF (5ng/ml), or with TNF in the presence of increasing concentrations of NGF. Nuclear proteins were extracted and analyzed by EMSA using a labeled NF-kB probe. (B) A875 cells were incubated for 2, 6 or 10 hours in DMEB alone or DMEB supplemented with TNF (5ng/ml) in the absence or presence of NGF at 25 ng/ml. Nuclear proteins were extracted and analyzed by EMSA (upper panel) using a labeled NF-kB probe. Cell lysates were analyzed for p75NTR content by immunoblotting to confirm p75NTR expression (lower panel). This experiment was repeated four times with similar results. (C) A875 cells were incubated with DMEB alone or DMEB supplemented with NGF (250 ng/ml) for 1 hour and then induced with TNF for an additional two hours. Nuclear proteins and EMSA were performed as above. This experiment was repeated three times with similar results. Abbreviations are P for probe alone (no cellular extract added), D for DMEB, T for TNF, N for NGF and TN for TNF + NGF.

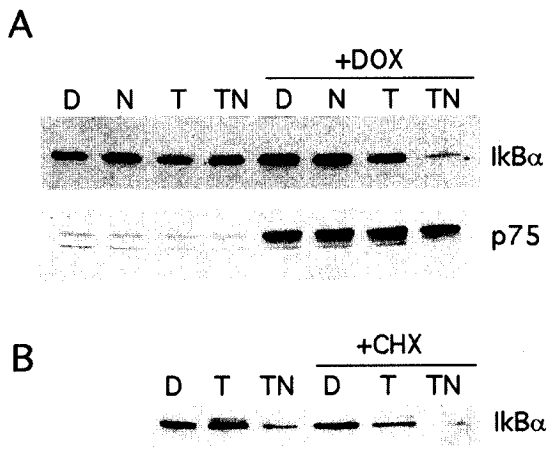


**Figure 2.5.** NGF does not directly reduce IkBα levels but instead facilitates IkBα degradation in the presence of TNF. (A) TIMP75-3 cells were treated with or without doxycycline for 18 hours and then treated with TNF (10 ng/ml) with or without NGF (100 ng/ml) for 10 hours as indicated. Immunoblotting of cell lysates show IkBα levels (top panel) and confirm p75NTR expression (bottom panel). (B) A875 cells were treated with DMEB alone or supplemented with TNF (10 ng /ml) and/or NGF (100 ng/ml) in the presence or absence of cycloheximide (10 μg/ml) for 2 hours as indicated. IkBα levels in cell lysates were determined by immunoblotting. Experiments shown in (A) and (B) were each repeated at three times with similar results. Abbreviations are D for DMEB, T for TNF, N for NGF and TN for TNF + NGF.

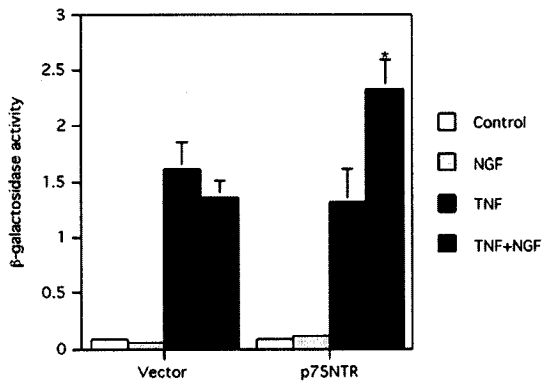


**Figure 2.6.** NGF has no direct effect on NF-kB transcriptional activity but increases TNF-mediated NF-kB transcriptional activity through a p75 NTR-dependent pathway. 293HEK cells transiently transfected with NF-kB dependent b-galactosidase and GFP reporter constructs with either a control vector or with a p75NTR expression vector. 24 hours after transfection, cells were switched to media containing either 5 ng/ml TNF, 100 ng/ml NGF or the two combined for 16 hours and then cell lysates were prepared and analyzed for LacZ activity as described in Materials and Methods. Multiple analysis of variance shows that cells expressing p75NTR, TNF and TNF+NGF treatment groups differ significantly (p value $< 0.0001$; indicated by asterisk). This experiment was repeated three times with similar results.

# PREFACE TO CHAPTER 3

Chapter Two has shown that p75NTR does not directly activate NF-kB under normal growth conditions. Instead, p75NTR functions to enhance TNF-dependent NF-kB activation in several cell lines. This finding indicates that p75NTR functions to modulate cytokine-initiated NF-kB responses outside the nervous system, but says little about the role p75NTR plays in regulating neuronal NF-kB. Regulation of neuronal NF-kB, indeed, is poorly understood. Reports vary on the degree and location of NF-kB activity within the nervous system, the ability of specific inducers to increase neuronal NF-kB, and the role of NF-kB in promoting or suppressing neuronal survival. Therefore, to clarify these issues, we generated transgenic NF-kB reporter mice that are sensitive to neuronal NF-kB activity. Chapter Three establishes the developmental pattern of NF-kB activity within these mice and asks whether NF-kB is necessary and sufficient for central neuron survival.

# CHAPTER 3

# CONSTITUTIVE NUCLEAR FACTOR-KB ACTIVITY IS REQUIRED FOR CENTRAL NEURON SURVIVAL

# Constitutive Nuclear Factor-kB Activity Is Required For Central Neuron Survival.

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**Running title:** NF-kB Activity Supports Neuronal Survival

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## ABSTRACT

The function of NF-kB within the developing and mature central nervous system is controversial. We have generated transgenic mice to reveal NF-kB transcriptional activity *in vivo*. As expected, constitutive NF-kB activity was observed within immune organs and TNF-inducible NF-kB activity was present in mesenchymal cells. Intriguingly, NF-kB activity was also prominent in the central nervous system throughout development, especially within neo-cortex, olfactory bulbs, amygdala, and hippocampus. NF-kB in the central nervous system was restricted to neurons and was blocked by overexpression of dominant-negative NIK or the IkBαM super-repressor. Blocking endogenous neuronal NF-kB activity in cortical neurons using recombinant adenovirus induced neuronal death whereas induction of NF-kB activity increased levels of anti-apoptotic proteins and was strongly neuroprotective. Together, these data demonstrate a physiological role for NF-kB in maintaining survival of central neurons.

## INTRODUCTION

Nuclear factor-kappa B (NF-kB) transcription factors are required for regulating cell survival and differentiation, and for inflammatory and immune responses. The five mammalian NF-kB subunits (RelA (p65), NFkB1 (p52/p100), NFkB2 (p50/p105), RelB, and c-Rel) each contain a Rel homology domain that allows these factors to dimerize and bind DNA (reviewed in (Gilmore, 1999; Perkins, 2000)) In lymphocytes and other activated immune cells, NF-kB is retained in the nucleus and is constitutively active. In most cells, however, NF-kB dimers are normally rendered inactive in the cytosol by virtue of their interaction with one of the inhibitory IkB proteins (IkBα, IkBβ, IkBδ, IkBε, Bcl-3). Translocation to the nucleus occurs only following stimuli-induced IkB protein degradation. This process requires the activation of kinase cascades that converge on IKK1 and IKK2, related catalytic kinase subunits that, together with NEMO/IKKγ, form a complex which phosphorylates IkB family members and subsequently targets them for ubiquitination and proteosomal degradation (reviewed in (Karin and Ben-Neriah, 2000))

In non-neuronal cells, three major functions have been ascribed to the NF-kB family. Inducible NF-kB activity is crucial for activating genes mediating the pro-inflammatory response, a key component of the host defense system (Hatada et al., 2000). NF-kB activation also induces the transcription of anti-apoptotic genes and thereby promotes survival (Van Antwerp et al., 1998; Wang et al., 1998; Barkett and Gilmore, 1999). Finally, NF-kB plays a crucial role in maturation of the skin and skeletal systems (Li et al., 1999a). The precise role(s) of NF-kB within the nervous system is less clear. Several studies have found that NF-kB activity facilitates neuronal survival (Barger et al., 1995; Guo et al., 1998; Lezoualc'h et al., 1998; Maggirwar et al., 1998; Hamanoue et al., 1999; Kaltschmidt et al., 1999) yet others report that NF-kB activation is required for neuronal death (Grilli et al., 1996; Post et al., 1998; Schneider et al., 1999). The use of genetically altered mice will no doubt resolve this issue but so far, the results have been equivocal. For example, mice lacking the p50/p105 NF-kB subunit show increased hippocampal damage in response to kainate-induced excitotoxicity (Yu et al., 1999) yet p50/p105 nulls also show reduced neuronal damage following focal cerebral ischemia (Schneider et al., 1999)

To clarify the role of NF-kB in the CNS, the pattern of transcriptionally active neuronal NF-kB needs to be established. To address this, we generated transgenic mice that provide a sensitive readout of NF-kB activity, particularly within the nervous system. Primary fibroblasts derived from these mice show appropriate reporter gene activation in response to known NF-kB inducers and this response is blocked by overexpression of IkBαM, a specific NF-kB repressor. Transgenic mice show constitutive NF-kB activation in peripheral lymphoid tissues and display high levels of NF-kB activity in developing epidermal appendages. Intriguingly, the reporter mice also reveal high levels of NF-kB activity within developing and mature neurons of the central nervous system. Reducing neuronal NF-kB activity through overexpression of an IkB super-repressor or dominant inhibitory NF-kB inducing kinase (NIK) induces cortical neuron death. Conversely, adenovirus-mediated overexpression of p65/RelA in primary neurons induces accumulation of Bcl-xL, IAP1 and IAP2 and confers strong protection against neuronal apoptosis induced by etoposide or camptothecin. Together, these studies demonstrate that active NF-kB activity is present throughout the developing and adult nervous system and indicate that NF-kB plays an important role in survival of CNS neurons.

## EXPERIMENTAL PROCEDURES

**DNA Construct and Production of Transgenic Mice.** To create a high fidelity NF-kB reporter minigene, an NF-kB tandem repeat derived from the long terminal repeat of HIV (HIV-LTR) was placed just upstream of a minimal SV40 promoter. Overlapping oligonucleotides were used to make additional identical NF-kB tandem repeats which when combined, produced three tandem NF-kB repeats upstream of the SV40 minimal promoter. The enhancer/promoter fragment was cloned upstream of an E. coli β-galactosidase gene modified to contain a mammalian Kozak consensus, an SV40 T-antigen derived nuclear localization signal, and a polyA tract and splicing signal derived from the protamine I gene (Mercer et al., 1991). The minigene cassette was isolated from parentel vector and injected into pronuclei to produce a total of 10 transgenic founder mice in a C3HxBalb/c background. Genotyping of transgenic mice was performed by PCR analysis from tail biopsies (Laird et al., 1991) using primers directed to β-galactosidase (5'-CTGCAGATAACTGCCGTCACTCC-3', 5'-CTTAATCGCCTTGCAGCACAT-3').

**Cell Culture and Reagents.** Primary mouse embryonic fibroblasts (MEF) were derived from the dorsal skin of E15-E16 transgenic embryos and maintained in DMEM containing 10% bovine calf serum, 2 mM l-glutamine, and 100 mg/ml penicillin/streptomycin. 293A cells were maintained in DMEM containing 10% bovine calf serum, 2 mM l-glutamine, and 100 μg/ml penicillin/streptomycin. Cortical cultures were prepared from E15-16 transgenic mouse telencephalon and maintained 8-10 DIV in Neurobasal media (Life Technologies) supplemented with a final concentration of 0.5X B27 supplement (Life Technologies), 0.5X N2 supplement (Life Technologies), 2 mM l-glutamine, and 100 μg/ml penicillin/streptomycin. IkBα, p100, c-IAP1, BcL-XL antibodies were from Santa Cruz Biotechnology, p65/RelA monoclonal antibody was from Transduction Laboratories, GFP polyclonal antibody from Clontech, β-galactosidase polyclonal antibody from ICN and the anti-hemagglutinin (HA) monoclonal antibody 12CA5 was from Boerhinger Mannheim. Secondary donkey anti-rabbit or donkey anti-mouse horseradish conjugates were from Jackson Immunochemicals.

**Generation of Recombinant Adenovirus.** The IkBαM cDNA (Van Antwerp et al., 1998) was provided by Inder Verma and subcloned into the CMV 5' transfer vector and recombinant adenovirus was generated in 293A cells using standard techniques (Hitt et al., 1997). Human p65/RelA cDNA was cloned into the pAdTrack-CMV shuttle vector and recombinant adenovirus was generated as previously described (He et al., 1998). The HA tagged dominant negative NIK (dnNIK) consists of a truncated protein where the kinase domain and TRAF interacting domain were deleted (amino acid 1-623 deletion) (Natoli et al., 1997). The adenoviral dnNIK was constructed using the Cre-lox recombination method as described previously (Russo et al., 2002). IkBαM, dnNIK and control GFP or β-galactosidase adenovirus were amplified in 293 cells, purified over sucrose gradients and stock titer values were obtained using the TCID method. For primary cell infections, appropriate titers of virus were diluted into 10% of the culture volume and added directly to MEFs or cortical neurons at the time of plating or on cells plated 2 or 5 days earlier.

**β-Galactosidase Assays and Immunofluorescence.** Embryos, organs or cultured cells were fixed for 20 minutes at 4° C in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) and then assayed for β-galactosidase activity by incubation in 37° C in 80 mM dibasic sodium phosphate, 20 mM monobasic sodium phosphate, 2 mM MgCl2, 0.2% NP40, 1 mg/ml sodium deoxycholate, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 800 μg/ml X-gal (4-chloro-5-bromo-3-indolyl-ß-galactoside, Sigma) for 4-16 hours. Samples were then washed in PBS and postfixed in 4% paraformaldehyde in PBS. Immunostaining was performed on parallel PFA fixed cultures using antibodies directed against β-galactosidase (polyclonal, ICN Biomedicals), MAP2 (monoclonal, clone AP-20 from Sigma), β-III-tubulin (monoclonal, Sigma clone SDL.3D10), GFAP (monoclonal, Boerhinger Mannheim), and using donkey anti-mouse conjugated-FITC and donkey anti-rabbit-CY3 (Jackson Labs) as fluorescent secondary antibodies.

**Transcriptional Assays.** For 293HEK cells, cells on 6 well plates were transfected with CaPO4 precipitates containing the reporter plasmid on day 0, induced with cytokines

beginning on day 1 and harvested in lysis buffer 16 hours later. β-galactosidase activity was assessed by o-nitrophenyl β-D-galactopyranoside conversion (Promega). To quantify β-galactosidase activity in primary transgenic MEFs, cells were placed into 96 well plates, left uninfected or infected with recombinant adenovirus for 24 hours, induced with TNFα for 16 hours, then harvested in RIPA buffer. Lysates were assayed for β-galactosidase activity using Galactostar, a chemiluminescent substrate (Tropix). The same chemiluminescent technique was performed using on transgenic cortical cultures which were plated at 20000 cells per well on a 96-well plate, infected with recombinant adenovirus on day 5 *in vitro*, and harvested 4 days later.

**Survival and Apoptotic Assays.** Dissociated cortical neurons were plated as above and infected with recombinant adenovirus. After the periods indicated in the figure legends, neurons were assayed for viability using 3(4,5-dimethylthio-zol-2-yl)2,5-diphenyltetrazolium bromide (MTT; Sigma), which was added at a final concentration of 1 mg/ml for 4 hours. 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 4-6 wells, experiments performed in triplicate and results were analyzed for statistical significance by multiple analysis of variance. For TUNEL assays, infected neurons were treated 24 hours later with 20 μM camptothecin or etoposide (Calbiochem) for 16 hours, fixed and permeabilized in 4% PFA and 1:1 acetone/methanol, incubated with Biotin-dUTP (Boehringer Mannheim) and TdT as per the manufacturer's protocol (Promega), and followed with streptavidin-CY3 (Boehringer Manneheim) for visualization.

## RESULTS

Previous studies have demonstrated that kB elements within the HIV-LTR promoter are sensitive to neuronal NF-kB activity *in vitro* (Rattner et al., 1993) and *in vivo* (Corboy et al., 1992; Buzy et al., 1995). To produce a reporter construct that would reflect endogenous NF-kB activity in neurons, we generated an NF-kB responsive minigene containing 3 tandem HIV-derived kB binding element repeats placed proximal to a minimal promoter derived from SV40. This construct drives expression of β-galactosidase that was modified to include an SV40 T-antigen derived nuclear localization sequence (Figure 3.1A). When transfected into 293HEK cells, the reporter construct exhibited low basal activity and could be readily induced to express β-galactosidase following treatment with TNFα well established NF-kB activator (Figure 3.1B). The NF-kB responsive minigene cassette was injected into pronuclei and a total of 10 transgenic founder mice were produced in a C3HxBalb/c background. Of these, five (17812, 17813, 17816, 17817, 17820) showed no developmental β-galactosidase expression, suggesting that the transgene was incorporated into areas of inactive chromatin. Of the remainder, three founders (17814, 17815, 17819) showed identical β-galactosidase expression patterns which are described in detail below. The two remaining founder lines (17818, 17821) showed differing subsets of the expression patterns observed in the 17814, 17815, and 17819 lines. The variable expression in the 17818 and 17821 lines likely represents local enhancer effects on transgene expression and these lines were not studied further.

To confirm appropriate *in vivo* activity of the incorporated minigene, primary fibroblast cells (MEFs) were derived from transgenic mouse embryos and analyzed for TNFα-induced β-galactosidase activity. Figure 3.1C shows that β-galactosidase activity was not detected in cultured transgenic MEFs under normal growth conditions, but was present following exposure to a low concentration of TNFα. The MEF cultures used in these experiments represent a mixture of cells derived from transgenic and non-transgenic embryos and therefore likely under-represents the responsiveness of a pure transgenic population. To confirm that the TNFα-mediated induction of β-galactosidase in transgenic MEFs reflects *bona fide* NF-kB transcriptional activity, we created a

recombinant adenovirus encoding IkBαM, a modified form of IkBα that is resistant to proteolytic degradation and represses NF-kB signaling by constitutively retaining NF-kB subunits in the cytosol in latent form. Figure 3.1D shows that endogenous IkBα is rapidly degraded in cells exposed to TNFα (compare first and fifth lanes) but IkBαM remains intact under these conditions and is therefore available to bind and retain cytosolic NF-kB subunits in the cytoplasm. Transgenic MEFs that were left uninfected or were infected with recombinant adenovirus encoding IkBαM were exposed to increasing concentrations of TNFα and then analyzed forβ-galactosidase activity. Figure 3.1E shows that the TNFα-mediated increase in β-galactosidase is attenuated in cells co-expressing IkBαM and demonstrates that β-galactosidase activity induced by TNFα in these primary cultures occurs through the NF-kB signaling pathway.

To examine NF-kB transcriptional activity during development, transgenic litters from lines 17814, 17815 and 17819 were fixed and whole-mount stained for β-galactosidase activity at different post-implantation stages. Results for these three lines are identical and only those from the 17814 line are shown in Figure 3.2A-3.2K. At E13, NF-kB activity is observed in prominent tactile and sinus hair follicles and in vibrissae primordia (Figure 3.2A). The telencephalon is prominently stained and NF-kB activity is present at the roof plate of the midbrain. Staining is also observed at the midbrain/hindbrain junction (Figure 3.2B), within mammary gland primordia, in the thoracic region and in the gonadal area (Figure 3.2C). Comparative NF-kB activity in transgenic versus non-transgenic negative control littermates is shown in Figure 3.2D.

At embryonic day E16, increased numbers of vibrissae are stained and NF-kB activity is prominent over the presumptive eyelid crease (Figure 3.2E). Prominent NF-kB activity is also visible in epidermis on plantar and palmer surfaces of forepaws and hindpaws (Figure 3.2F). In neonates, high levels of NF-kB activity was observed in the CNS within the cortex, olfactory lobes, roof plate of the midbrain (Figure 3.2H), and the midbrain/hindbrain junction (data not shown). The dermal surface of skin also had NF-kB activity, with a beaded appearance which likely represents staining within multi-nucleated muscle fibers (Figure 3.2G).

NF-kB is retained in the cytoplasm in an inactive form in most cells, but constitutive NF-kB activity occurs in a variety of immune cells and immuno-competent organs (Lernbecher et al., 1993; Carrasco et al., 1994; Weih et al., 1994). Consistent with this, high β-galactosidase activity was observed in trachea and bronchial tubes, areas of primary immune defense that have high numbers of lymphocytes (Figure 3.2I), and in lymphoid organs of transgenic positive animals (Figure 3.2J-K). Together these results show that these transgenic mice provide accurate reporting of endogenous NF-kB activity and indicate that NF-kB activity is present within the developing brain during murine development.

Figure 3.3 shows that NF-kB activity remains elevated in the CNS into adulthood, particularly in the forebrain. Serial brain sections reveal NF-kB activity in the olfactory bulbs (granule cell layer of the main olfactory bulb and the anterior olfactory nucleus - Figure 3.3A), the olfactory tubercle (islands of Calleja - Figure 3.3A-C), in all layers of the neocortex (Figure 3.3A-H), in amygdala and claustrum, and within the dentate gyrus and the hippocampus (Figures 3.3D-G). Lower levels of NF-kB activity are present in the piriform and entorhinal cortices (Figure 3.3I) and within the hypothalamus (Figure 3.3J). Cingulate and parietal cortex contain abundant blue nuclei in all cortical layers, with staining most prominent in layers 2, 4, and 5 (Figure 3.3K). In the hippocampus, β-galactosidase positive nuclei are visible throughout the deep and superficial pyramidal layers of Ammon's horn and robust activity is present in the CA1 and CA2 regions. Neurons within the CA3 region of the hippocampus show markedly reduced NF-kB activity compared to those in CA1 and CA2 (Figures 3.3E, K). NF-kB positive nuclei can also be found throughout the stratum oriens and radiatum (Figure 3.3K) and within a layer of interneurons along the stratum lacunosum-moleculaire (Figure 3.3F-G, K).

To confirm that the β-galactosidase activity observed within the embryonic telencephalon reflects neuronal NF-kB activity, cortical cultures were prepared from E16 transgenic embryos, maintained in vitro for 8-10 days and then analyzed for β-galactosidase accumulation by immunohistochemistry. Figure 3.4A shows that cells exhibiting typical

pyramidal neuronal morphology contain β-galactosidase activity and Figure 3.4B shows that the β-galactosidase-positive cells in these cultures co-express the neuronal markers βIII-tubulin and MAP2. GFAP-positive glial cells consistently lacked β-galactosidase activity (data not shown).

To confirm that β-galactosidase levels within transgenic neurons are regulated by NF-kB transcriptional activity, NF-kB activity was disrupted in transgenic neurons using recombinant adenovirus encoding elements of the NF-kB signaling cascade. Figure 3.4C shows that expression of the IkBαM super-repressor, which retains NF-kB subunits in the cytosol, reduced levels of β-galactosidase in transgenic neurons whereas β-galactosidase levels were elevated in transgenic neurons infected with an adenovirus encoding the p65/RelA NF-kB subunit, which increases NF-kB transcriptional activity. Together, these results indicate that the constitutive β-galactosidase activity observed within transgenic neurons is regulated by NF-kB activity.

In many cells, activation of NF-kB induces transcription of anti-apoptotic genes and the presence of NF-kB transcriptional activity in central neurons therefore raised the possibility that constitutive neuronal NF-kB activity may play a role in maintenance of central neuron survival. To address this, NF-kB activity was reduced in cortical neurons using adenovirus encoding the IkBαM super-repressor and, 48 hours later, assessed for survival using MTT assays. Figure 3.4D shows that infection with a control β-galactosidase adenovirus had no significant effect on neuronal survival whereas infection with the IkBαM super-repressor significantly reduced survival, at each multiplicity of infection tested. To address if this neuronal loss occurs through activation of apoptotic cascades, cortical neurons were infected with IkBαM and treated with zVAD, a broad spectrum caspase inhibitor. Under these conditions, cortical neuron survival was significantly enhanced ($p<0.03$, data not shown).

These results indicated that constitutive NF-kB activation is necessary for the survival of primary cortical neurons. To confirm this and to begin to address the signaling events that might contribute to this effect, NF-kB signaling was disrupted in primary cortical neurons

using a recombinant adenovirus encoding a dominant negative form of NF-kB inducing kinase (dn-NIK), a MAP3K implicated in NF-kB activation in non-neuronal cells (Malinin et al., 1997; Regnier et al., 1997). To first confirm that dominant-negative NIK reduced NF-kB activity in primary neurons, transgenic cortical neurons were infected with dominant-negative NIK adenovirus and assessed for reductions in β-galactosidase activity. Figure 3.5A shows that infection with a control adenovirus encoding GFP had no effect whereas infection with equivalent titers of dominant-negative NIK adenovirus virus resulted in a significant reduction in β-galactosidase activity. At adenovirus titers greater than 5 MOI, NIK expression appeared to cause neuronal cell death. To investigate this effect, primary cortical neurons were infected with increasing titers of adenovirus encoding either dominant-negative NIK or GFP and assessed for survival using MTT assays. Figure 3.5B shows that the adenovirus encoding GFP had no significant effect on neuronal survival whereas infection with adenovirus encoding dominant-negative NIK resulted in substantial neuronal death. These results indicate that constitutive NF-kB signaling plays an important role in the maintenance of primary cortical neuron survival.

The profound reduction in survival induced by the IkBαM super-repressor and by dominant-negative NIK suggests that NF-kB activity may normally regulate a pro-survival response in neurons. We therefore tested whether activation of NF-kB confers a survival advantage in cortical neurons. Adenovirus encoding GFP alone or encoding both GFP and p65/RelA were used to infect primary cortical neurons prior to treatment with camptothecin and etoposide, inhibitors of topoisomerase I and II that are highly neurotoxic and which cause apoptotic cell death of 50-80% of primary cortical neurons (see Figure 3.6I). After 18 hours of drug treatment, infected cells were assessed for apoptotic death using TUNEL assays and by examination of nuclear morphology. Figure 3.6A-D shows that essentially all neurons which were infected with control adenovirus encoding GFP alone displayed pyknotic nuclei and were TUNEL-positive, indicating widespread apoptotic cell death. In contrast, Figure 3.6E-H shows neurons infected with adenovirus encoding p65/RelA and GFP that were uniformly TUNEL-negative and contained healthy nuclei (whereas uninfected neurons in the same field were apoptotic). Quantification of these studies (Figure 3.6I) revealed that greater than 90% of neurons

infected with p65/RelA were protected from apoptosis normally induced by etoposide or camptothecin. Together, these data indicate that NF-kB activation confers a profound survival advantage to cortical neurons.

To determine if protein products of anti-apoptotic genes were induced by NF-kB in cortical neurons, cells were infected with adenovirus encoding GFP alone or encoding both GFP and p65/RelA for 48 hours, lysed and analyzed by immunoblot. Genes encoding NF-kB signaling elements are themselves very sensitive to NF-kB activation and therefore provide useful internal controls to demonstrate NF-kB activation. Levels of endogenous IkBαand NFkB1 protein were unaffected in neurons infected with GFP alone but both were strongly induced in cells expressing p65/RelA and GFP (Figure 3.6J), indicating that NF-kB activity was induced in cortical neurons by p65/RelA overexpression. Anti-apoptotic genes of the Bcl-2 family and inhibitor of apoptosis proteins (IAPs) represent two main classes of anti-apoptotic genes that are regulated by NF-kB in non-neuronal cells and we therefore examined Bcl-$X_L$, IAP1, and IAP2 as representative members of these two families. Figure 3.6J shows that levels of each of these genes were increased in cells overexpressing p65/RelA and GFP, but not in cells overexpressing GFP alone. Therefore, activation of NF-kB in primary cortical neurons appears to increase levels of anti-apoptotic proteins and thereby elevate the survival threshold of primary cortical neurons.

## DISCUSSION

In this study, we have produced transgenic reporter mice that provide a reliable means for assessing NF-kB transcriptional activity in vivo. Mouse embryonic fibroblasts derived from these animals display an inducible NF-kB transcriptional readout that is inhibited by IkBαM, a repressor of NF-kB signaling, while transgenic lymphoid cells display constitutive NF-kB activity. Intriguingly, the transgenic reporter mouse reveals prominent constitutive NF-kB activity within neurons of the developing and mature CNS. Blockade of NF-kB activity results in neuronal death whereas p65/RelA overexpression confers protection against insults and induces expression of anti-apoptotic gene products that include Bcl-Xl, IAP1 and IAP2, indicating an important role for NF-kB activity in the regulation of neuronal survival.

NF-kB refers to transcriptional activity that is mediated by the Rel family of gene products through NF-kB *cis* elements (see (Karin and Ben-Neriah, 2000)) for review). There is considerable diversity in the DNA binding properties of the different NF-kB proteins and the NF-kB consensus sequence has over 60 variants with different binding properties. The DNA binding potential of Rel family members is regulated by IkB binding and by IkB independent post-translation mechanisms. Transcription regulated through NF-kB cis elements will therefore reflect the presence and affinities of subsets of Rel family members that are present in various tissues during specific developmental windows. The NF-kB enhancer element that we used to generate the transgenic mice reported here was derived from a fragment of the HIV-LTR that is particularly sensitive to neuronal NF-kB activity (Corboy et al., 1992; Buzy et al., 1995). p65/RelA and p50/p105 bind this NF-kB element in cultured neurons (Rattner et al., 1993) but the precise complement of NF-kB proteins that mediate the activation of this element in neurons remains unknown. Our identification of constitutive NF-kB activity within CNS neurons expands on previous studies that have used EMSA and immunological methods to identify constitutive NF-kB in developing cortex and in CA1 and CA3 regions of the hippocampus, with much lower activity in the cerebellum (Bakalkin et al., 1993; Kaltschmidt et al., 1993; Rattner et al., 1993; Kaltschmidt et al., 1994).

Other groups have used distinct NF-kB cis-elements to generate reporter mice that identify endogenous NF-kB activity. Using the kB motif from the immunoglobin kB enhancer, Lernbercher et al (1993) identified NF-kB activity only in lymphoid tissues whereas the use of the p105 Rel enhancer or regions of the immunoglobin kB enhancer revealed β-galactosidase activity in lymphoid tissues as well as in developing rhombencephalon, spinal medulla and blood vessels (Schmidt-Ullrich et al., 1996). It is likely any single NF-kB element will provide a readout of only a subset of endogenous NF-kB activities and it is therefore not surprising to see differences between animals generated using distinct NF-kB cis elements. Indeed, generation of mice null for various members of the Rel family have revealed that the physiological sites of NF-kB action extend well beyond those revealed in a single transcriptional reporter mouse line (Lernbecher et al., 1993; Beg et al., 1995; Weih et al., 1995; Beg and Baltimore, 1996; Schmidt-Ullrich et al., 1996; Franzoso et al., 1997; Iotsova et al., 1997).

A crucial step in validating the transgenic reporter mice is to ablate NF-kB signaling and demonstrate concomitant reductions in β-galactosidase reporter gene activity. Primary fibroblasts derived from the transgenic reporter mouse revealed TNFα-induced β-galactosidase activity which was strongly attenuated in cells expressing the IkBαM repressor, a mutated form of IkBα which cannot be phosphorylated by IKK proteins and which therefore retains NF-kB dimers in the cytosol. Several recent studies have shown that TNFα-dependent NF-kB induction in MEFs occurs through an IKK2-dependent signaling mechanism and our results suggest that peripheral cells derived from these transgenic animals provide a sensitive transcriptional readout of this pathway.

Primary cortical neurons showed constitutive β-galactosidase activity which was reduced by infection with adenovirus encoding the IkBαM repressor or using a dominant inhibitory form of NIK, a MAP3K that binds both TRAF and IKK1 proteins and which normally regulates activation of NF-kB in response to some, but not all, cytokines (Yin et al., 2001). IkBαM repressor should retain Rel family members in the cytosol whereas the dominant negative NIK variant used in our studies is a deletion mutant that lacks the kinase domain but contains the TRAF and IKK binding domains that functions by

blocking the recruitment of endogenous NIK and by titrating upstream and downstream effectors (Malinin et al., 1997; Natoli et al., 1997; Ling et al., 1998; Van Antwerp et al., 1998; Delhase et al., 1999; Foehr et al., 2000). Together, these distinct approaches show that the β-galactosidase activity present in primary cortical neurons is indeed regulated by NF-kB activity. The precise signaling elements that contribute to NF-kB dependent activation of β-galactosidase in these animals are not certain and likely to be complex. It will be particularly interesting to examine the roles of the IKK proteins in this regard; mice rendered null for IKK1 or IKK2 display no apparent neuronal phenotype (Li et al., 1999a; Li et al., 1999b) yet mice lacking both genes show enhanced apoptosis in the neuroepithelium and a defect in neurulation (Li et al., 2000), consistent with the hypothesis that together these genes regulate NF-kB dependent survival pathways in developing neurons.

We have shown that expression of the IkBαM repressor or dominant negative NIK results in profound reduction in cortical neuron viability, consistent with the hypothesis that NF-kB normally plays an important role promoting central neuron survival. These results are consistent with several studies that indicate that NF-kB promotes survival of peripheral neurons. In sympathetic neurons, overexpression of a mutated derivative of c-Rel lacking the transactivation domain blocks neurotrophin-dependent survival whereas c-Rel overexpression facilitates survival (Maggirwar et al., 1998), in part by inducing gene products that block Cytochrome c release (Sarmiere and Freeman, 2001). Similarly, in developing dorsal root sensory neurons, members of the neurotrophin and CNTF families activate NF-kB dependent survival pathways that require p65/RelA (Hamanoue et al., 1999; Middleton et al., 2000).

The impact of NF-kB on the survival of CNS neurons is more controversial, with some studies suggesting a role for NF-kB in the promotion of survival whereas others indicate that NF-kB may facilitate apoptosis (reviewed in (Mattson and Camandola, 2001)). For example, NF-kB activation appears to protect central neurons against amyloid β-peptide toxicity (Barger et al., 1995) and excitotoxic or oxidative stress (Goodman and Mattson, 1996; Mattson et al., 1997) yet NF-kB exerts a pro-apoptotic effect that facilitates

glutamate-induced toxicity (Grilli and Memo, 1999). Further, mice rendered null for p50/p105 show increased death in response to kainate-induced excitotoxicity but are resistant to damage induced by ischemia (Schneider et al., 1999; Yu et al., 1999). Our data showed that induction of NF-kB mediated by p65/RelA overexpression effectively protected primary cortical neurons from death induced by etoposide or camptothecin, likely through upregulation of IAPs and anti-apoptotic Bcl2 family members. This finding are in agreement with recent results showing that Jak2-dependent activation of NF-kB resulted in accumulation of XIAP and IAP2 proteins and conferred neuroprotection to toxic concentrations of S-nitrosocystein, a nitric oxide donor (Digicaylioglu and Lipton, 2001). Together, these results suggest that physiological stimula that increase NF-kB activation in neurons will confer neuroprotection and these data are consistent with recent findings that show that preconditioning stimuli that confer neuroprotection on central neurons *in vivo* result in increased neuronal NF-kB activation (Blondeau et al., 2001; Ravati et al., 2001). Thus, our results support the hypothesis that constitutive NF-kB is necessary for neuronal survival and that further increases in NF-kB activation are neuroprotective. However, the complexity of NF-kB signaling pathways that results in specific gene activation events under physiological situations should not be underestimated and we cannot rule out the possibility that there may be pathological conditions that activate sets of NF-kB dependent genes that may induce distinct effects that include facilitating apoptosis.

The precise stimuli that contribute to constitutive NF-kB activation within neurons are unclear. NF-kB is activated by numerous stimula and it is possible that constitutive paracrine or autocrine activation loops act to increase NF-kB activity. It is also possible that NF-kB is a retrograde signal that links synaptic events to transcription (Kaltschmidt et al., 1993; Meberg et al., 1996). Glutamate, kainic acid and NO all activate neuronal NF-kB (Guerrini et al., 1995; Simpson and Morris, 1999) and recent studies have demonstrated activity-dependent translocation of p65/RelA from neurites to the nucleus of living neurons stimulated with glutamate, kainate, or potassium chloride (Wellmann et al., 2001). These studies therefore raise the intriguing possibility that NF-kB activity may link neuronal activity to cell survival pathways.

In summary, we have established that NF-kB transcriptional activity is prominent in the developing and adult nervous system. We have shown that NF-kB activity is necessary for neuronal survival and found that overexpression of NF-kB in primary neurons confers a high degree of neuroprotection through production of anti-apoptotic genes. Together, these studies demonstrate an important role for NF-kB in the development and maintenance of the nervous system.

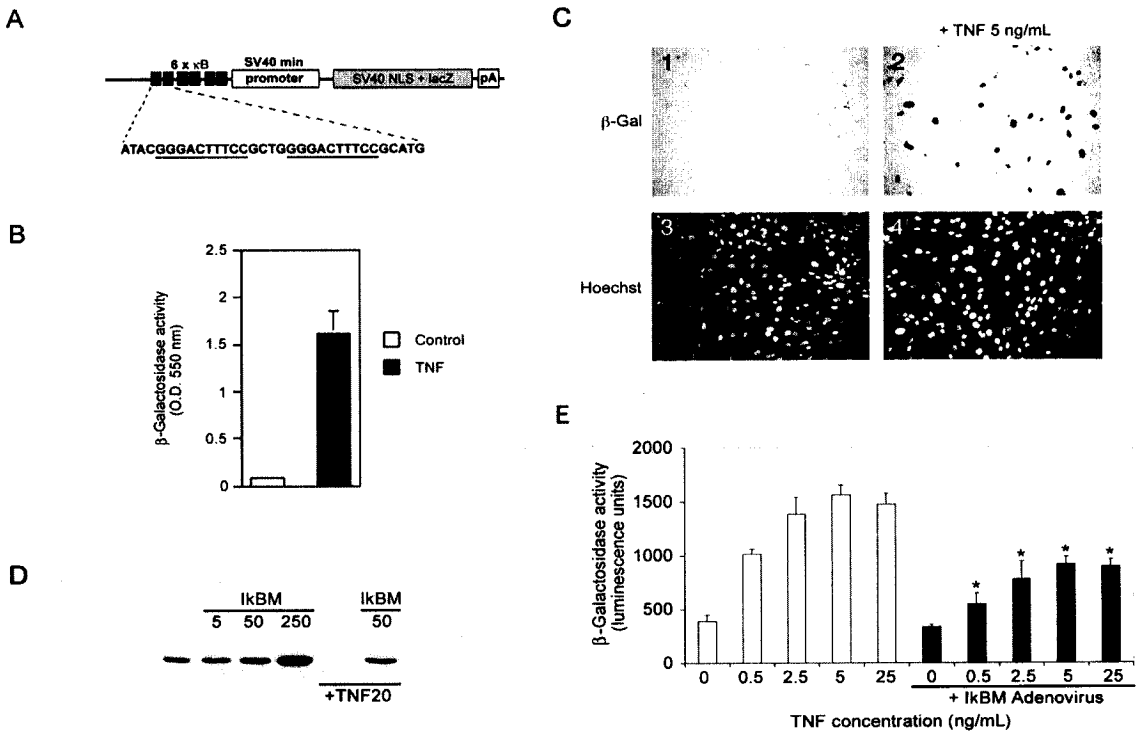


**Figure 3.1.** Transgene design and in vitro validation of the κB-dependent β-galactosidase construct. (A) The NF-κB r eporter minigene contains three tandem HIV-LTR repeats upstream of the SV40 minimal promoter, an E. coli β-galactosidase cDNA modified to contain a mammalian Kozak consensus, an SV40 T-antigen derived nuclear localization signal and a polyA tract derived from the protamine I gene. (B) HEK293 cells were transiently transfected with the minigene and induced with DME + TNFα (5 ng/ml) or with DME alone (indicated as control) for 16 hours and analyzed for β-galactosidase activity. (C) Primary MEFs derived from transgenic mice were incubated with (Panels 2, 4) or without (Panels 1, 3) TNFα(5 ng/ml) for 16 hours and then assessed for β-galactosidase activity. Cultures were counterstained with Hoechst 33342 (Panels 3, 4) to show cell nuclei. (D) MEFs were infected with 0, 5, 50 or 250 MOI of recombinant IkBαM adenovirus for 24 hours and total cell lysates were prepared and analyzed by immunoblotting for IkBα. Wells that were mock-infected or infected with 50 MOI of recombinant IkBαM adenovirus were exposed to TNFa (20 ng/ml) for 10 minutes. Endogenous IkBα is completely degraded by t his treatment but IkBαM is unaffected. (E) Transgenic MEFs were incubated with 0, 0.5, 2.5, 5, and 25 ng/ml TNFα for 16 hours in the absence (white bars) or presence (black bars) of IkBαM adenovirus (approximately 50% i nfection efficiency). β-galactosidase activity was quantified using a ch emiluminescent assay (Galacto-Star, Tropix). Each data point represents the average of 6 wells of a 24-well plate and error bars indicate standard deviation. Results were analyzed for statistical significance by ANOVA (Tukey HSD multiple comparison) and statistically significant differences of $p<0.001$are indicated by '*'.

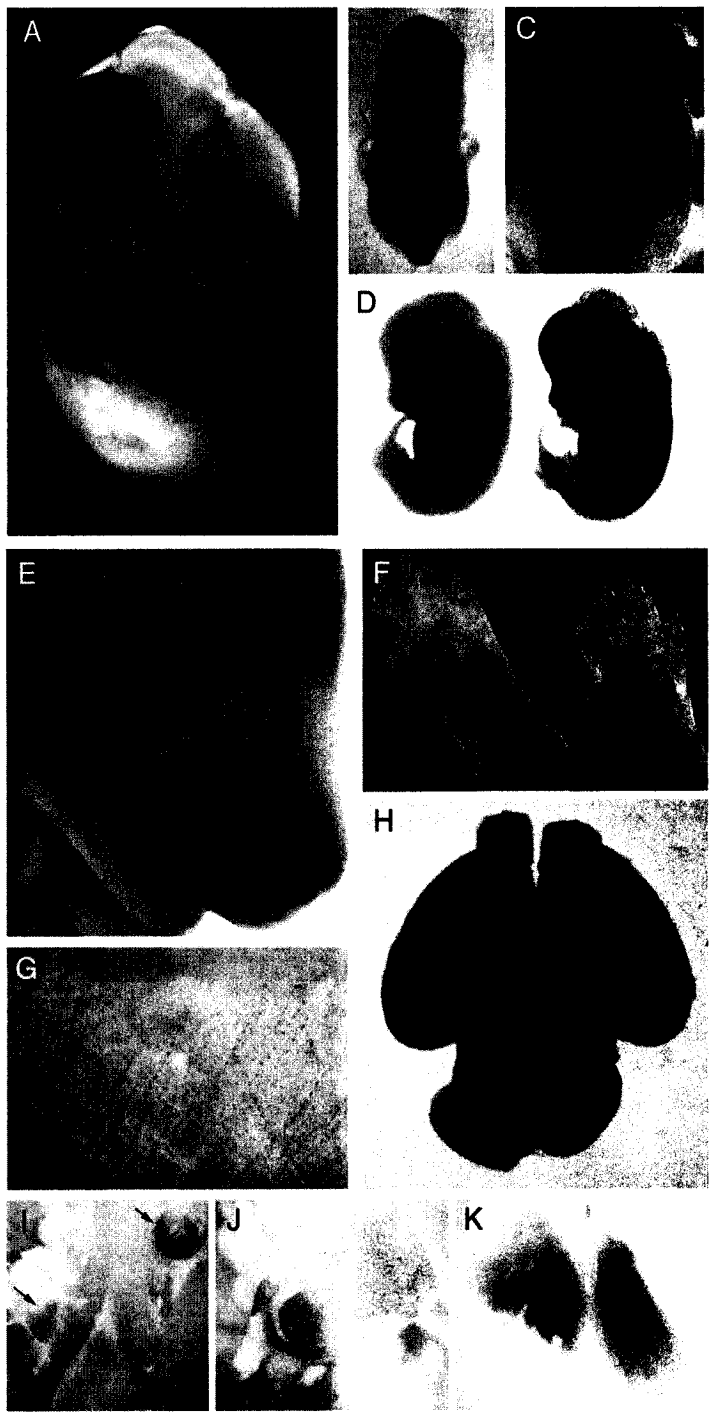


**Figure 3.2.** β-galactosidase expression pattern in discrete locations in embryonic and adult transgenic reporter mice. (A) Whole-mount X-gal staining of an E13 transgenic mouse shows high basal NF-κB activity in the telencephalon and along the roof plate of the midbrain. Facial staining is visible within the primordia of the vibrissae (5 parallel rows) and in the prominent tactile hair follicles. (B) Dorsal view of E13 transgenic embryos shows staining at the roof plate of the midbrain and at the midbrain-hindbrain junction. (C) Close-up of thoracic region. NF-κB activity is present in mammary gland primordia and in the gonadal area. (D) β-galactosidase staining in transgenic (right) and in control littermate (left). (E) E16 transgenic embryo showing prominent staining in vibrissae of the snout, in the olfactory lobes and in the developing eyelid. (F) NF-κB activity in the pads of the plantar surface of the E16 hindpaw (left) and of the palmer surface of the E16 forepaw (right). (G) NF-κB activity within nuclei, likely multinucleated muscle fibers, beneath superficial layers of P1 skin. (H) Robust NF-κB activity in P1 cortex, olfactory lobes, and roof plate of the midbrain. (I-K) Lymphoid organs from P60 transgenic mice were analyzed for β-galactosidase activity as described in the Materials and Methods. Constitutive NF-κB activity was detected along the trachea and bronchial tubes (I), in the thoracic lymph nodes (J and indicated by arrows in I), and in the thymus (K).

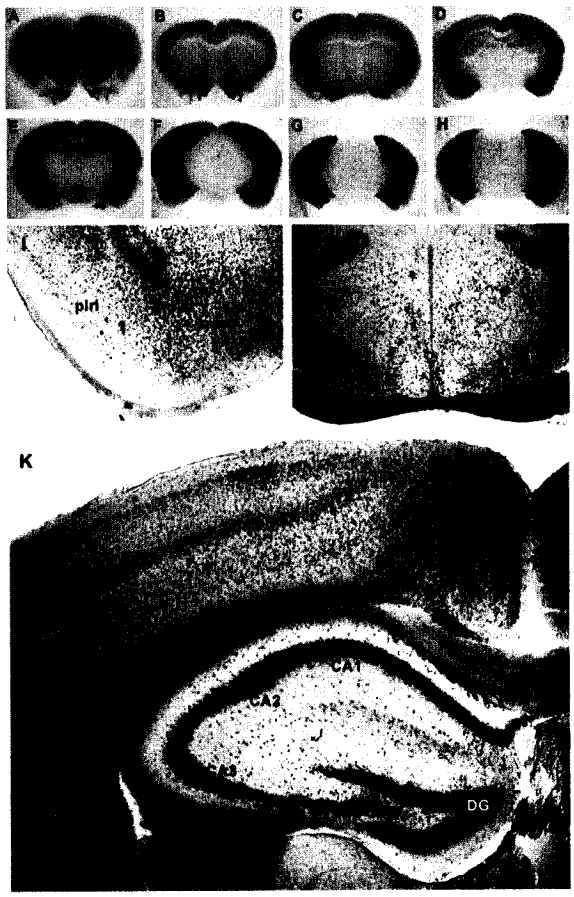


**Figure 3.3.** NF-kB activity in the adult brain. (A-H) 3 mm serial sections of P180 transgenic brain were stained for β-galactosidase activity. Robust activity is visible in cortical layers 2, 4, and 5 (A-G), in the outer layers of the olfactory lobes (A), and in the islands of Calleja (olfactory tubercle) (A-C). Lower levels of β-galactosidase activity are present in the entorhinal and piriform cortices (D-F), and in the amygdala (D-E), claustrum (C-F), dentate gyrus and the hippocampus (D-G). (I) NF-kB activity is present in the piriform/entorhinal cortex (piri) and is prominent in the amygdala. (amyg). (J) NF-kB activity is present in cells throughout the hypothalamus. (K) NF-kB activity is prominent in the dentate gyrus (DG) and in CA1 and CA2 regions of the hippocampus. NF-kB activity is present in the CA3 region but lower than in CA1 and CA2 (also see 3D, 3E). Positive nuclei are also found in the stratum oriens, radiatum and lacunosum-moleculaire of Ammon's horn. Within the cingulate and parietal cortex, positive nuclei are found in all cortical layers but layers 2, 4, and 5 are most prominently stained.

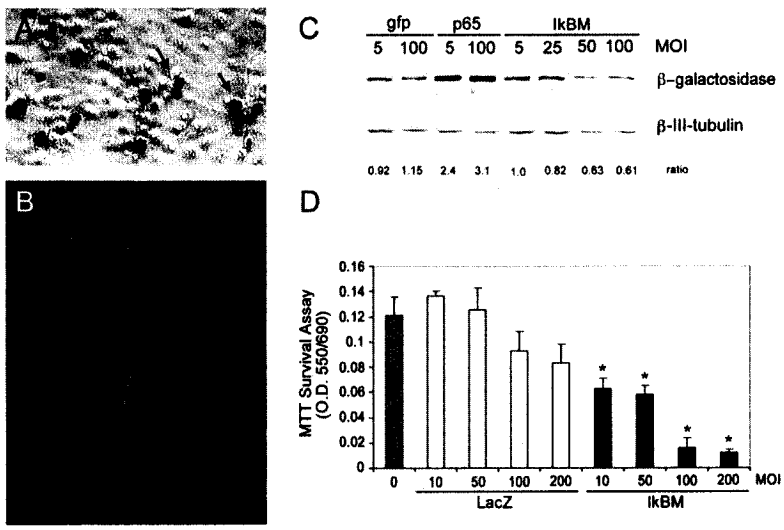


**Figure 3.4.** NF-kB transcriptional activity is abundant in cultured primary cortical neurons. (A) E16 primary cortical neurons derived from a heterozygote litter were grown for 10 DIV and then fixed and assessed for β-galactosidase activity. Arrows indicate transgenic nuclei. (B) E16 primary cortical neurons were immunostained for β-galactosidase and β-III-tubulin. β-galactosidase immunoreactivity is shown in red, bIII-tubulin is green, and nuclei stained with Hoescht 33342 are blue. (C) Transgenic E16 cortical neurons derived from a heterozygote litter were infected with indicated MOIs of recombinant adenovirus expressing GFP, GFP and p65/RelA or IkBαM for 48 hours, lysed, normalized for protein content and analyzed by for b-galactosidase content by immunoblot. Levels of βIII-tubulin assessed in parallel blots confirmed equivalent protein loading between lanes. (D) Primary cortical neurons were infected with adenovirus encoding β-galactosidase (white bars) or IkBαM (black bars) and survival was measured by MTT conversion 48 hours later. Error bars indicate standard deviation. Results were analyzed for statistical significance by ANOVA (Tukey HSD multiple comparison). Statistically significant differences of $p<0.001$are indicated by '*'. For A-D, each experiment was performed at least three times.

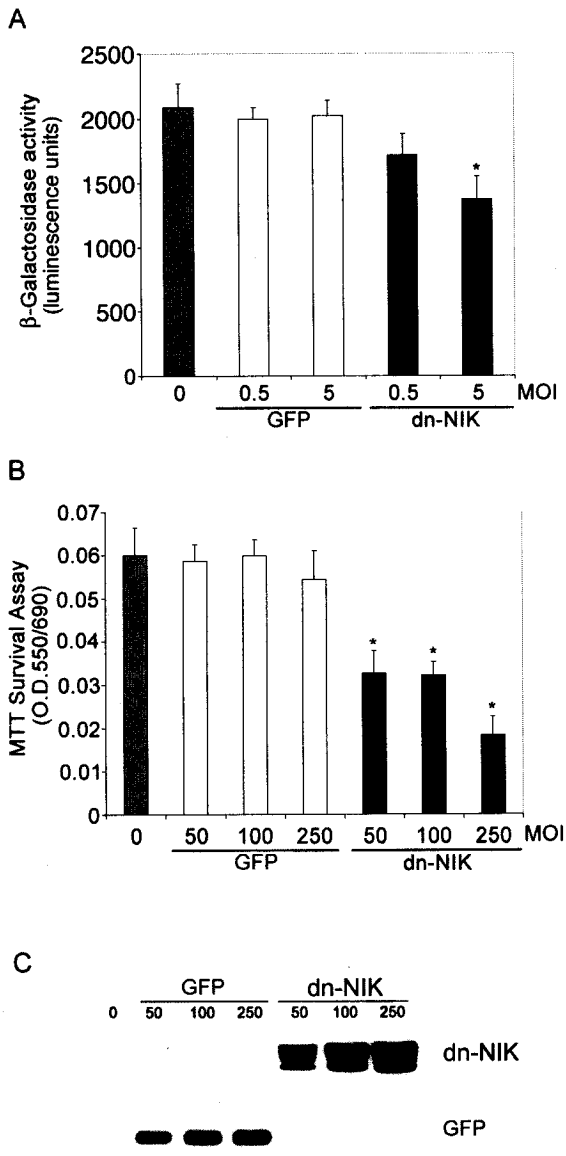


**Figure 3.5.** NIK signaling is required for NF-κB transcriptional activity and for neuronal viability in primary cortical neurons. (A) E16 primary cortical neurons derived from a heterozygote litter were mock infected (grey bar) or infected with control GFP adenovirus (white bars), or dn-NIK adenovirus (black bars) at 0.5 or 5 MOI and harvested 4 days later. Lysates were analyzed for β-galactosidase activity using a chemiluminescence assay (Tropix). β-galactosidase activity was significantly reduced in cells infected with 5 MOI of dn-NIK ($p < 0.03$, indicated by '*'). (B-C) Cortical neurons were infected with 0 (grey bar), 50, 100, or 250 MOI of recombinant adenovirus encoding GFP (white bars) or dn-NIK (black bars) for 72 hours and t hen analyzed for viability by MTT dye conversion (B) and for GFP and dn-NIK expression by imm unoblotting (C). β-galactosidase overexpression had no significant effect on neuronal survival but overexpression of dn-NIK reduced survival at each MOI tested ($p<0.001$, indicated by '*'). For both (A) and (B), 6 wells were analyzed per condition and results were analyzed for statistical significance by ANOVA (Tukey HSD multiple comparison). For A-C, each experiment was repeated at least three times.

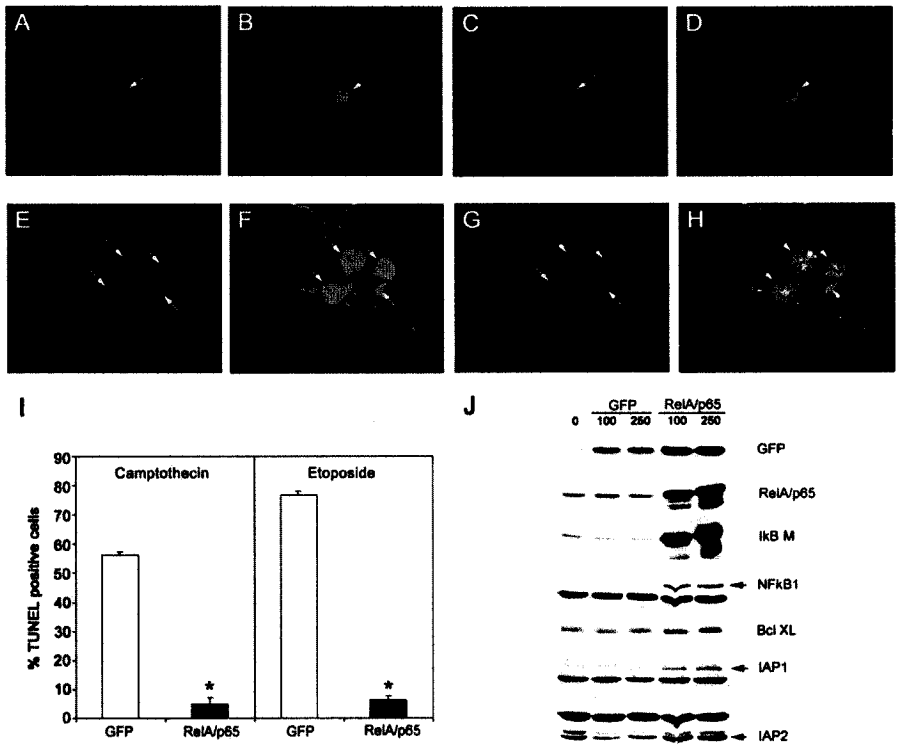


**Figure 3.6.** p65/RelA protects cortical neurons from apoptotic death. E15-16 cortical neurons were infected with 75 MOI of recombinant adenovirus encoding GFP alone (panels A-D) or with recombinant virus encoding both p65/RelA and GFP (panels E-H) for 24 hours. Cells were then exposed to etoposide (20 mM) for a further 18 hours and then fixed and analyzed for GFP fluorescence (B, F - green), and for apoptosis using Hoescht 33342 nuclear staining (A, E - blue), and TUNEL labeling (C, G - red). D and H are rmerged images of A -C and E-G respectively. Cells infected with GFP alone (and uninfected cells) rapidly underwent apoptosis when exposed to etoposide whereas neurons infected with p65/RelA were robustly viable under t hese conditions. (I) Cells were infected with 75 MOI of adenovirus expressing either GFP or exp ressing GFP together with p65/RelA for 48 hours and then treated with camptothecin (20 mM) or with etoposide (20 mM) for 18 hou rs. GFP-positive cells were scored for TUNEL positive nuclei. Expression of p65/RelA conferred robust protection from apoptosis due to campthothecin ($p < 0.001$; indicated by '*') or etoposide ($p<0.0001$; indicated by '*'). At least 300 cells were assessed for each condition and results were analyzed for statistical significance by Student's T-test. (J) E16 cortical neurons were either left uninfected or were infected with 75 MOI of recombinant adenovirus expressing GFP or expressing both GFP and p65/RelA for 48 hours. Neurons were then lysed and analyzed by immunoblot. Levels of endogenous IkBa, NFkB1, IAP1, IAP2, and Bcl-Xl were specifically increased by p65/RelA overexpression.

# PREFACE TO CHAPTER 4

Chapter Three has demonstrated that NF-kB activity is prominent in the CNS throughout development and is required for central neuron survival. The precise signaling elements that contribute to neuronal NF-kB activity, however, remain to be determined. Therefore, to identify contributing signaling components, we have been breeding our NF-kB reporter mice to mice deficient in potential regulators including p75NTR and TRAF6. This part of the study is currently ongoing.

Since p75NTR can function as an apoptotic receptor *in vitro* and *in vivo*, and since p75NTR re-expression in pathological situations often occurs within neurons that are undergoing apoptosis (Roux et al., 1999; Troy et al., 2002), the third objective of this thesis is to identify the signaling pathways required for p75NTR-induced death. In Chapter Four, recombinant p75NTR adenovirus has been used to constitutively activate p75NTR and reliably compare p75NTR-initiated death events in a number of cell lines and central neurons. Chapter 4 also determines 1) whether p75NTR uses an intrinsic or extrinsic death mechanism, 2) whether JNK activity is required, and 3) whether BH3-domain only proteins are activated and essential for p75NTR-induced death.

# CHAPTER 4

# APOPTOSIS INDUCED BY P75NTR REQUIRES JUN KINASE-DEPENDENT PHOSPHORYLATION OF BAD.

# Apoptosis Induced by p75NTR Requires Jun Kinase-dependent Phosphorylation of Bad.

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## ABSTRACT

The p75 neurotrophin receptor (p75NTR), a member of the TNF receptor superfamily, facilitates apoptosis during development and following injury to the central nervous system. The signaling cascades activated by p75NTR that result in apoptosis remain poorly understood. In this study, we show that activation of p75NTR in primary cortical neurons, in PC12 cells and in glioma cells results in activation of Jun kinase (JNK), accumulation of Cytochrome c within the cytosol, and activation of Caspases 9, 6 and 3. To link p75NTR-dependent JNK activation to mitochondrial Cytochrome c release, regulation of BH3-domain-only family members was examined. Transcription of BH3-domain-only family members was not induced by p75NTR but p75NTR-dependent JNK activation resulted in phosphorylation and oligomerization of the BH3-domain-only family member, Bad. Loss of function experiments using Bad dominant negatives or RNA interference demonstrated a requirement for Bad in p75NTR-induced apoptosis. Together, these studies provide the first data linking apoptosis induced by cell surface receptor activation to the post-translational regulation of BH3-domain-only family members.

## INTRODUCTION

The four mammalian neurotrophins comprise a family of related growth factors required for differentiation, survival, development, and death of specific populations of neurons and non-neuronal cells. The effects of the neurotrophins are mediated by binding to cell surface TrkA, TrkB and TrkC tyrosine-kinase receptors and to the p75 neurotrophin receptor (p75NTR). Roles for Trk receptors in neurotrophin action in neuronal survival, growth and synaptic modulation are now well established (Kaplan and Miller, 2000; Patapoutian and Reichardt, 2001). The functions of the p75NTR receptor are complex and have been more difficult to ascertain (Dechant and Barde, 2002; Roux and Barker, 2002). It is clear that p75NTR functions as a Trk co-receptor that increases neurotrophin binding affinity (Barker and Shooter, 1994; Ryden et al., 1997; Esposito et al., 2001) and recent studies suggest that it may be a critical element in a receptor complex that responds to myelin-based growth inhibitory signals (Wang et al., 2002; Wong et al., 2002) and regulates myelination (Cosgaya et al., 2002). p75NTR also has autonomous signaling roles, particularly in facilitating apoptosis. In vitro analyses have shown that p75NTR induces cell death in primary trigeminal (Davies et al., 1993), hippocampal (Friedman, 2000; Brann et al., 2002), and sympathetic neurons (Lee et al., 1994; Bamji et al., 1998), as well as retinal precursor (Frade et al., 1996; Frade and Barde, 1998), Schwann (Soilu-Hanninen et al., 1999; Syroid et al., 2000; Petratos et al., 2003), oligodendrocyte (Casaccia-Bonnefil et al., 1996; Yoon et al., 1998) and neuroblastoma cells (Bunone et al., 1997). In vivo, p75NTR plays a prominent role in apoptosis that occurs in glia and neurons following traumatic injury to the spinal cord (Casha et al., 2001; Beattie et al., 2002) or brain (Roux et al., 1999; Troy et al., 2002) and has been implicated in developmental apoptosis in somites, (Cotrina et al., 2000) retina and spinal cord (Frade and Barde, 1999) and in the peripheral nervous system (Bamji et al., 1998).

The signaling events that link p75NTR activation to apoptosis are beginning to emerge and p75NTR-dependent apoptosis is associated with an increase in Rac and Jun kinase (JNK) activity and Caspase activation (Tournier et al., 2000; Harrington et al., 2002). The precise ligand requirements for p75NTR apoptotic signaling are not clear but recent studies have shown that unprocessed NGF (proNGF) is a more efficacious p75NTR

ligand than mature NGF (Lee et al., 2001; Beattie et al., 2002). A plethora of p75NTR interacting proteins have been identified (Roux and Barker, 2002) and some of these, including NRAGE (Salehi et al., 2000), NRIF (Casademunt et al., 1999) and NADE (Mukai et al., 2000), facilitate p75NTR-dependent apoptosis. We have recently shown that NRAGE activates a mitochondrial death pathway involving JNK-dependent Cytochrome c release and the activation of Caspases (Salehi et al., 2002) but establishing the precise roles of each of the cytosolic interactors of p75NTR remains a significant challenge.

Despite this progress, several important questions remain unresolved. The proximal elements that connect p75NTR to apoptotic pathways remain uncertain and it is not clear whether JNK activation is a prerequisite for p75NTR-induced apoptosis in all responsive cells. Further, the mechanisms employed by p75NTR to induce mitochondrial Cytochrome c release and Caspase activation are unknown. In this report, we addressed the mechanism of p75NTR-induced apoptosis in primary mouse cortical neurons and in pheochromacytoma, glioma, neuroblastoma and medulloblastoma cells. Our findings reveal that activated p75NTR invariably causes JNK activation, mitochondrial Cytochrome c release and Caspase 9, 6 and 3 activation. We show that JNK activation is necessary for p75NTR-dependent Caspase cleavage in all responsive cell types. To link p75NTR-induced JNK activation to mitochondrial dysfunction, we examined the ability of p75NTR to increase expression of BH3-domain-only proteins but found that p75NTR did not activate transcription of BH3-domain-only genes. Instead, we demonstrate that p75NTR activation results in JNK-dependent phosphorylation of the BH3-domain-only protein Bad and show that Bad is required for p75NTR-induced apoptosis.

## EXPERIMENTAL PROCEDURES

**Materials.** Cell culture reagents were purchased from BioWhittaker, unless otherwise indicated. The p75NTR antibody αP1 and the phospho-Ser128 antibody have been previously described (Roux et al., 1999). The phospho-Thr83 p53 antibody was a kind gift of Ze'ev Ronai. The JNK1 antibody (C-17, cat# sc-474), the two Bad antibodies (C-20, cat# sc-943 and N-19 cat# sc-6542) and the actin antibody (C-2, cat#sc-8432) were purchased from Santa Cruz Biotechnology. Anti-Flag antibody (M2, cat# F-3165) was obtained from Sigma, Cytochrome c antibody was purchased from Pharmingen (cat# 556433), β-galactosidase (LacZ) antibody was purchased from Promega (cat# 23781) and anti-HA antibody (12CA5, cat# 1583816) was purchased from Roche. Phospho-$Thr^{183}/Tyr^{185}$ JNK (G9, cat# 9255), phospho-$Ser^{63}$ c-Jun (cat# 9261), phospho-$Ser^{73}$ c-Jun (cat# 9164S), c-Jun (cat# 9162), Caspase-9 (cat# 9502), cleaved Caspase-3 (Asp175, cat# 9661), cleaved Caspase-6 (Asp198; cat# 9761S), and cleaved PARP (Asp214; cat# 9541) specific antibodies were obtained from Cell Signaling Technology. Horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories. Immunoreactive bands were detected using enhanced chemiluminescence purchased from Perkin Elmer Life Sciences. All other reagents were from Sigma, Calbiochem, or ICN Biochemicals, unless otherwise indicated.

**Plasmids and recombinant adenovirus.** Preparation of recombinant adenovirus expressing enhanced green fluorescence protein (AdGFP), β-galactosidase (AdLacZ), full-length p75NTR (Adp75NTR) , the Flag-tagged JNK-binding domain of JIP1 (AdJBD) and HA-epitope tagged MLK-3 (adMLK3) have been previously described (Roux et al., 2002). All adenoviruses were amplified in 293A cells and purified on a sucrose gradient, as previously described (Roux et al., 2002). Viruses were titered by optical density and using the tissue culture infectious dose 50 (TCID) assay in 293A cells. Titers are expressed in term of plaque forming units. The Bad dominant negative plasmid consisting of GFP fused to a Bad nonapeptide in which Ser128 was substituted by Ala and the parental GFP vector have both been previously described (Konishi et al., 2002). The Bad RNAi construct was generated as previously described (Gaudilliere et al., 2002).

**Cell culture, infection and transfection.** Human glioma (U343, U373, U87, and U251) and medulloblastoma (UW228-1, UW228-3, UW228-3, and Daoy) cell lines were provided by Dr. Roland Del Maestro (McGill University) and maintained in 5% $CO_2$ at 37° C in either Dulbecco's modified Eagle's medium (DMEM) or RPMI medium and supplemented with 10% fetal calf serum (FCS, Clontech), 2 mM L-glutamine, 100 ug/ml penicillin/streptomycin. Neuroblastoma cell lines (SY5Y, SKNAS, 15N, and NGP) were provided by Dr. David Kaplan and maintained as above. The rat pheochromocytoma cell line, PC12, was maintained as previously described (Roux et al., 2001) and the PC12rtTA cell line (PC12) was purchased from Clontech and maintained in 10% $CO_2$ at 37° C in DMEM supplemented with 10% FCS, 5% horse serum, 2 mM L-glutamine, 100 ug/ml penicillin/streptomycin and 100 μg/ml G418. Cell lines were plated 18 to 24 hours prior to transfection and typically harvested 24 to 48 hours after infection. Primary cortical cultures were prepared from E14-16 CD1 mouse telencephalon as described previously (Bhakar et al., 2002). Neuronal cultures were infected prior to plating and then maintained *in vitro* for 2 days in Neurobasal media (Life Technologies) supplemented with 1X B27 supplement (Life Technologies), 2mM L-glutamine, and 100 μg/ml penicillin/streptomycin. PC12 cells were plated on poly-L-lysine coated plates and transfected using Lipofectamine2000 as directed by the manufacturer (Invitrogen). Cells lines were infected with adenovirus 24 hours after plating.

**Cytochrome c release assay.** Cytosol enriched subcellular fractions were prepared as described in (Salehi et al., 2002). In brief, five million cells were harvested, washed once in Tris-buffered saline (10 mM Tris (pH 8.0), 150 mM NaCl), once in Buffer A (100 mM sucrose, 1 mM EGTA, 20 mM MOPS (pH 7.4)), and then resuspended in 500 ul Buffer B (Buffer A plus 5% Percoll, 0.01% digitonin, 1 ug/ml aprotinin, 1 ug/ml leupeptin, 1 ug/ml pepstatin, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride). A sample of this suspension was retained as total cell lysate. The remainder was incubated on ice for 15 minutes and then centrifuged at 2500 g for 10 minutes to remove intact cells and nuclei. The supernatant was then centrifuged at 15 000 g for 15 min to pellet mitochondria. The final supernatant was designated cytosol.

**Immunoblotting.** Cells were lysed in RIPA buffer (10 mM Tris (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1 ug/ml Aprotinin, 1 ug/ml leupeptin, 1 ug/ml pepstatin, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride) and analyzed for protein content using the BCA assay (Pierce). Samples were normalized for protein content, suspended in Laemmli sample buffer, separated by SDS-polyacrylamide gel electrophoresis, and electroblotted onto nitrocellulose. Blocking and secondary antibody incubations of immunoblots were performed in Tris-buffered saline/Tween (10 mM Tris (pH 8.0), 150 mM NaCl, 0.2% Tween 20) supplemented with 5% (w/v) dried skim milk powder or 5% (w/v) bovine serum albumin (BSA) (Pierce). All primary antibody incubations were performed in the blocking solution, except for those involving phospho-specific antibodies which were performed in Tris-buffered saline/Tween supplemented with 5% BSA. Immunoreactive bands were detected by chemiluminescence (Perkin Elmer Life Sciences), according to the manufacturer's instructions.

**Survival assay.** Analysis of cell survival was performed by MTT assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which was added at a final concentration of 1 mg/ml for the last four hours of a 48 hour infection. The reaction was ended by the addition of one volume of solubilization buffer (20% SDS, 10% dimethylformamide, and 20% acetic acid). After overnight solubilization, specific and non-specific absorbencies were read at 570 and 690 nm, respectively. Each data point was performed in triplicate or quadruplicate, and experimental results were analyzed by multiple analyses of variance with statistical probabilities assigned using the Tukey test for multiple comparisons. Each experiment was performed independently at least three times.

**RT-PCR.** 450,000 U373 cells or primary cortical neurons were infected with virus and 24 hours later mRNA was isolated using the RNEasy Mini kit according to the manufacturer's instructions (Qiagen). cDNA was generated using the Omniscript RT kit (Qiagen) and random hexamers (Roche) as primers. PCR was performed for 30 cycles using 300 nM of the following primers for U373 cells:

| | | |
|---|---|---|
| actin | sense, 5' | CACCACTTTCTACAATGAGC |
| | antisense, 5' | CGGTCAGGATCTTCATGAGG |
| hBIMEL | sense, 5' | TGGCAAAGCAACCTTCTGATG |
| | antisense, 5' | AGTCGTAAGATAACCATTCGTGGG |
| hBMF | sense, 5' | CTTGCTCTCTGCTGACCTGTTTG |
| | antisense, 5' | AAGCCGATAGCCAGCATTGC |
| hHrk/Dp5 | sense, 5' | TCGGCAGGCGGAACTTGTAG |
| | antisense, 5' | GCTGTATGTAAATAGCATTGGGGTG |
| hBIK | sense, 5' | AACCCCGAGATAGTGCTGGAAC |
| | antisense, 5' | GCTGGAAACCAACATTTTATTGAGC |
| hPUMA | sense, 5' | ACTGTGAATCCTGTGCTCTGCC |
| | antisense, 5' | ACCCCCCAAATGAATGCCAG |
| hNOXA | sense, 5' | CCAAACTCTTCTGCTCAGGAACC |
| | antisense, 5' | CGGTAATCTTCGGCAAAAACAC. |

For mouse cortical neurons, PCR was performed using the same conditions as above using the following primers:

| | | |
|---|---|---|
| mBimEL | sense, 5' | CCCCTACCTCCCTACAGACAGAA |
| | antisense, 5' | CCAGACGGAAGATAAAGCGTAACAG |
| mBMF | sense, 5' | CTTGCTCTCTGCTGACCTCTTTG |
| | antisense, 5' | GTTGCGTATGAAGCCGATGG |
| mHrk/Dp5 | sense, 5' | TGGAAACACAGACAGAGGAAGCC |
| | antisense, 5' | AAAGGAAAGGGACCACCACG |
| mBIK | sense, 5' | TCACCAACCTCAGGGAAAACATC |
| | antisense, 5' | AGCAGGGGTCAAGAGAAGAAGG |
| mNOXA | sense, 5' | TGATGTGATGAGAGAAACGCTCG |
| | antisense, 5' | AAAGCAATCCCAAACGACTGCC |
| p75NTR | sense, 5' | TGAATTCTGGAACAGCTGCAAAC |
| | antisense, 5' | CCTTAAGTCACACTGGGGATGTG |

5% of the cDNA prepared was used in a 25ul PCR reaction and the reaction product was separated on an 8% polyacrylamide gel, stained with ethidium bromide and visualized under UV light.

**Single Cell Caspase-3 Activation Assay.** PC12 cells were transfected with plasmids encoding either GFP alone, GFP fused to a dominant interfering Bad nonapeptide, or GFP plasmid and pU6/BS-Bad RNA interference plasmid at a 1:2 ratio. Cells were infected with either adLacZ or with adp75NTR 48 hours after transfection and fixed 24 hours later using 4% paraformaldehyde (PFA) in PBS. Cells were blocked in TBS supplemented with 5% donkey serum and 0.3% Triton-x100 for 30 minutes and then incubated for 18 hours at 4°C with control rabbit sera or with antibodies directed against cleaved Caspase 3. Secondary antibodies (donkey anti-rabbit conjugated Cy3) and Hoescht 33248 were applied for 2 hours at 4° C. GFP-positive cells were scored for the presence of activated Caspase 3 by a blinded observer, with 300 cells counted per condition. This experiment was repeated three times and the composite data was analyzed for statistical significance by ANOVA (Tukey HDS multiple comparison).

## RESULTS

The physiological conditions that result in activation of p75NTR apoptotic pathways are complex and likely regulated by multiple ligands and co-receptors. We have previously shown that recombinant adenovirus expressing full-length p75NTR or the p75NTR intracellular domain efficiently induces apoptosis in the absence of added ligand (Roux et al., 2001) and this approach was used to define apoptotic signaling pathways activated by p75NTR. We began by testing cell lines and primary cell types for susceptibility to p75NTR-induced death. Figure 4.1 shows that primary mouse cortical neurons, PC12 pheochromacytoma cells and U343 and U373 glioma lines all showed reduced viability when infected with adenovirus expressing p75NTR whereas infection with control adenovirus expressing β-galactosidase (LacZ) had no significant effect. Other lines tested, including other glioma lines (U251 and U87), various medulloblastoma lines (Daoy, UW288-1, UW288-2, and UW288-3), and neuroblastoma lines (SY5Y, 15N, NGP, and SKNAS) were resistant to p75NTR-induced apoptosis in this assay (data not shown). For the remainder of this study, we focused our attention on p75NTR-dependent apoptosis in primary mouse cortical neurons, rat PC12 cells and human U343 and U373 glioma lines.

Activation of the extrinsic apoptotic pathway by death receptors that are structurally related to p75NTR results in autocleavage and activation of Caspase 8. Activation of the intrinsic apoptotic pathway results in release of mitochondrial contents and activation of Caspase 9. We therefore determined the activation status of apical Caspases 8 and 9 and effector Caspases 3 and 6 during p75NTR-induced apoptosis. Expression of p75NTR resulted in a reduction in levels of full-length Caspase 9, a corresponding increase in activated Caspase 9, Caspase 3, and Caspase 6 and accumulation of the cleaved form of PARP, a Caspase 3 substrate (Figure 4.2A-B). In contrast, p75NTR-dependent Caspase 8 cleavage was not observed in any of the cell types examined (data not shown). p75NTR-dependent Caspase activation was not due to adenoviral toxicity since cells infected with comparable quantities of LacZ adenovirus did not exhibit Caspase activation. These data indicate that p75NTR-induced apoptosis occurs primarily through an intrinsic death pathway that involves release of mitochondrial contents and activation of Caspase 9.

Caspase 9 activation requires formation of an apoptosome complex consisting of Caspase 9, Apaf-1 and cytosolic Cytochrome c. Release of Cytochrome c from mitochondria into the cytosol is a key regulatory step in this process. To determine if Cytochrome c is released during p75NTR-induced apoptosis, cells were left uninfected or were infected with p75NTR or a control adenovirus, then lysed, subjected to subcellular fractionation and cytosolic fractions were analyzed for Cytochrome c levels by immunoblot. Figure 4.2C shows that Cytochrome c was not detected in the cytosol of uninfected cells or in cells infected with control adenovirus whereas cytosolic Cytochrome c was readily detected in the cytosol of cells expressing p75NTR. Thus, p75NTR induces Cytochrome c release from mitochondria of multiple cell types.

Activation of the JNK pathway is an important regulator of apoptotic events in several neuronal death paradigms and JNK can be activated by p75NTR in several cell types. Consistent with this, we found that p75NTR expression in primary mouse cortical neurons and in PC12 and U373 cells consistently resulted in phosphorylation of JNK (Figure 4.3A-B) or induced a dose-responsive increase in the phosphorylation of c-Jun, a JNK target, (Figure 4.3C). These results indicate that 75NTR–induced JNK activation is a consistent feature of a variety of p75NTR-responsive cell types.

To begin to address the role of the JNK pathway in p75NTR-induced apoptosis, we tested the effect of CEP1347, a MAP3K inhibitor that exhibits anti-apoptotic effects in several neuronal and non-neuronal systems (Saporito et al., 2002). We first tested the ability of CEP1347 to block c-Jun phosphorylation in PC12 cells overexpressing MLK3, a MAP3K identified as a target of CEP1347. Figure 4.4A shows that the compound almost completely blocked the robust c-Jun phosphorylation induced by this kinase at 200nM, a concentration typically used in protein overexpression paradigms. We next examined whether CEP1347 reduced c-Jun phosphorylation or Caspase 3 activation which was induced by p75NTR. CEP1347 did indeed reduce p75NTR-dependent c-Jun phosphorylation and Caspase 3 activation but only at high concentrations (500-1000 nM; Figure 4.4B-C; data not shown). Consistent with this, p75NTR-mediated decreases in cellular viability was also blocked by high CEP1347 concentrations (data not shown).

These findings indicate that reductions in MAP3K and JNK signaling attenuates apoptosis induced by p75NTR yet suggest that blockade of a non-preferred target of CEP1347 is required for this effect.

To directly assess the role of JNK activity in p75NTR-induced death, an adenovirus expressing the JNK binding domain of the JIP scaffolding molecule (AdJBD) was used to inhibit JNK activity in vivo. This JIP fragment is believed to sequester JNK and thus acts as an effective dominant inhibitor of JNK signaling (Harding et al., 2001). We first confirmed that AdJBD is capable of blocking JNK-dependent target phosphorylation by demonstrating that it blocked c-Jun phosphorylation induced by TNFα, a well characterized JNK pathway inducer (Figure 4.5A). Subsequent studies established that AdJBD was equally effective in blocking c-Jun phosphorylation induced by p75NTR expression (Figure 4.5B). To determine if JNK inhibition blocked apoptotic signaling induced by p75NTR, cells were infected with p75NTR in the absence or presence of AdJBD and assessed for Caspase 3 activation. Expression of AdJBD effectively blocked Caspase 3 activation in all responsive cell types, indicating a crucial role for JNK activation in p75NTR-induced apoptosis (Figure 4.5C and data not shown).

These data demonstrate that JNK activation is a prerequisite for p75NTR-induced apoptosis but substrates of JNK that play a role in p75NTR-induced apoptosis are unknown. To begin to characterize targets of JNK involved in p75NTR-induced death, we first compared c-Jun phosphorylation induced by p75NTR or MLK3, a potent inducer of JNK activity (see above). Figure 4.6A shows that p75NTR and MLK3 induced robust phosphorylation of JNK. However, there was considerable discordance between the JNK activation, c-Jun phosphorylation and Caspase-3 activation induced by p75NTR versus MLK3. p75NTR and MLK3 induced comparable JNK phosphorylation but only MLK3 produced a substantial increase in c-Jun phosphorylation whereas only p75NTR induced substantial cleavage of Caspase 3. To determine if our experimental design may have missed an early peak in p75NTR-induced c-Jun phosphorylation, JNK activation and c-Jun phosphorylation were examined at 12, 18, 24 and 30 hours after adenovirus infection. Figure 4.6B shows that phosphorylated JNK was first detected 18 hours after p75NTR

infection and increased further by 24 and 30 hours. Cleaved Caspase 3 was detectable 24 hours after infection but c-Jun phosphorylation showed a significant lag, and an elevation in phospho-Jun levels were detected only after 30 hours infection. These data show that JNK activation correlates with p75NTR-induced death and suggests that c-Jun is not a preferred substrate of the JNK complex which is activated by p75NTR.

BH3-domain-only proteins directly and indirectly induce the association of Bax and Bak, which in turn facilitates release of mitochondrial proteins such as Cytochrome c into the cytosol. Transcriptional activation of BH3-domain-only genes through c-Jun or p53 dependent pathways is important in apoptosis in several neuronal and non-neuronal settings. We therefore examined whether p75NTR-induced apoptosis correlated with accumulation of BH3-domain-only gene products. PC12 and U373 cells and cortical neurons were infected with LacZ or p75NTR adenovirus and alterations in mRNA levels of the BH3-domain-only family members Bim, Bmf, Hrk, Bik, Puma, and Noxa were determined by RT-PCR. mRNA corresponding to each of these family members were readily detected in both cell types examined but p75NTR-dependent increases in their levels were not detected (Figure 4.7 and data not shown). This indicates that JNK activation induced by p75NTR does not induce transcription of BH3-domain-only genes and suggests that alternate pathways are responsible for p75NTR-induced Cytochrome c release and Caspase 3 activation.

BH3-domain-only proteins can, in some instances, be regulated by post-translational mechanisms. Akt-dependent phosphorylation of Bad on Ser112 and Ser136 allows it to associate with 14-3-3 proteins and thereby suppresses its pro-apoptotic activity. Apoptotic kinases including JNK directly activate the cell death machinery by phosphorylating Bad at Serine 128 (Donovan et al., 2002). The phosphorylation of Bad at this residue disrupts the interaction of Bad with 14-3-3 proteins thus allowing Bad to induce apoptosis (Konishi et al., 2002). We therefore determined if p75NTR activation resulted in phosphorylation of Bad on Ser128. PC12 and U373 cells were infected with LacZ or p75NTR adenovirus and alterations in Bad phosphostatus was examined by immunoblot. Figures 4.8A and 4.8B show that p75NTR expression had little effect on the levels or

phosphostatus of monomeric Bad (~25 kD) but rather induced the accumulation of a higher molecular weight species (~75 kD). This product was detected by two antibodies directed against distinct epitopes in Bad (N19, C20) as well as by a phospho-specific antibody directed against the JNK phosphorylation site within Bad. The 75 kDa product therefore appears to represent a stable oligomeric complex containing Bad phosphorylated on Serine 128. To determine if JNK activity contributes to p75NTR-dependent Bad phosphorylation and oligomerization, PC12 cells were infected with adenovirus expressing p75NTR in the absence or presence of AdJBD, lysed and examined by Bad immunoblot. Figure 4.8C shows that inhibiting JNK activity with AdJBD prevented formation of the Bad complex, indicating that JNK activity is required for p75NTR-dependent Bad phosphorylation and oligomerization.

To determine if phosphorylation of Serine 128 within Bad is necessary for p75NTR-induced apoptosis, PC12 cells were transfected with a dominant negative Bad serine 128 mutant allele (Konishi et al., 2002) and then infected with p75NTR or control virus. The ability of the dominant negative Bad construct to inhibit p75NTR-dependent Caspase 3 activation was assessed after twenty-four hours of virus infection by scoring transfected cells for the presence of cleaved Caspase 3. Figure 4.9 shows that expression of the dominant negative Bad serine 128 mutant allele confers significant protection from p75NTR-induced apoptosis, indicating that Bad phosphorylation is necessary for p75NTR-induced apoptosis. To confirm that Caspase 3 cleavage induced by p75NTR requires Bad, p75NTR-induced apoptosis was assessed in cells in which the endogenous level of Bad were reduced using RNA interference. The ability of the RNAi construct to reduce Bad levels was first validated in 293 cells (Figure 4.10) and then used to reduce Bad levels in PC12 cells. PC12 cells were transfected with GFP alone or with GFP together with the Bad-RNAi plasmid and, 48 hours later, were infected with either p75NTR or LacZ adenovirus for 24 hours. Figure 4.9 shows that PC12 cells transfected with Bad-RNAi are highly resistant to p75NTR-induced apoptosis, indicating a crucial role for Bad in the p75NTR apoptotic pathway.

## DISCUSSION

The mechanisms utilized by p75NTR to induce apoptosis are unique and bear little similarity to cell death signaling pathways employed by other pro-apoptotic members of the TNF receptor superfamily. In this report, we show that p75NTR-induced death correlates with cytosolic accumulation of Cytochrome c and activation of Caspase 9 and Caspase 3. Using the JNK binding domain of JIP as a dominant suppressor of JNK activity, we show that JNK is required for p75NTR-induced Caspase 3 activation. Under conditions in which p75NTR induces JNK phosphorylation and death, p75NTR does not increase mRNA levels of BH3-domain-only family members that are transcriptionally regulated by c-Jun or p53. Instead, we demonstrate that p75NTR specifically increases phosphorylation and oligomerization of Bad and show that Bad plays a crucial role in p75NTR-induced death.

Ligand binding to cell surface apoptotic receptors such as Fas and DR3 induces cell death by initiating formation of a DISC complex that facilitates FADD-dependent Caspase 8 aggregation and activation. Other death stimuli induce apoptosis primarily via Cytochrome c-dependent activation of Caspase 9 (Shi, 2002). Activation of Caspase 8 versus Caspase 9 is therefore a distinguishing regulatory event that provides insight into the precise apoptotic pathways invoked by an extracellular stimulus. We have found that in glioma cells, PC12 cells and primary cortical neurons, p75NTR-induced apoptosis is invariably accompanied by the activation of Caspase 9, Caspase 6, and Caspase 3. p75NTR-dependent Caspase 8 activation was never observed. This suggests that activation of the intrinsic death pathway is crucial for p75NTR-induced apoptosis and indicates that cytosolic mitochondrial Cytochrome c accumulation is an important regulatory step in p75NTR-induced death. These findings are in substantial agreement with other studies examining p75NTR-dependent Caspase activation and are consistent with a recent study showing that blockade of Caspase 9 activity significantly attenuates p75NTR-induced apoptosis (Gu et al., 1999; Wang et al., 2001; Troy et al., 2002). Together, these results show that p75NTR induces apoptosis through an intrinsic death pathway that results in mitochondrial Cytochrome c release and Caspase 9 activation.

The JNK signaling cascade plays a crucial role in apoptosis induced by a variety of stimuli (Kuranaga and Miura, 2002). We examined the role of JNK in p75NTR-induced apoptotic signaling by expressing a fragment of the JIP scaffolding molecule that directly binds to JNK and thus acts as a dominant JNK suppressor. This approach revealed that JNK signaling is a critical prerequisite for p75NTR-dependent Caspase activation in all cell types examined. We also report that CEP1347 reduces p75NTR-dependent death but only at high concentrations, suggesting that inhibition of p75NTR-induced death by CEP1347 likely results from blockade of a non-preferred target distinct from MLK3. Together with other recent studies (Friedman, 2000; Harrington et al., 2002), these data therefore indicate a crucial role for JNK activation in p75NTR-induced apoptosis in all cell types examined to date and raises the possibility that enzymes in the JNK pathway may provide feasible targets for inhibiting p75NTR-induced apoptosis following traumatic CNS injury.

BH3-domain-only family members inhibit the action of anti-apoptotic Bcl-2 family members such as Bcl-2 and Bcl-xL and facilitate the action of Bax and Bak at the mitochondria (Letai et al., 2002). The regulation of BH3-domain-only proteins is a key step linking proximal signaling events to the induction of cell death (Huang and Strasser, 2000). In sympathetic neurons, JNK activation results in phosphorylation of c-Jun which in turn results in transcription of the BH3-domain-only family members Bim and Hrk (Harris and Johnson, 2001; Putcha et al., 2001; Whitfield et al., 2001). In other systems, p53 activation results in transcription of Noxa and Puma, also pro-apoptotic BH3-domain-only family members (Wu and Deng, 2002). We therefore hypothesized that p75NTR-dependent apoptosis was associated with transcription of known BH3-domain-only family members. However, p75NTR does not appear to enhance transcription of BH3-domain-only family members, suggesting that alternative pathways are responsible.

BH3-domain-only family members are present in normal cells in the absence of apoptotic stimuli and must be rendered inactive to prevent apoptosis. One mechanism that accomplishes this is their sequestration through protein-protein interactions. For example, the BH3-domain-only protein Bad is bound to 14-3-3 (Zha et al., 1996; Datta et al., 2000)

and Bim and Bmf can be sequestered in the cytosol by binding to dynein light chain or myosin V (Puthalakath et al., 1999; Puthalakath et al., 2001). Significantly, recent findings have revealed that the sequestration of these three BH3-domain-only proteins can be negatively regulated by JNK. UV irradiation of HEK293 cells results in JNK-dependent phosphorylation of Bmf and Bim, releasing these proteins from their sequestration and allowing them to contribute to the apoptotic cascade (Lei and Davis, 2003). The Serine 128 phosphorylation of BAD activates BAD specifically by inhibiting the interaction of Serine 136-phosphorylated BAD with 14-3-3 proteins (Konishi et al. 2002). Serine 136 is a target of survival factor-induced kinases including Akt in neurons. That p75NTR induces cell death in part by inducing the phosphorylation of BAD at Serine 128 suggests that p75NTR promotes apoptosis by opposing survival factor signals that suppress the cell death machinery. Further, p75NTR activation results in the oligomerization of Bad through a JNK-dependent pathway. Aside from Bad itself, the components of this stable oligomeric complex remain unknown but may include anti-apoptotic proteins such as Bcl-2 and Bcl-Xl (Letai et al., 2002). These findings provide the first data linking cell surface receptor activation to the post-translational regulation of BH3-domain-only family members and indicate that p75NTR regulates apoptosis through a JNK pathway that is independent of transcription.

Palmada et al (2002) have recently found that c-Jun is not required for p75NTR-induced cell death. Consistent with this, our data show that levels of c-Jun phosphorylation induced by p75NTR are modest and do not induce transcription of c-Jun targets that include Bim and Hrk. Thus, although c-Jun phosphorylation is a useful surrogate to assess JNK activation, it does not appear to play a significant role in p75NTR-induced apoptosis. However, alternative JNK-dependent pathways may contribute to p75NTR-dependent apoptosis. One candidate pathway involves p53, which can be activated by direct JNK phosphorylation and has been implicated in p75NTR-induced apoptosis in one study (Aloyz et al., 1998). However, and p75NTR readily induces apoptosis in cells lacking functional p53 (eg. U373 cells, Figure 4.1) and phosphospecific antibodies directed against Thr 81, a JNK target residue in p53 (Buschmann et al., 2001), or against Ser15 or Ser20 (Dumaz et al., 2001) did not reveal significant p75NTR-dependent

phosphorylation of p53 (data not shown). Nonetheless, we cannot rule out the possibility that p53 or related family members may play a role in p75NTR-induced apoptosis in specific circumstances. Further examination of transcriptional pathways in p75NTR action is warranted.

p75NTR plays a prominent role in nervous system apoptosis, particularly following trauma, and a detailed picture of the pro-apoptotic signal transduction mechanisms activated by the receptor is required. In this study, we show that p75NTR-dependent JNK activation is invariably required for Caspase activation and find that p75NTR-dependent JNK activation induces phosphorylation and activation of the BH3-domain-only protein Bad and that Bad is required for p75NTR-induced apoptosis.

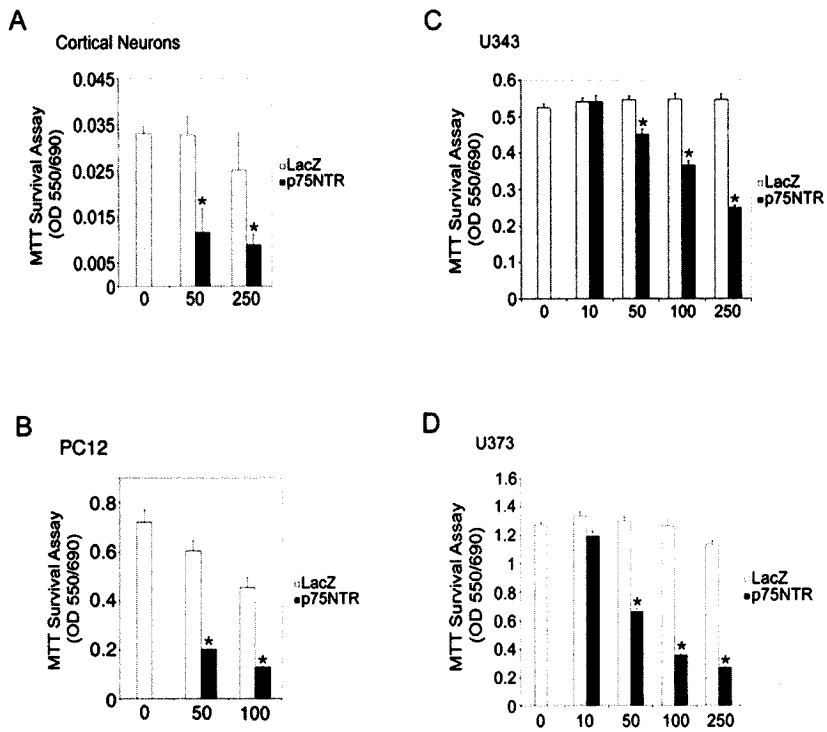



**Figure 4.1.** Overexpression of p75NTR induces cell death in a variety of cell types. (A) cortical neurons, (B) PC12, (C) U343 (wild type p53), and (D) U373 (mutant p53) cells were infected with increasing multiplicities of infection (MOI) of LacZ or p75NTR recombinant adenovirus and then analyzed for survival by the MTT assay (see Materials and Methods). Error bars indicate SD. Results were analyzed for statistical significance by ANOVA (Tukey HSD multiple comparison). Statistically significant differences of $p<0.001$ are indicated by an asterisk.

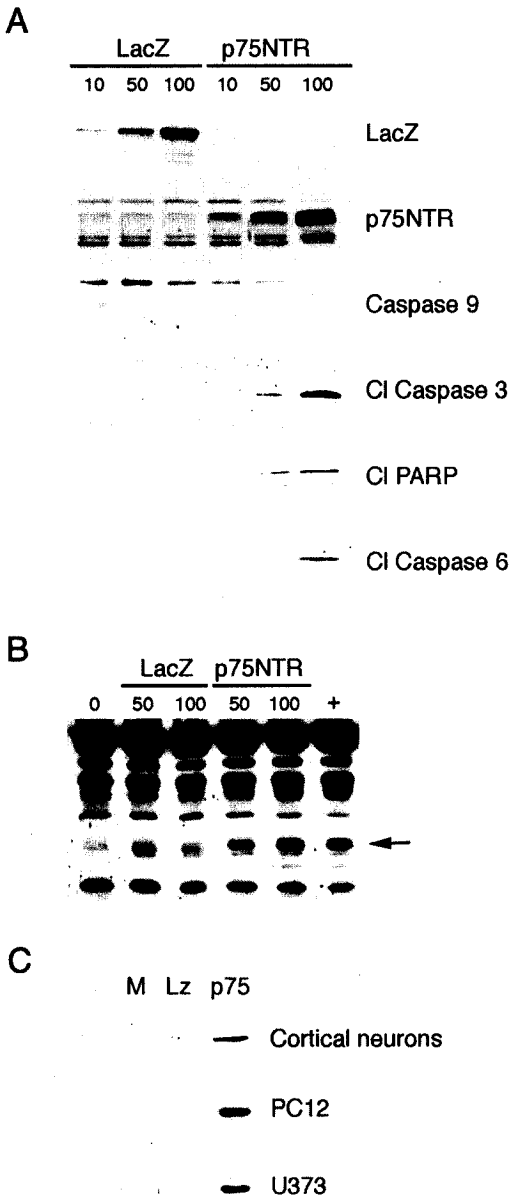
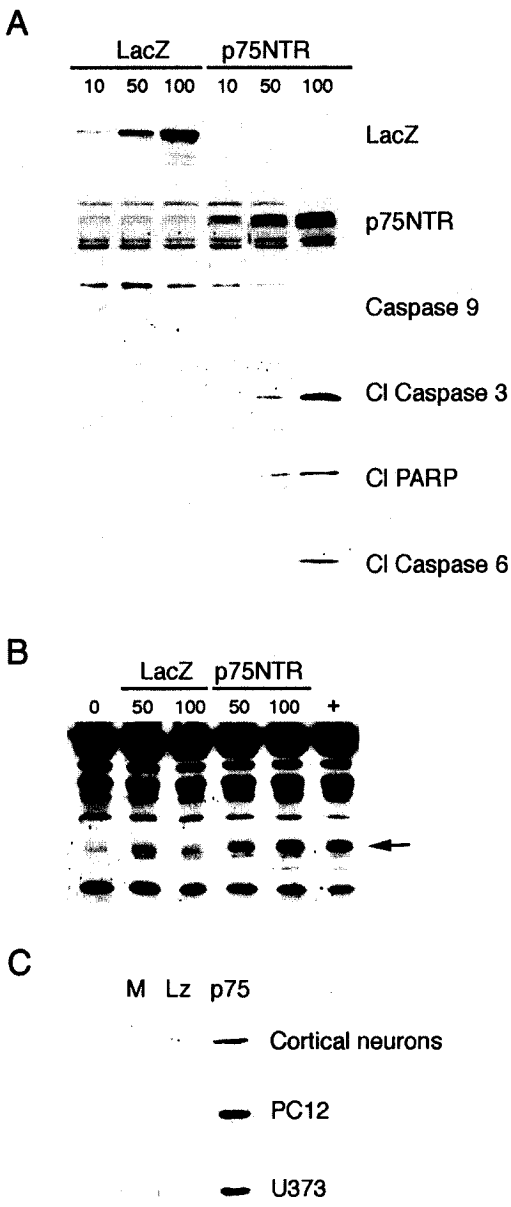



**Figure 4.2.** p75NTR activates Caspases and induces accumulation of cytosolic Cytochrome C. (A) Cortical neurons infected with 10, 50, or 100 MOI of LacZ or p75NTR recombinant adenovirus were lysed and analyzed by imm unoblot for levels of LacZ, p75NTR and full-length Caspase 9 protein or, using cleavage-specific antibodies, for levels of cleaved Caspases 3 and 6 and cleaved Poly(ADP-ribose) polymerase (PARP). (B) U373 cells were infected with 50 or 100 MOI of either LacZ or p75NTR adenovirus for 48 hours, or treated with etoposide 50 uM (+), and then lysed and analyzed for increases in cleaved Caspase 9. (C) E15 cortical neurons, U373, and PC12 cells were left uninfected (0) or were infected with 100 MOI of LacZ (Lz), or p75NTR (p75) recombinant adenovirus. 30 hours later cells were fractionated for cytosolic components as described in "Materials and Methods". Cytosolic fractions normalized for protein content were analyzed by immunoblotting with an antibody directed against Cytochrome C.

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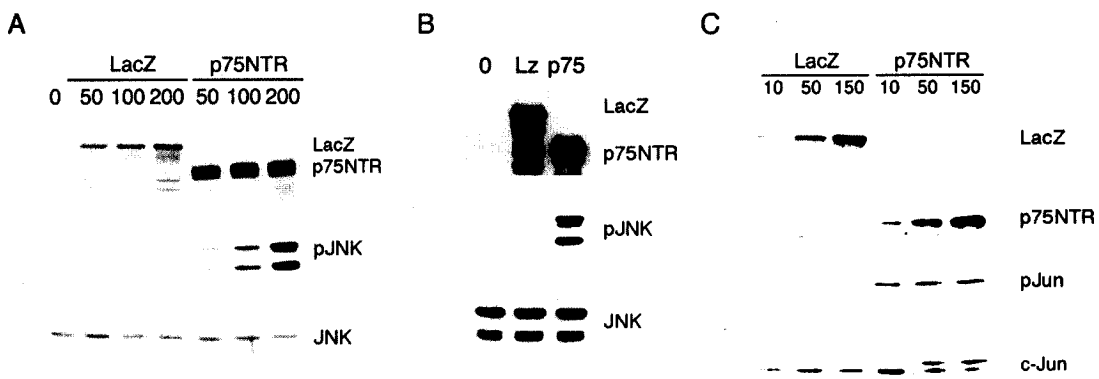


**Figure 4.3.** p75NTR activates the JNK pathway. (A) U373 cells were infected with 0, 50, 100, or 200 MOI of control AdLacZ or with Adp75NTR, (B) PC12 cells were injected with 0 or 50 MOI of AdLacZ or Adp75NTR, and (C) cortical neurons were infected with 10, 50, or 150 MOI of AdLacZ or Adp75NTR. Lysates were prepared 30-48 hours after infection and examined by immunoblot for LacZ, p75NTR, phosphorylated JNK (pJNK), total JNK, phosphorylated c-Jun (pJun) and total c-Jun as indicated.

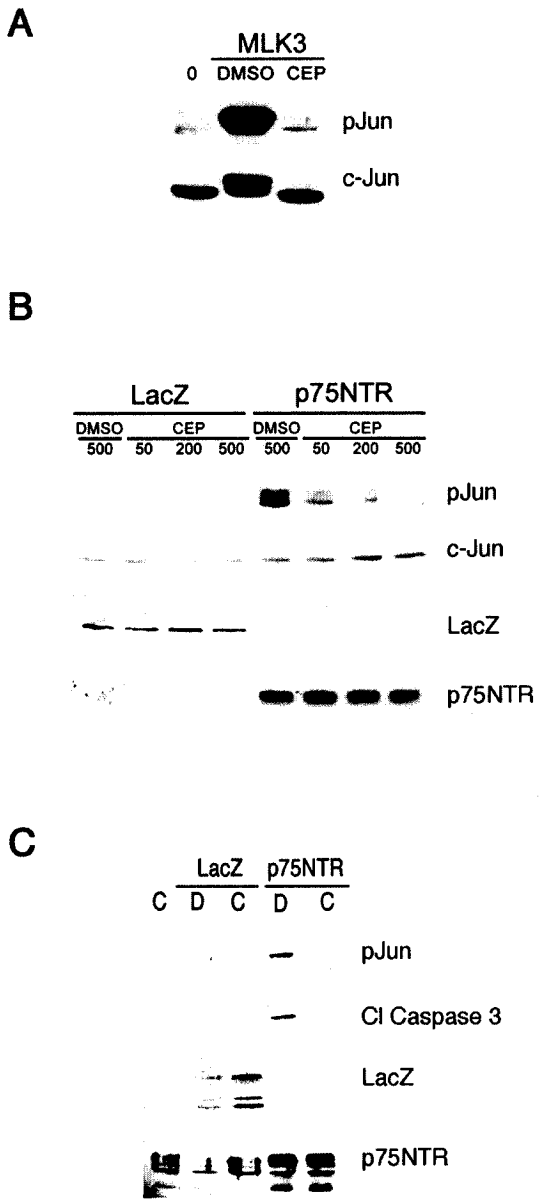


**Figure 4.4.** Inhibition of MAP3K signaling attenuates apoptosis induced by p75NTR. (A) U373 cells infected with 100 MOI of MLK3 adenovirus or left uninfected (0), were treated 47 hours later with DMSO or CEP1347 at 200 nM for 1 hour. Cells were harvested and lysates subjected to immunoblot analysis for phospho-Ser63 c-Jun (pJun) and t otal c-Jun protein. (B) Cortical neurons infected with 50 MOI of LacZ or p75NTR adenovirus were treated with DMSO or 50, 200, or 500 nM CEP1347 for 1 hour as in (A). Lysates were analyzed by immunoblot as indicated (pJun, c-Jun, LacZ, p75NTR). (C) AdLacZ or Adp75NTR-infected cortical neurons were treated with 500 nM of CEP1347 [C] or DMSO [D] at the time of infection and lysates were prepared 48 hours later and analyzed by immunoblot for levels of p75NTR, LacZ, phospho-Ser63 c-Jun (pJun), and c leaved Caspase 3 (cl. Caspase 3).

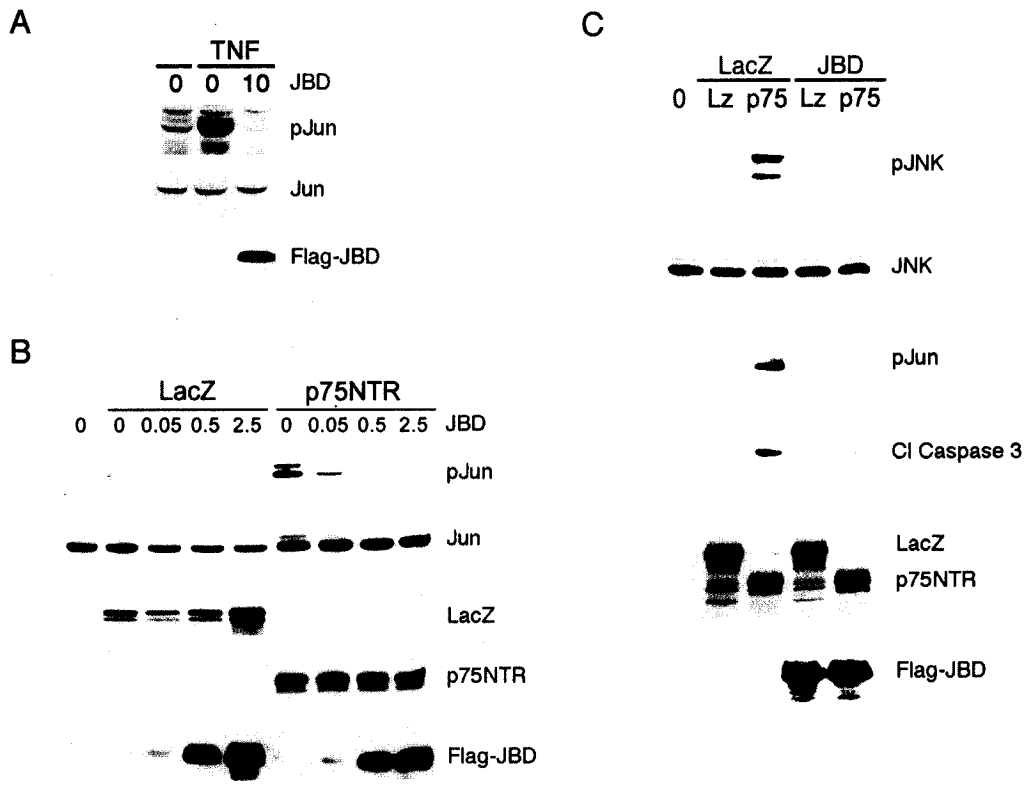


**Figure 4.5.** Activation of the JNK pathway is required for p75NTR-mediated Caspase activation. Immunoblots for phospho-Ser63 c-Jun (pJun), c-Jun, Flag-JIP, LacZ, p75NTR, phospho-Thr183/Tyr185-JNK (pJNK), JNK, and cleaved Caspase 3 were performed as indicated on lysates from (A) U373 cells treated with TNF 20ng/ml that were either left uninfected (0) or infected with JBD-JIP adenovirus (JBD) at 10 MOI, (B) cortical neurons infected with 50 MOI of LacZ or p75NTR adenovirus together with increasing amounts (0, 0.05, 0.5, 2.5 MOI) of JBD-JIP adenovirus, and (C) PC12 cells infected with 50 MOI of LacZ or p75NTR adenovirus supplemented with LacZ or JBD-JIP (JBD) adenovirus (both at 5 MOI).

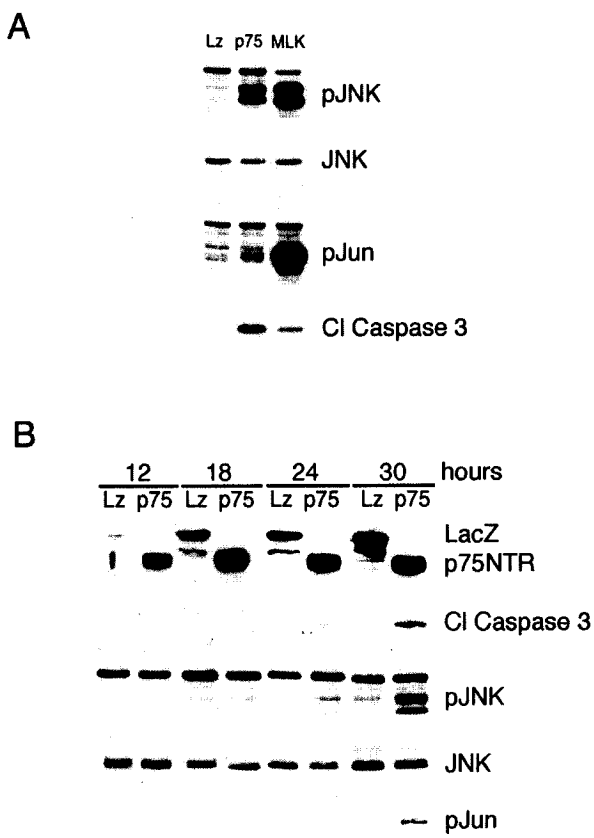


**Figure 4.6.** p75NTR-induced Caspase 3 cleavage does not correlate with phosphorylation of c-Jun. PC12 cells were infected with 50 MOI of AdLacZ (Lz), Adp75NTR (p75), or AdMLK3 (MLK) recombinant adenovirus and lysates were prepared at 30 hours post-infection (A and C), or at 12, 18, 24, and 30 hours post-infection (B). Lysates normalized for protein content were analyzed for LacZ, p75NTR, cleaved Caspase 3, phospho-Thr183/Tyr185-JNK (pJNK), total JNK, phospho-Ser63 c-Jun (pJun) and total c-Jun protein levels by immunoblot as indicated.

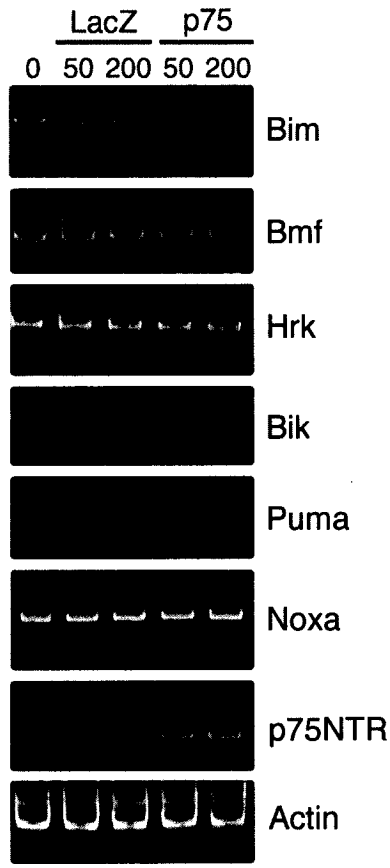


**Figure 4.7.** p75NTR does not transcriptionally regulate BH3-domain-only proteins. Cortical neurons were infected with 0, 50, or 200 MOI of LacZ or p75NTR (p75) adenovirus and 24 hours later mRNA was isolated as described in Materials and Methods. RT-PCR was performed using primers directed against Bim, Bmf, Hrk, Bik, Puma, Noxa, p75NTR and Actin as indicated.

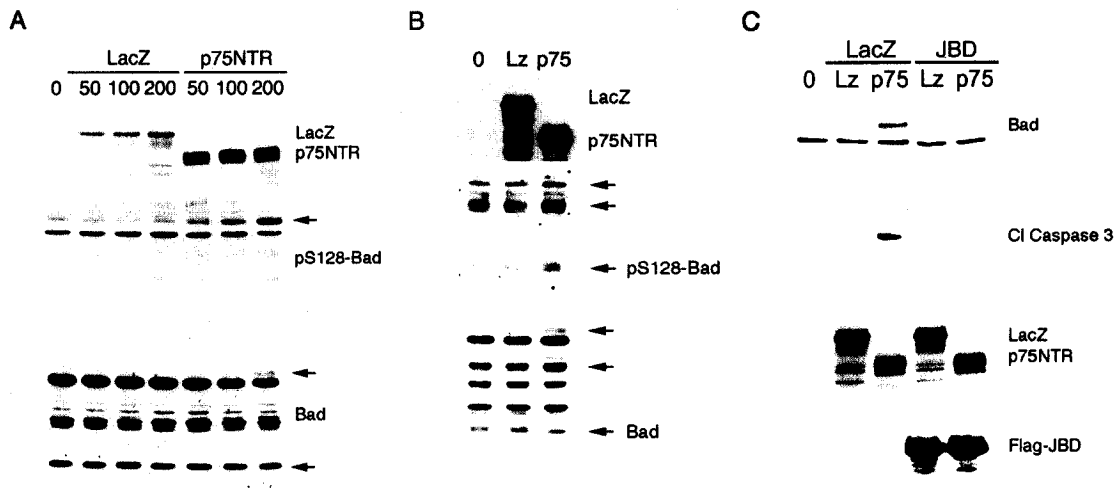


**Figure 4.8.** p75NTR activates JNK-dependent phosphorylation and oligomerization of Bad. (A) U373 cells were infected with 0, 50, 100, or 200 MOI of LacZ or p75NTR adenovirus and lysates were analyzed by immunoblot for LacZ, p75NTR, phospho-Ser128 Bad, and Bad (C-20 – shown; N19 – data not shown). (B) PC12 cells were left uninfected (0) or were infected with LacZ (Lz) or p75NTR (p75) adenovirus aqt 100 MOI and lysates were analyzed by immunoblot for LacZ, p75NTR, phospho-Ser128 Bad, and Bad (C-20). (C) PC12 cells were infected with nothing (0), LacZ (Lz), or p75NTR (p75) adenovirus together with either 5 MOI of LacZ or JBD-JIP (JBD) adenovirus. Lysates were compared for expression of Bad, cleaved Caspase 3, LacZ, p75NTR, and Flag-JIP (Flag) by immunoblot as indicated.

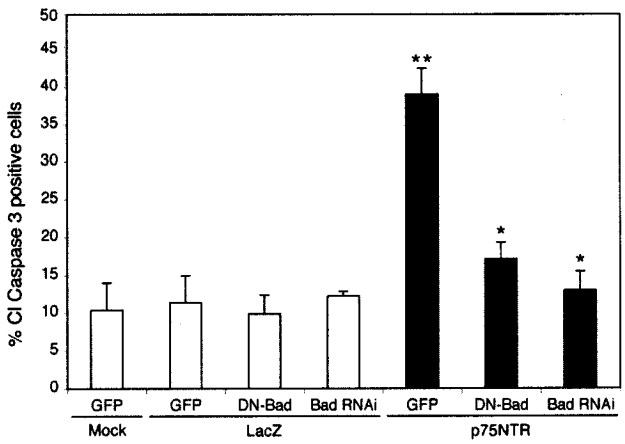


**Figure 4.9.** Bad is required for p75NTR-induced apoptosis. PC12 cells were transfected with GFP plasmid alone or w ith GFP plasmid together with plasmids encoding DN-Bad (S128A) or expressing Bad-RNAi. Cells were infected 48 hours later with LacZ or p75NTR adenovirus and, at 24 hours post-infection, were fixed and immunostained for cleaved Caspase 3 as des cribed in Materials and Methods. Transfected cells were scored for Caspase 3 cleavage by a blind observer (n =300 cells/condition). '**' indicates a difference of $p<0.001$ between GFP/Mock (Bar 1) and GFP/p75NTR (Bar 5) and '*' indicates a difference of $p<0.001$ between GFP/p75NTR (Bar 5) and both DN-Bad/p75NTR (Bar 6) and with Bad RNAi/p75NTR (Bar 7), indicated by ANOVA.

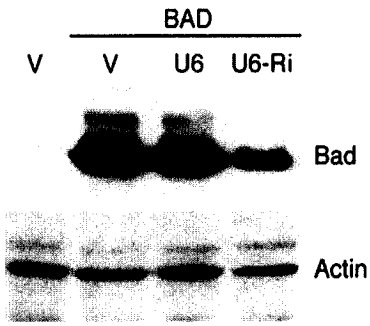


**Figure 4.10.** Va lidation of Bad RNA Interference vector. Bad RNAi was validated by transfection of 293 cells with a Bad expression plasmid in the absence or presence of the U6-driven Bad RNAi plasmid, followed by lysis and analysis by immunoblot. Upper panel was analyzed with an anti-Bad antibody (N-20) and lower panel with an antibody directed against actin. M= Mock transfection, V= pcDNA3, U6= pcDNA+U6 promoter, U6-R1i= pcDNA3+U6 promoter driving Bad RNAi.

# CHAPTER 5

# GENERAL DISCUSSION AND CONCLUSIONS

# 5.0 GENERAL CONCLUSION

Apoptosis, a genetically regulated cell death program, is essential to the normal functioning of the nervous system, particularly during development and in neurodegenerative disease. In recent years, multiple factors associated with the execution of apoptosis, such as Caspases and Bcl-2 family members, have been discovered and their signaling and molecular interactions have been demonstrated (reviewed in Shi, 2002). In the nervous system, however, the precise mechanistic basis for regulating these apoptotic factors has only recently begun to emerge. One example of key regulators include the neurotrophins which tranduce pro- and anti-apoptotic signals through interactions with the Trk and p75NTR receptors.

By investigating and reporting findings related to p75NTR-mediated signaling events, this thesis has furthered our understanding of the mechanisms regulating nervous system apoptosis.

## 5.1 MAJOR FINDINGS

1. We have demonstrated that p75NTR directly activates NF-kB only under conditions of cellular stress. However, we have also found that under normal growth conditions, p75NTR modulates TNF-dependent NF-kB activation to ultimately increase the levels of active NF-kB within the cell (Bhakar et al., 1999, Chapter 2). Indeed, these data were among the first to demonstrate that p75NTR cooperates with receptors with which it shares functional and structural homologies, such as members of the TNFR superfamily. These data also suggest that previous reports of p75NTR-mediated NF-kB activation are analogous to the stress-paradigms used in our studies and hence that p75NTR may function as an effective sensor for cellular stress.

2. We have created transgenic NF-kB reporter mice sensitive to neuronal NF-kB activity (Chapter 3; Bhakar et al., 2002). We have clearly identified constitutive NF-kB activity within neurons of the developing and mature CNS. We have also demonstrated that blocking endogenous neuronal NF-kB activity in cortical neurons results in dramatic reductions in neuronal viability, whereas inducing NF-kB activity increases levels of anti-apoptotic proteins and is strongly neuroprotective. Together, these findings demonstrate a physiological role for NF-kB in maintaining the survival of central neurons. In addition, we have recently identified TRAF6 as an important regulator of the constitutive NF-kB activity within all non-neuronal tissues detected in our reporter mice (see below). This finding indicates that these mice will also be useful for the study of TRAF/NF-kB functions outside the nervous system, throughout development, and during disease.

3. We have demonstrated that p75NTR-induces apoptosis in cortical neurons and several cell lines through an intrinsic death pathway (Chapter 4; Bhakar, manuscript in preparation). These effects are accompanied by 1) JNK activation, 2) mitochondrial release of Cytochrome c, 3) activation of Caspases 9, 3 and 6,

and 4) the phosphorylation of several JNK targets, including c-Jun, ATF-2 (see below), and Bad. We have also shown that JNK activation is invariably required for p75NTR-dependent Caspase activation, but that JNK activation does not increase the transcription of BH3-domain-only proteins. Instead, we have demonstrated that JNK activity results in the phosphorylation and oligomerization of the BH3-only member Bad. In addition, we have demonstrated, that Bad is required for p75NTR-dependent apoptosis. While these findings provide the first link between cell surface receptor activation and the pro-apoptotic phosphorylation of Bad, these data also show how p75NTR-dependent JNK activation can contribute to apoptosis.

## 5.2 P75NTR AND STRESS

### 5.2.1 p75NTR as a stress and modulatory cytokine receptor

NGF binding to p75NTR was initially reported to activate NF-kB in primary Schwann cells and PCNA cells (Carter et al., 1996). p75NTR-dependent NF-kB activation has since been reported 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; Ye et al., 1999; Gentry et al., 2000; Hughes et al., 2001; Cosgaya et al., 2001; Bui et al., 2001, 2002; Khursigara et al., 1999; Foehr et al., 2000; Wooten et al., 2001; Mamidipudi et al., 2002; Burker et al., 2003). In Chapter 2 we show that p75NTR does not directly activate NF-kB but instead, increases NF-kB activation initiated by conditions of severe stress or treatment with TNF. This result is surprising given that the p75NTR homolog, TNFR1, consistently induces robust NF-kB responses within the same cell types. However, a stress or cytokine requirement for p75NTR to modify NF-kB activation is consistent with previous and more recent reports demonstrating that NGF can induce NF-kB activation only after a temperature stress or serum-free conditions within Schwann cells (Carter et al., 1996, Khursigara et al., 1999), oligodendrocytes (Ladiwala et al., 1998; Yoon et al., 1998), P19 cells (Burke et al., 2003), and PCNA cells (Cosgaya et al., 2001; Carter et al., 1996). Furthermore, NGF-dependent NF-kB increases have been modest and sometimes are detectable only within the first few days of culture (Khursigara et al., 1999) or when cells are dying (Ladiwala et al., 1998; Yoon et al., 1998). Therefore, all these findings suggest that p75NTR does not directly activate NF-kB under normal growth conditions. Instead, these findings suggest that p75NTR modulates cytokine receptor signaling and more generally, functions to respond to cell stress.

In fact, p75NTR may function as an efficient sensor to stress since many of the pathways activated by p75NTR can be characterized as stress response signals (for review see Dobrowsky and Carter, 2000). p75NTR can regulate the NF-kB and JNK pathways, for example, and NF-kB is often activated following stressful changes including serum-starvation, viral toxicity, and DNA damage (reviewed in Wang et al., 2002b). Similarly, JNK, originally termed SAPK (stress-activated protein kinase), was identified as a kinase responsive to osmotic stress and UV damage. Indeed, NF-kB and JNK activation are

responses to stress that typically link environmental changes to the regulation of apoptotic pathways (reviewed in Davis, 2000). Moreover, p75NTR activation can also lead to increases in the production of the sphingolipid metabolite, ceramide, which is a third signal often characterized as a stress response (Dobrowsky et al., 1994, 1995; Blochl et al., 1996; Culmsee et al., 2002; DeFreitas et al., 2001; Brann et al., 2002; Brann et al., 1999).

Interestingly, we have recently found that the expression of p75NTR interacting protein, TRAF4, can lead to the retention of p75NTR in the endoplasmic reticulum (ER) (Vaillantcourt, manuscript in preparation). It is tempting to speculate that since the retention of proteins within the ER is one form of cellular stress (Ferrri and Kroemer, 2001), the retention of p75NTR in the ER may constitute an additional stress signal. This may be significant physiologically since a) the bulk of p75NTR is in the ER (Barker, unpublished results), b) the ER is a major site of signal integration for sensing damage (reviewed in Ferri and Kroemer, 2001), and c) homologs of p75NTR interacting proteins such as MAGE-A3 can bind to and regulate Caspases found within the ER (Morishima et al., 2002).

p75NTR signaling may also respond to different types of cell stresses by providing a cell with options. In cells subjected to transient stress, for example, p75NTR responds with signals to survive. In cells undergoing prolonged stress, on the verge of dying, p75NTR signals manifest in apoptosis. This hypothesis is supported by recent work demonstrating that the physiological responses to signals activated by p75NTR differ with the length of time in culture (DeFreitas et al., 2001; Brann et al., 99; Brann et al., 2002). p75NTR activation and ceramide generation regulate neurite formation and outgrowth of hippocampal neurons at early stages of culture. In contrast, as the cultures mature, p75 expression levels and ceramide generation result in apoptotic effects. Similarly, p75NTR activation in Schwannoma cells gives rise to increases in NF-kB activity in newly plated cultures. However, p75NTR activation in older cultures results in increases in ATF-2 activity (Khursigara et al., 2001).

### 5.2.2 Mechanisms of p75NTR modulatory/sensor functions

The mechanisms by which cellular stress may increase p75NTR responsiveness to NF-kB (Chapter 2) are unknown. However, one possibility may include the increased production of TNF or cytosolic signaling elements. In this scenario, NGF acting through p75NTR is not a primary inducer of the pathway but rather a synergizing component of a stress-induced signal to increase NF-kB activity in an autocrine manner. Supporting this interpretation of our data, other researchers have found that NGF treatment of p75NTR-expressing cells can increase TNF production and release (Barouch et al., 2001). Similarly, cytokines, including LIF and TNF, or the inflammatory Ab peptide, have been shown to use and sometimes require, p75NTR for amplification of cellular responses (Savitz et al., 2000; MacEwan et al., 1995; Kuner et al., 1998; Perini et al., 2002). Indeed, many cytokines are produced in the CNS after injury or disease, when p75NTR production tends to be dramatically increased, and this response may represent a physiologically relevant stage for stress-induced p75NTR function (Dowling et al., 1999; Beattie et al., 2002; Stoll et al., 2002). Even in antigen provoked models of asthma, necessary cytokine and inflammatory responses are prevented when p75NTR is absent (Tokuoka et al., 2001).

Crosstalk between p75NTR and cytokine or stress receptors may also occur at other levels. Both TNFR1 and p75NTR bind and recruit TRADD and TRAF proteins (see introduction; reviewed in Baker and Reddy, 1996), suggesting that concentration of interacting proteins at the receptor's ICD could increase TNF responsiveness. Alternatively, p75NTR may activate signaling pathways that converge on NF-kB components used by TNF. For example, p75NTR activates the Akt pathway, and Akt could synergize with TNF-mediated NF-kB signals by directly phosphorylating IKK (Kane et al., 1999; Ozes et al., 1999; Romashkova et al., 1999). Supporting this model, p75NTR has previously been shown to enhance TNFR-initiated cellular responses implicated in NF-kB signaling. These responses include activation of cPLA2 (cytosolic phospholipase A2) and generation of oxygen radicals (MacEwan, 1996). In fact, given the expanding number of receptors that can interact with p75NTR (Trks, NgR, ARMS, PLAIDD, and GT1b) (Gargano et al., 1997; Bibel et al., 1999; Salehi et al., 2000; Wang

et al., 2002; Wong et al., 2002; Yamashita et al., 2000; Frankowski et al., 2002) and the number of p75NTR interactors that can be shared by other receptors (TRADD, TRAFs, MAGE proteins) (see introduction; Baker and Reddy, 1996; Williams et al., 2003), it is likely that p75NTR cooperates with several receptor types by recruiting common effectors and activating convergent signaling pathways.

## 5.3 NF-KB AND NEURONAL SURVIVAL

### 5.3.1 Detection of neuronal NF-kB activity

In Chapter 3, we discussed generating a transgenic NF-kB reporter mouse to clarify the role and pattern of transcriptionally active NF-kB within the nervous system. We pursued this line of research because previous methods for measuring NF-kB activity were complicated and provided weak, unreliable results. For example, gelshift assays, using identical kB elements as those found within our mice, detect only modest NF-kB activation. This modest detection is likely due to the difficulties in using standard high salt nuclear extraction procedures on neuronal cultures with high membrane content. In fact, to facilitate visualization, some researchers add detergents like DOC or SDS to the extraction procedure (Bakalkin et al., 1993; Kaltschmidt et. al 1993, 1994; Rattner et al., 1993; Jarosinski et al., 2001).

As we have shown in Chapter 3, our transgenic mouse data demonstrates high levels of constitutive NF-kB activity within specific neurons of the developing and mature CNS. We have been able to suppress this activity with the IkBαM repressor or with a dominant negative NIK construct, and we have been able to increase this activity by overexpressing RelA. These results demonstrate that our NF-kB reporter mice measure bonafide NF-kB activity within neurons, and therefore provide a more accurate and robust means to measure neuronal NF-kB activity than previously used in vitro binding assays.

### 5.3.2 The restricted pattern of neuronal NF-kB activity

In Chapter 3, we found high levels of constitutive NF-kB activity specifically associated with neurons in forebrain areas throughout mouse development (Chapter 3). We have also shown that this neuronal NF-kB activity is necessary for the survival of cortical neurons. However, we have found that other brain regions do not demonstrate detectable constitutive NF-kB activity within our transgenic mice. Therefore, it remains to be determined what distinguishes neurons within the frontal cortex from neurons within other brain areas.

Specific and differential activation of NF-kB has been previously reported between several neuronal populations and between different nervous system cell types (Bakalkin et al., 1993; Kaltschmidt et al., 1993, 1994; Rattner et al., 1993). Different degrees of NF-kB activation between distinct neuronal populations and different nervous system cell types suggests the rather pedantic interpretation that forebrain neurons require NF-kB activity for cell-type specific regulation.

Alternatively, the distinct NF-kB activity pattern in our transgenic mice (Chapter 3) could reflect the specificity and limitations of our reporter construct such that other active NF-kB dimers in the brain might not be detected without alternate kB DNA-binding sites. We chose an NF-kB enhancer element sensitive to neuronal NF-kB activity to generate our reporter construct (Corboy et al., 1992; Buzy et al., 1995; Rattner et al., 1993). There are, however, several variants of kB elements, several functional NF-kB dimer combinations, and a variety of environmental cues that regulate NF-kB activity (reviewed in Baldwin, 1996). It is likely, therefore, that our NF-kB element only effectively measures a subset of endogenous NF-kB activities, and thus NF-kB activities in other brain areas may be present and necessary but not detectable. Supporting this interpretation, two other NF-kB reporter mice have been generated using different kB DNA binding elements and both mice show little NF-kB activation throughout the brain (Lernbecher et al., 1993; Schmidt-Ullrich et al., 1996).

### 5.3.3 Regulation of constitutive neuronal NF-kB activity

The physiological signals that regulate constitutive neuronal NF-kB activity are poorly understood. As discussed below, these signals include the extracellular regulators and the intracellular signal transducing mechanisms.

#### 5.3.3.1 Extracellular regulators of neuronal NF-kB activity

Although Chapter 3 demonstrates that NF-kB is active and necessary for cortical neuron survival, it is not clear what signals regulate this constitutive neuronal NF-kB activity. Standard NF-kB activators in non-neuronal cells are typically ineffective at altering NF-kB activity in neurons. However, recent work suggests potential neuron-specific NF-kB

stimuli. Glutamate and membrane depolarization, for example, induce NF-kB activation in hippocampal pyramidal neurons, cortical neurons, and cerebellar granule neurons in cell culture (Guerrini et al., 1995; Kaltschmidt et al., 1995; Burr et al., 2002). Cytokines, neurotrophic factors (see below), neurotransmitters, and oxidative stress can also increase neuronal NF-kB activity, although the degree and location of thses effects have varied between reports (for review see Mattson and Camandola, 2001). These differences are particularly evident with regards to TNF stimulation. TNF stimulation was first reported to increase NF-kB activation in neurons (Barger et al., 1995; Furukawa et al., 1998) but is now believed to increase NF-kB activity only within CNS glia (Digicaylioglu and Lipton, 2001; Bhakar, unpublished results). Future genetic studies will be needed to confirm the relevance of these stimuli in the regulation of constitutive neuronal NF-kB activity.

To address the relevance of neurotrophins as neuronal NF-kB regulators, we have treated neurons and PC12 cells with neurotrophins or p75NTR-specific antibodies and have not found neurotrophin or p75NTR specific inductions in NF-kB activity (Bhakar, unpublished results). Additionally, overexpression of p75NTR in neurons using our recombinant p75NTR adenovirus or a p75NTR plasmid containing the Ta1-a-tubulin promoter does not induce NF-kB activation, even when TNF is added (Bhakar, unpublished results; Majdan et al., 1997). Our only success has been within NGF-treated TNFR1-containing primary oligodendrocytes (Ladiwala et al., 1998), where cultures were stressed due to conditions of serum-starvation. This result is consistent with our findings in Chapter 2 demonstrating that stress or cytokine treatment is required prior to NGF-mediated NF-kB activation. Accordingly, more recent studies demonstrate NGF- and cytokine-mediated NF-kB inductions in cultured peripheral neurons only following serum-deprivation (Hamanoue et al., 1999; Maggirwar et al., 1998; Taglialatela et al., 1997; Middleton et al., 2000). Together, these data suggest that, similar to non-neuronal cell types (Chapter 2), p75NTR activation is not required for direct regulation of constitutive NF-kB activation within the CNS. Instead, p75NTR cooperates with cytokine-initiated signals to enhance neuronal NF-kB activity.

### 5.3.3.2 Intracellular mechanisms of NF-kB activation in the CNS

The extracellular signals regulating constitutive neuronal NF-kB activity are elusive, and the necessary intracellular regulators unclear. In cultured cortical neurons, Burr and colleagues (2002) suggest that basal (constitutive) levels of activated NF-kB, which are maintained by synaptic activity and involve N-methyl-D-aspartate (NMDA) and AMPA/kainate glutamate receptors, are coupled to activation of a Src-family tyrosine kinase and a Ras-like GTPase in a cGMP-dependent manner (Burr et al., 2002). Since stimulating glutamate receptors can increase intracellular Ca2+ concentrations, Ca2+ has also been implicated in regulating NF-kB activity in neurons. Israel and colleagues (Lilienbaum et al., 2003) suggest that Ca2+ regulation of basal NF-kB activity within cerebellar granule neurons occurs through 1) the direct opening of L-type voltage-sensitive Ca2+ channels at the plasma membrane and 2) through the indirect opening of In3P receptors associated with intracellular stores of Ca2+. Subsequent steps in signal transduction are thought to involve the major cellular sensors of Ca2+ levels. These sensors include the Ca2+ and calmodulin-dependent phosphatase calcineurin, PKC family members, and the Ras/PI3-K/Akt kinase pathway. Interestingly, the combined inhibition of PI3-K and PKC, using chemical inhibitors, reduces NF-kB activity significantly more than the inhibition of one pathway alone. This data suggests that constitutive NF-kB activity requires a complex interplay of signals from several pathways.

Some of these pathways may also include or converge upon more classical NF-kB pathways that are used in non-neuronal cells. The major regulators of NF-kB activation in non-neuronal cells are expressed in the nervous system (reviewed in Mattson and Camandola, 2001; Grilli and Memo, 1999b). This includes TRAF proteins, IKK1, IKK2, IkB inhibitors, and the most common NF-kB subunits p65, p50 and c-Rel (Li et al., 2000; Lomaga et al., 2000; Reegnier et al., 2002). In fact, the recent deletion of some of these regulators suggests their requirement in nervous system development (see below, section 5.3.4). Additionally, several groups have demonstrated the activation of canonical NF-kB pathways within cultured peripheral neurons or within neuronal cell lines. For example, some groups report NF-kB activation following the recruitment of membrane proximal proteins including the TRAFs, TRADD, IRAK, MyD88, p62, aPKC, IKK2, and IKK1

(Khursigara et al., 1999; Wooten et al., 2000; Mamidipudi et al., 2002). Similarly, NF-kB activation within neurons has been reported subsequent to NIK activation, phosphorylation of IKK, and phosphorylation of IkBα (Foehr et al., 2000a, b). Recent work using erythropoietin, an inducer of red blood cell production, or NGF-treated PC12 cells and hippocampal neurons has also shown that activating neuronal NF-kB can require a novel Jak/STAT-dependent pathway and an unusual tyrosine phosphorylation of IkBα (Digicaylioglu and Lipton, 2001, Bui et al., 2002).

In Chapter 3, we used two classical pathway inhibitors, an IkBαM repressor and a dominant negative form of NIK (dn-NIK), to address if classical pathways contribute to the regulation of constitutive neuronal NF-kB activity. We found that expression of either the IkBαM or the dn-NIK inhibitor significantly decreased constitutive NF-kB activity within cortical neurons and consequently, cortical neuron viability. The IkBαM repressor constitutively retains NF-kB dimers within the cytosol to prevent activation of NF-kB. The effectiveness of this repressor, therefore, suggests that the activity detected by our reporter construct reflects bonafide NF-kB activity. However, this result provides little information regarding the intracellular signaling pathways involved upstream.

The dn-NIK inhibitor, on the other hand, is a truncated, kinase-dead mutant of NIK that binds both TRAF and IKK proteins (Russo et al., 2002; Yin et al., 2001). When dn-NIK is expressed in neurons, neuronal NF-kB activity is reduced and this result suggests that NIK or targets of NIK might be key regulators. Supporting this interpretation, NIK expression has previously been shown to protect PC12 cells from serum-withdrawal induced apoptosis through NF-kB anti-apoptotic activity (Foehr et al., 2000).

The mechanisms by which NIK may regulate constitutive neuronal NF-kB activity are unknown. In non-neuronal cells, NIK activity is largely responsible for initiating non-canonical NF-kB activation through IKK1-activated p100 processing (Deng et al., 2000; Wang et al., 2001; Yang et al., 2001). Therefore, to further our understanding of the mechanisms regulating constitutive neuronal NF-kB activity, we examined the regulation of p100 processing by NIK within central neurons. In the presence of our dn-NIK mutant,

however, no changes in p100 processing are detected (Bhakar, unpublished observations). This result suggests that either central neurons use alternative NIK targets to activate NF-kB or NIK is not directly involved and our dn-NIK protein titrates out relevant activators of NF-kB. Ultimately, crosses of our NF-kB reporter mice to animals lacking various NF-kB signaling components will be needed to confirm the necessary regulators of constitutive NF-kB activity in central neurons.

We have recently crossed our NF-kB reporter mice to mice lacking one potential regulator, TRAF6. Interestingly, TRAF6 deletion completely attenuates the constitutive NF-kB activity within all detected non-neuronal tissues but does not affect the neuronal NF-kB activity within our NF-kB reporter mice (Figure 5.1) (K.Dickson, manucript in preparation). TRAF6-deficient mice display defects in bone remodeling and osteopetrosis along with defective development of epidermal appendices and glands, problems in tooth eruption and lymph node organogenesis, and brain exencephaly (Lomaga et al., 1999, 2000; Naito et al., 1999, 2002). Most of these tissues correspond to areas displaying strong NF-kB staining in our mice. TRAF4 deletion also results in defects corresponding to areas with significant constitutive NF-kB staining in our mice. These areas include the neural tube, the respiratory tract and various skeletal formations (Regnier et al., 2002). Current work in our lab is addressing the issue of whether TRAF4 alone or in combination with TRAF6 regulates the constitutive NF-kB activity within the CNS.

### 5.3.4 NF-kB functions in the CNS

The functions of NF-kB outside of the nervous system are well understood. In general, NF-kB is critical for inflammation and immune responses, morphogenesis of skin and bone, and protecting cells from apoptotic stimuli. In the nervous system, however, NF-kB functions have been controversial.

So far, research has suggested three main, seemingly contradictory, functions for NF-kB in the CNS. First, NF-kB can facilitate neuronal survival. Second, NF-kB can, in some instances, induce neuronal apoptosis. Third, NF-kB has been implicated in the regulation of neuronal growth and plasticity.

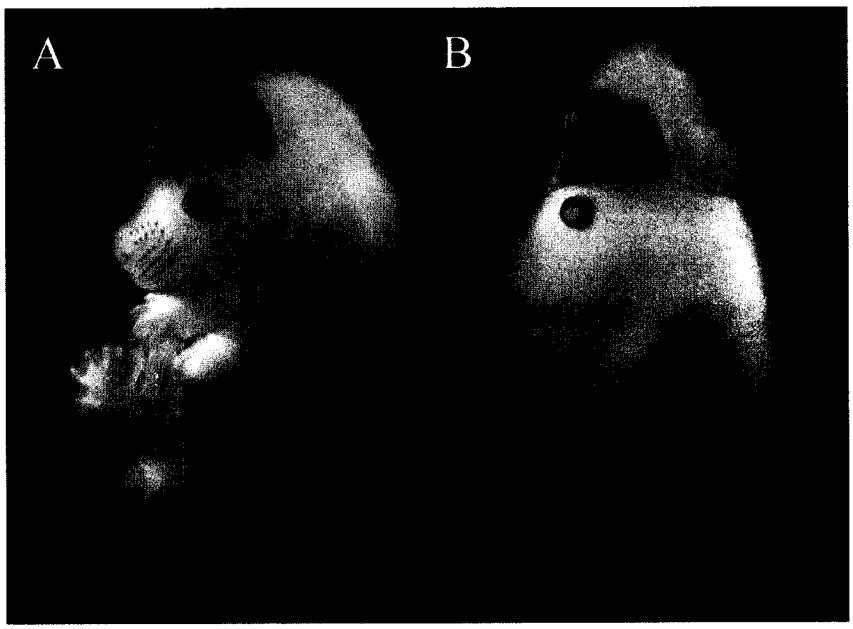


**Figure 5.1.** TRAF 6 is not required for constitutive NF-kB activity in the brain. Whole-mount X-gal staining of embryonic day13 wild-type (A) and TRAF 6-deficient (B) transgenic NF-kB reporter mice. (A) High basal NF-kB activity is found in the telencephalon. Facial staining is visible within the primordial of the vibrissae (5 parallel rows), around the eye, and in the tactile hair follicles. In the thoracic region, NF-kB activity is present in mammary gland primordial and around the gonads. (B) TRAF 6-deficient embryos display hindbrain exencephaly and high basal NF-kB activity within the telencephalon. No NF-kB activity can be detected within non-neuronal tissues stained in (A).

### 5.3.4.1 NF-kB facilitates neuronal survival

The controversy on neuronal NF-kB function is partly due to the difficulty of working with neurons, but mostly it is due to the lack of genetic models with obvious neurological phenotypes. In the past five years, however, several new NF-kB regulators have been discovered and the deletion or compound ablation of some of these proteins has provided interesting clues. For example, mice deficient in both the IKK1 and 2 catalytic components of IKK show defects in neurulation, neural tube closure, and telencephalon size, in addition to the skin, bone and liver phenotypes specific to the individual IKK members (Li et al., 2000). These severe neurological problems are associated with the increased apoptosis of central neurons, which suggests that IKK1 and 2 promote neuronal survival.

Similarly, TRAF4- and TRAF6-deficient mice also display failures in neural tube closure and exencephaly associated with increased apoptosis (Lomega et al., 2000; Regnier et al., 2002). Moreover, p65 null mice demonstrate significant losses in neuron number in several different sensory ganglia (Hamanoue et al., 1999; Middleton et al., 2000). As well, in Drosophila, deleting the NF-kB homologue Dorsal, or removing the interleukin-like receptor Toll, leads to developmental anomalies of specific motorneurons (Halfon et al., 1995).

Consistent with all the above studies, we have found that blocking constitutive NF-kB activity within cortical neurons results in dramatic reductions in viability (Chapter 3, Bhakar et al., 2002). Moreover, we can overexpress active NF-kB (RelA) within cortical neurons, and this provides cells with a survival advantage (Chapter 3). Thus the results of these studies, taken together, underscore the importance of NF-kB in the nervous system, and in general, they indicate that NF-kB functions to facilitate the survival of developing neurons.

An NF-kB pro-survival role may be useful for more than just developmental neuroprotection. In fact, increasing NF-kB activity prior to lethal insults to the brain, often results in significant inhibition of apoptosis. For example, short pulses of kainite or

lithium, within adult rodents, activates NF-kB, which prevents larger lethal neurotoxic injuries to the brain (Blondeau et al., 2001; Ravati et al., 2001). Likewise, preconditioning neurons with short hypoxia treatments activates NF-kB-dependent gene production, which prevents apoptosis (Digicaylioglu and Lipton, 2001). Therefore, NF-kB also facilitates the survival of adult neurons.

### 5.3.4.2 NF-kB promotes neuronal apoptosis

Paradoxically, activation of NF-kB has also been reported to induce neuronal apoptosis in vitro and in vivo (Grilli et al., 1996, 1999; O'Neill et al., 1997; Schneider et al., 1999). Additionally, under pathological conditions, including acute or chronic neurodegenerative disorders, NF-kB activity is often increased (Rong et al., 1996; Clemens et al., 1997; Hunto et al., 1997; Lukiw et al., 1998; Gabriel et al., 1999). Lezoualc'h and colleagues suggest one explanation for these contradictory functions is that acute increases in NF-kB contribute to an apoptotic pathway, whereas preconditioning stimuli that lead to increases in steady-state NF-kB activity provides neuroprotection (Lezoualc'h et al., 1998).

Alternatively, other researchers suggest that detrimental versus useful activation of NF-kB is determined by the type of cell responding to the stimuli (Mattson and Camandola, 2001). For example, increases in NF-kB activation within microglia and astrocytes (Qin et al., 1998; Laflamme et al., 1999; Schwaninger et al., 1999) results in immune-like increases in cytotoxic agents such as nitric oxide and reactive oxygen species, which damage neurons. In contrast, increases in NF-kB activation within neurons, typically increases the production of prosurvival genes such as Bcl-xL (Bhakar et al., 2002; Bui et al., 2002). Such differences will likely be important for future therapeutic approaches especially since NF-kB activity is increased within both neurons and glia in several neurodegenerative disorders (Hunot et al., 1997; Kaltschmidt et al., 1994b; Lukiw et al., 1998; Nickols et al., 2003; Rong et al., 1996; Clemens et al., 1997; Gabriel et al., 1999).

### 5.3.4.3 Other NF-kB functions in the CNS

Recent work suggests that, in addition to regulating neuronal apoptosis, NF-kB activity also regulates the growth and plasticity of neurons. In Drosophila, for example, Dorsal is

enriched in the postsynaptic densities of glutamatergic synapses. Deletion of Dorsal results in neuromuscular junction (NMJ) abnormalities including defects in sprouting, extra-axonal accumulation of synaptic vesicles, and the transformation of axon terminals into growth-cone like structures (Guerrini et al., 1995; Cantera et al., 1999). In addition, active NF-kB can be found in mammalian axons and synapses (Povelones et al., 1997; Kaltschmidt et al., 1993b), and gfp-RelA or endogenous NF-kB is translocated from synaptic locations to the nucleus following glutamate treatment (Wellman et al., 2001), or in long-term memory paradigms (Freudenthal et al., 2000).

### 5.3.5 Mechanisms of neuroprotection by NF-kB

It is well established that, in non-neuronal cells, NF-kB regulates the expression of genes encoding proteins that block apoptotic pathways (Van Antwerp et al., 1996; Wang et al., 1996; Barkett and Gilmore, 1999). In Chapter 3, increasing NF-kB activity within cortical neurons was shown to provide cells with a high degree of neuroprotection, and this protection correlated with dramatic increases in the production of the anti-apoptotic proteins Bcl-xL, c-IAP1 and c-IAP2. Previous reports that demonstrate NF-kB-dependent increases in Bcl-2 proteins within neurons, are in agreement with our data (Bui et al., 2002; Culmsee et al., 2002). Indeed, the anti-apoptotic Bcl-2 family members and the IAP proteins are powerful death-suppressing proteins. Anti-apoptotic Bcl-2 proteins are best known to inhibit apoptosis through heterodimeric interactions with pro-apoptotic members of the Bcl-2 family (reviewed in Moskowitz and Lo, 2003; Merry and Korsmeyer, 1997). In contrast, IAP proteins block apoptosis by binding to and directly inhibiting cleaved and unprocessed members of the Caspase family (reviewed in Richter et al., 2000).

The mechanisms by which constitutive NF-kB activity within the developing and adult brain supports neuron survival is, therefore, likely through the constitutive expression of anti-apoptotic Bcl-2 and IAP family members. Consistent with this hypothesis, overexpression of the anti-apoptotic Bcl-2 proteins can override neurotoxic death signals ranging from trophic factor deprivation to pathological stimuli (Garcia et al., 1992; Martinou et al., 1994; Dubois-Dauphin et al., 1994; Sagot et al., 1995; Cao et al., 2003).

Similarly, overexpression of IAP family members can protect neurons from degeneration induced by ischemia (Trapp et al., 2003), trophic-deprivation (Yu et al., 2003), and axotomy (Perrelet et al., 2002; Perrelet et al., 2000). In fact, the in vivo deletion of Bcl-2 results in dramatic losses in motor, sensory and sympathetic neurons and Bcl-xL null mice die very early due to massive cell death in the developing nervous system (Motoyama et al., 1995; Michaelidis et al., 1996). Therefore, in vitro and genetic evidence demonstrates critical roles for these proteins in the maintenance of neuronal survival.

## 5.4 P75NTR AND CELL DEATH

### 5.4.1 p75NTR is an atypical death receptor

It is now well accepted that p75NTR induces apoptosis in a number of cell types (see introduction) throughout development (Cotrina et al., 2000; Botchkarev et al., 2000; Frade and Barde, 1999; Syroid et al., 2000; Aloyz et al., 1998; Agerman et al., 2000) and following injury (Cheema et al., 1996; Ferri et al., 1998, 1999; Syroid et al., 2000; Beattie et al., 2002; Troy et al., 2002). The mechanisms p75NTR uses to activate cell death, however, have remained obscure. The difficulty in understanding arises, in part, because the signaling paths for p75NTR are regulated by several distinct ligands and co-receptors. As well, there is a shortage of appropriate cellular models to use. Consequently, to identify p75NTR signaling mechanisms we developed, in our final study, an adenovirus-based overexpression paradigm that results in constitutive p75NTR activation (Chapter 4). High p75NTR expression levels obtained with our p75NTR adenovirus consistently resulted in apoptotic cell death within several cell lines and within primary cortical neurons (Chapter 4). These results indicate that our adenoviral system provides a reliable means to study p75NTR-initiated apoptotic events.

In Chapter 4, we demonstrate that p75NTR uses an intrinsic cell death pathway requiring JNK activity, which is an unusual killing mechanism for a TNF receptor superfamily member. Most forms of apoptosis engage the cell death machinery by triggering one of two pathways. In cell surface-regulated apoptosis, death receptor (DR) activation recruits and activates FADD for Caspase 8-dependent death. The alternate pathway, relying on cell-intrinsic cues, induces apoptosis primarily through cytosolic Cytochrome c-dependent activation of Caspase 9 (reviewed in Joza et al., 2002). Typically DRs of the TNFRSF use the cell surface extrinsic pathway requiring Caspase 8. In contrast, most forms of neuronal death support roles for Caspase 9 activity and the intrinsic death machinery.

The initial identification of a DD within p75NTR, common to DRs, suggested that p75NTR might recruit FADD and initiate apoptosis through an extrinsic pathway. More recent results, however, suggest that p75NTR does not interact with FADD nor does it

activate Caspase 8 (Chapter 4, Wang et al., 2001; Coulson et al., 1999). Instead, p75NTR interacts with several distinct adaptor proteins and induces apoptosis with mitochondrial release of Cytochrome c and activation of Caspases 9, 3 and 6 (Chapter 4, Gu et al., 1999; Wang et al., 2001; Troy et al., 2002; Jover et al., 2002, Coulson et al., 2000, Beattie et al., 2002). In addition, Rac (Harrington et al., 2001) and JNK activities are increased during p75NTR-induced death (Chapter 4, Casaccia-bonnefil et al., 1996; Yoon et al., 1998; Aloyz et al., 1998; Bamji et al., 1998; Roux et al., 2001; Friedman et al., 2000). One group has demonstrated that Rac is necessary for p75NTR-induced death (Harrington et al., 2001). As well, we have shown in Chapter 4 that blocking the JNK pathway using a dominant inhibitory JIP mutant or the broad based Mixed Lineage Kinase (MLK) inhibitor, CEP1347, completely attenuates p75NTR-dependent Caspase activation. Therefore, more parallels are found between p75NTR apoptotic pathways and the signaling pathways activated in neuronal death paradigms than in DR-initiated apoptotic pathways (Figure 5.2).

### 5.4.2 p75NTR-initiated apoptotic events upstream of JNK

Although our results in Chapter 4 demonstrate that JNK activity is necessary for p75NTR-induced death, how p75NTR activates JNK, remains to be answered. Neuronal death paradigms suggest that small G proteins (such as Cdc42), MAP3Ks, and MAP2Ks are likely to be crucial components (Fan et al., 1996; Hirai et al., 1996; Rana et al., 1996; Tibbles et al., 1996; Sakuma et al., 1997; Bazenet et al., 1998; Kanamoto et al., 2000; Xu et al., 2001). Indeed, p75NTR-dependent JNK activation and Caspase activation is blocked by high concentrations of CEP1347, and these results support a role for a MAP3K component since CEP1347 can inhibit other MAP3Ks. Interestingly, recent results from our lab (Salehi et al., 2002) demonstrate that the p75NTR-interacting protein, NRAGE, induces apoptosis through a mitochondrial-dependent pathway requiring JNK and Caspase 9, 3 and 7 activities. These findings are the first to link a p75NTR-interacting protein to activation of JNK and subsequent cell death. Therefore, the membrane proximal components of the p75NTR apoptotic pathway might include NRAGE.

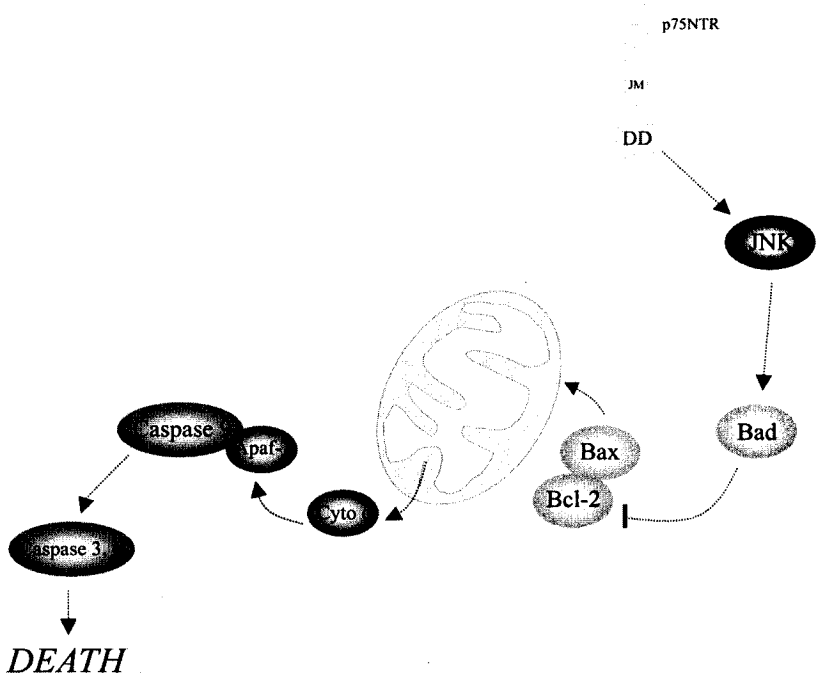


**Figure 5.2.** Schematic model of p75NTR-mediated apoptotic events. p75NTR activates c-Jun kinase (JNK) for subsequent phosphorylation of the BH3-domain only protein Bad, mitochondrial release of Cytochrome c, activation of Caspases 9, 3, and 6, and ultimately cell death.

Although p75NTR activation can lead to direct induction of JNK activity in a number of different cell types (Chapter 4; Casaccia-Bonnefil et al., 1996; Yoon et al., 1998; Aloyz et al., 1998; Bamji et al., 1998; Friedman et al., 2000; Roux et al., 2001), activation of the JNK pathway might also be the result of inactivation of a TrkA-dependent signaling pathway that antagonizes JNK. Ras, which is activated following the binding of NGF to TrkA, can suppress the JNK pathway in sympathetic neurons (Mazzoni et al., 1999) and similarly, ERK activity, which can oppose JNK signaling, is decreased in differentiated PC12 cells undergoing apoptosis after NGF deprivation (Xia et al., 1995). Therefore, in cell types that express both p75NTR and TrkA, p75NTR-mediated JNK activation might also require reduced TrkA signaling.

### 5.4.3 p75NTR-mediated apoptotic events downstream of JNK

Determining how JNK activation leads to mitochondrial release of Cytochrome c in p75NTR-induced apoptosis, has also been the subject of intense investigation. In most forms of intrinsic apoptosis, the Bcl-2 family of proteins are key regulators (for review see Opferman and Korsmeyer, 2003). After an apoptotic stimulus, for example, the pro-apoptotic BH3-domain only members accumulate and activate. Once active, BH3-domain only proteins function by inhibiting pro-survival Bcl-2 members, including Bcl-2 and Bcl-xL. Active BH3-domain only proteins also directly trigger the activation and oligomerization of pro-apoptotic multidomain Bcl-2 members such as Bax (Letai et al., 2002). Oligomerized Bax proteins are then believed to create pore-like structures, which allow for release of Cytochrome c and mitochondrial disruption. The activation of BH3-domain-only proteins is, therefore, a key regulatory step in mitochondrial release of Cytochrome c.

There are two main mechanisms for activation of BH3-domain only proteins. The first is transcriptional up-regulation of these pro-apoptotic members for their sufficient accumulation. The second activating mechanism is modification by post-translational means, including proteolytic cleavage or direct phosphorylation. An example of post-translational activation is found with the BH3-only protein, Bad. In healthy cells, Bad is phosphorylated at Ser 136 and 112 by prosurvival kinases, which promotes Bad

interaction with 14-3-3 to keep Bad inactive (Zha et al., 1996; Datta et al., 2000; Opferman and Korsmeyer, 2003). Upon exposure to an apoptotic stimulus, however, proapoptotic kinases such as Cdc2 or JNK, can directly phosphorylate Bad at Ser 128, and this phosphorylation event releases Bad from sequestration by 14-3-3 (Donovan et al., 2002; Konishi et al., 2002).

In neuronal death paradigms that require Cytochrome c release, JNK activition has been shown to regulate the activation of BH3-domain only proteins by both transcriptional and post-translational mechanisms. In NGF-deprived sympathetic neurons, for example, JNK activation results in the phosphorylation of c-Jun for c-Jun-dependent transcriptional increases in the BH3-domain only proteins, Bim and Hrk. Indeed, when Bim or Hrk is removed, the death of these sympathetic neurons is severely delayed (Harris et al., 2001; Putcha et al., 2001; Whitfield et al., 2001). More recent work demonstrates that JNK can also activate BH3-domain only proteins by direct phosphorylation. JNK activity within UV-treated 293 cells, NGF-deprived sympathetic neurons, or growth factor-deprived cerebellar neurons, results in the phosphorylation and activation of BH3-domain only proteins, Bad, Bim, and Bmf (Donovan et al., 2002; Lei et al., 2003; Putcha et al., 2003). Moreover, the pro-apoptotic phosphorylation of Bad and Bim contribute to trophic-deprivation-induced death of cerebellar granule and sympathetic neurons, respectively (Donovan et al., 2002; Konishi et al., 2002; Putcha et al., 2003).

In Chapter 4, we asked if p75NTR-mediated JNK activation led to activation of BH3-domain only proteins through transcriptional or phosphorylation mechanisms. We found that p75NTR-mediated JNK activation results only in modest phosphorylation of the most common JNK substrate, c-Jun, and no transcriptional increases in any BH3-domain only proteins. The modest phosphorylation of c-Jun is surprising given the robust activation of JNK itself and the dramatic phosphorylation of c-Jun by activated MLK3 within the same cell types (Chapter 4). This finding, however, is in agreement with previous studies demonstrating no obvious role for c-Jun in p75NTR-activated death (Palmada et al., 2002). Therefore, the phosphorylation of c-Jun is not likely to be sufficient or necessary to propagate a p75NTR death signal. Interestingly, these results are reminiscent of the

reduced developmental apoptosis detected in the neural tubes of Jnk1, Jnk2 double knockout mice (Kuan et al., 1999; Sabapathy et al., 1999), but which is not detected within the neural tubes of c-jun knockout or the JunAA knock-in mice (Hilberg et al., 1993; Behrens et al., 1999).

Although no transcriptional increases in BH3-domain only proteins were detected, we have found that p75NTR activation results in JNK-dependent Ser 128 phosphorylation and oligomerization of the BH3-domain only protein Bad (Chapter 4; Bhakar, manuscript submitted). We also demonstrate that reducing endogenous Bad using RNAi or a phospho-specific, dominant inhibitory Bad peptide, completely attenuates p75NTR-mediated death (Chapter 4; Bhakar, manuscript submitted). These results indicate that p75NTR induces apoptosis through a JNK-dependent, transcription–independent pathway requiring Bad. Moreover, these findings demonstrate that cell surface death receptors can mediate apoptosis through direct phosphorylation of BH3-only proteins.

Palmada and colleagues (2002) recently reported that p75NTR-induced death may also require transcriptional events. Indeed, there may be alternative JNK-dependent, but c-Jun-independent, transcriptional pathways that contribute to p75NTR-induced apoptosis. One candidate pathway involves p53. p53 can be directly phosphorylated and activated by JNK (Fuchs et al., 1998; Milne et al., 1995; Hu et al., 1997; Dumaz et al., 2001; Buschmann et al., 2001), and one group suggests that p53 is necessary for p75NTR to induce death (Bamji et al., 1998). In addition, in non-neuronal systems, p53 activation can result in transcriptional increases in the BH3-only members Noxa and Puma (Wu and Deng, 2002). However, no consistent p75NTR-activated increases in p53 phosphorylation (Bhakar, unpublished results) were detected in our cellular systems nor were any transcriptional increases in Noxa and Puma (Chpater 4). Moreover, p75NTR potently induces apoptosis in cells lacking functional p53 (Chapter 4).

Another candidate pathway that might contribute to p75NTR-induced apoptosis is the transcription factor ATF-2. ATF-2 is a substrate for JNK, and we have found that in PC12 cells and U373 cells, p75 activation can lead to JNK-dependent phosphoryltaion of ATF-

2 (Figure 5.3, Bhakar, unpublished results). ATF-2, a c-Jun binding partner, can be activated after neurotrophin withdrawal of sympathetic neurons (Eilers et al., 2001), and during injury-induced neuronal apoptosis (Walton et al., 1998; Ferrer et al., 2001). Moreover, Khursigara and collegues (2001) have shown that in the presence of the p75NTR-binding protein, TRAF6, p75NTR can increase ATF-2 transcriptional activity.

When activated, ATF-2 has been shown to induce the death of undifferentiated PC12 cells. However, the mechanism of cell death is currently unknown (Leppa et al., 2001). One mechanism might involve ATF-2 binding to AP-1 elements within the promoters of gene encoding proteins that are required for cell death. For example, c-Jun and ATF-2 heterodimers can activate the c-jun promoter and the Fas ligand promoter contains an AP-1 site (Kasibhatla et al., 1998). As well, increases in Fas ligand signaling can contribute to trophic factor withdrawal-induced death within PC12 cells, cerebellar granule neurons and motor neurons (Le-Niculescu et al., 1999; Raoul et al., 1999). ATF-2 can also regulate the transcription of cyclinD1 and TNF, and both products have been implicated in p75NTR signaling events and in forms of neuronal death (Bhakar et al., 1999; Venters et al., 2000; Ino et al., 2001; Khwaja et al., 2003). Therefore, it is plausible that p75NTR may use ATF-2 signaling and transcriptional events to mediate apoptosis. In fact, previous results suggesting that c-Jun is not involved in p75NTR-induced death may reflect functional compensation by ATF-2. Further examination of transcriptional pathways in p75NTR action is thus warranted.

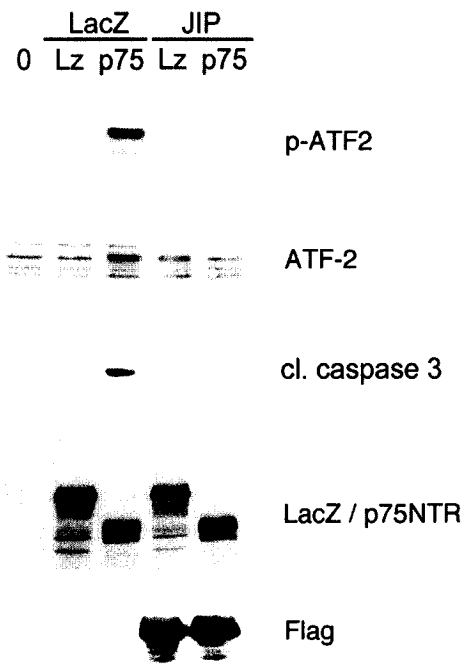


**Figure 5.3.** p75NTR activates JNK-dependent phosphorylation of ATF-2. PC12 cells were infected with nothing (0), LacZ (Lz), or p75NTR (p75) adenovirus together with either 5 MOI of LacZ or JBD-JIP (JBD) adenovirus. Lysates were compared for expression of phospho-ATF-2, ATF-2, cleaved Caspase 3, L acZ, p75NTR, and Flag-JIP (Flag) by immunoblot as indicated.

## 5.5 FUTURE P75NTR STUDIES

### 5.5.1 Proneurotrophins

The next stages of p75NTR study will be exciting. Experiments will be needed to confirm whether the pro-neurotrophin ligands activate JNK-dependent cell death and other p75NTR-mediated responses.

It will also be necessary to establish whether the pro-forms of BDNF, NT-3 and NT-4/5 also have enhanced affinity for p75NTR. One would expect an enhanced affinity, since a proportion of all neurotrophins are secreted in the pro-form and the pro-domains are highly conserved. It is also unclear whether proneurotrophins are secreted as dimers, what p75NTR contacts are needed, and what conformational changes are likely to occur. Furthermore, the Neurotrophic Hypothesis will need to be re-evaluated. Scarce quantities of neurotrophin are no longer the only initiating stimulus for neuronal cell death. Hence, the type and combination of pro- versus mature neurotrophins secreted by a target tissue will play a critical role in the decision process.

Interestingly, in the adult human brain, NGF and BDNF exist almost exclusively in the pro-form (Lee et al., 2001b), which suggests that p75NTR activation by proneurotrophins maybe important in the adult CNS. For example, oligodendroctyes surrounding sites of spinal cord injury have increased quantities of pro-NGF and are susceptible to p75NTR-mediated apoptosis (Beattie et al., 2002). Likewise, Alzheimer patients express elevated levels of proNGF in their cortices (Fahnestock et al., 2001). Therefore, proneurotrophin-p75NTR interactions may also significantly contribute to disease pathology. Furthermore, since BDNF plays an important role in synaptic plasticity and the majority of BDNF is secreted in the pro-form (Mowla et al., 2001), p75NTR responsiveness to pro-BDNF may be important for regulating long-term potentiation and memory.

### 5.5.2 Resolving the survival/death paradox

Significant work demonstrates that p75NTR can activate signaling pathways that facilitate survival or promote apoptosis (see introduction). Sometimes both effects occur

within the same cell types (Roux et al., 2001; Bhakar, unpublished results). Therefore, another remaining question is how p75NTR chooses between activating death and promoting survival.

Recent work suggests that the signaling options for p75NTR might involve changes within the receptor's oligomeric structure, and consequently, the specific recruitment of intracellular adaptor proteins. In the past, for example, initiating receptor activation was thought to require ligand binding. Now, however, an alternative model has been proposed. The alternative model is that TNFRSF members pre-assemble into oligomers before a ligand interaction occurs (Siegel et al., 2000; Chan et al., 2000). In this model, ligand binding to the assembled receptor complexes induces a conformational shift, and this change dictates a corresponding change in the receptor signaling response.

This alternative model has been demonstrated for TNFR1 receptor signaling events. For example, TNF binding to TNFR, releases the ICD bound inhibitory protein SODD (silencer of death domains), and the resulting receptor aggregate then initiates cellular responses by recruiting different adaptor proteins including TRADD, FADD, and TRAFs (Jiang et al., 1999).

Since overexpression of p75NTR or p75NTR fragments alone can activate signaling cascades without ligand treatment, the formation of different p75NTR oligomeric structures to regulate different cellular responses may be particularly relevant for p75NTR to function physiologically (Chapter 4; Paul, unpublished results; Rabizadeh et al., 1993; Bunone et al., 1997; Lievremont et al., 1999; Roux et al., 2001; Ye et al., 1999a, b; Majdan et al., 1997; Coulson et al., 2000). Consistent with this hypothesis, adaptors including TRAF4 and TRAF2 bind p75NTR constitutively in the absence of ligand (Vaillancourt, manuscript in preparation; Ye et al., 1999), whereas pro-apoptotic adaptors such as NADE, only associate when p75NTR is bound by ligand (Mukai et al., 2000). Similarly, we have found that low expression levels of p75NTR results in ligand-independent increases in cellular survival (Roux et al., 2001; Bhakar, unpublished results). The induction of apoptosis, however, requires much higher levels of p75NTR

within the same cell (Roux et al., 2001; Chapter 4). Accordingly, p75NTR is typically found in both monomeric and oligomeric forms on SDS-PAGE (Grob et al., 1985; Wang et al., 2001). As well, the CRD1 region necessary for TNFRSF pre-assembly is present with a high degree of conservation within p75NTR (Locksley et al., 2001). Therefore, significant evidence suggests that different p75NTR oligomeric states may dictate different p75NTR signaling outcomes to regulate the choice between survival and death.

An additional level of complexity is provided by recent findings demonstrating that p75NTR activity is regulated by both processed and unprocessed neurotrophin ligands. Proneurotrophins, for example, can activate p75NTR regardless of the presence of TrkA (Beattie et al., 2002; Lee et al., 2001). Hence, the ratio of pro- to mature neurotrophin will also emerge as a critical regulatory factor for the balance between survival and death (Chao and Bothwell, 2002). This balance was a property previously limited to the choice and amount of mature neurotrophins expressed.

## 5.6 SUMMARY

Numerous studies now indicate that the functions of p75NTR are varied and complex. p75NTR modulates Trk receptor signaling and cellular responses that regulate apoptosis and axon growth. p75NTR also regulates several receptor types and functions as an autonomous signaling unit. Consequently, in this thesis, I have discussed and presented work characterizing p75NTR signal tranducing mechanisms pertaining to the cellular decision of survival or apoptosis. I have established that p75NTR can function to modulate TNF receptor signaling and enhance NF-kB activation. I have also demonstrated that NF-kB activity is present in developing and adult forebrain neurons and is necessary for central neuron survival. Finally, I have shown that p75NTR induces apoptosis through an intrinsic death pathway requiring JNK activity and the phosphorylation and oligomerization of the BH3-domain only Bcl-2 member, Bad.

Since the cellular decision to live or to die is made by the coordinated action and balancing of many different pro- and antiapoptotic factors, not surprisingly then, the mechanisms of p75NTR function are highly redundant, tightly regulated, and very complex. Understanding the precise molecular components regulating these pathways will be essential in healing a variety of human diseases where control of this coordination and balance is defective. Indeed, a common hallmark of the stress/injury response in the nervous system, is massive up-regulation of p75NTR expression. Therefore, the findings in this thesis provide information leading to potential therapeutic intervention for treating central nervous system disease as well as shed light on the cellular requirements regulating apoptosis in the nervous system.

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Thank you,

Asha Bhakar
Centre for Neuronal Survival
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3801 University Ave., Rm MP-038
Montreal, Quebec, H3A 2B4
Canada
abhakar@hotmail.com
(514)490-1516
fax: (514)398-1319

Dr. David S. Park
Neuroscience Research Institute
University of Ottawa
451 Smyth Rd
Ottawa, ON
K1H 8M5
Canada
Tel# (613) 562-5800 ext 8816
Fax# (613) 562-5403

**From :** laura-lee tannis <lltannis@yahoo.com>
**To :** Asha Bhakar <abhakar@hotmail.com>
**Subject :** Re: permission to reprint Bahakr et.al. JN2002
**Date :** Thu, 21 Aug 2003 07:07:14 -0700 (PDT)

I accept.

August 20, 2003

Re: Copyright waivers for McGill University from
Co-authors on
published
papers

To All Co-authors,

I am writing to obtain your authorization to reprint
the article:
Bhakar AL,
Tannis LL, Zeindler C, Russo MP, Jobin C, Park DS,
MacPherson S, and
Barker
PA. "Constitutive Nuclear Factor-kB Activity Is
Required for Central
Neuron
Survival", Journal of Neuroscience (2002)
22(19):8466-8475, in my Ph.D.
thesis. Please email back "I accept" with this text
inserted in the
email if
you are in agreement.

Thank you,

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> 22(19):8466-8475, in my Ph.D.

## Copyright waivers from the co-authors for:

**Bhakar AL**, Howell JL, Paul CE, Salehi AH, Becker EBE, Said F, Bonni A and Barker PA. Apoptosis Induced by p75NTR Requires Jun Kinase-dependent Phosphorylation of Bad. **In preparation.**

**From :** "Jenny Howell" <howell_jenny86@hotmail.com>
**To :** abhakar@hotmail.com
**Subject :** Re:
**Date :** Thu, 21 Aug 2003 08:56:49 -0400

I, Jenny Howell, accept the reprint of the manuscript:
Bhakar AL, Howell JL, Paul CE, Salehi AH, Becker EBE, Said F, Bonni A and Barker PA. "Apoptosis Induced by p75NTR Requires Jun Kinase-dependent Phosphorylation of Bad", in Asha Bhakar's Ph.D. thesis.

From: "Asha Bhakar" <abhakar@hotmail.com>
To: amirsalehi@hotmail.com, christine.paul@mail.mcgill.ca, howell_jenny86@hotmail.com, phil.barker@mcgill.ca, azad_bonni@hms.harvard.edu, asfarid@hotmail.com
Date: Thu, 21 Aug 2003 06:45:22 +0000

August 20, 2003

Re: Copyright waivers for McGill University from Co-authors

To All Co-authors,

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Thank you,

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3801 University Ave., Rm MP-038
Montreal, Quebec, H3A 2B4
Canada
abhakar@hotmail.com
(514)490-1516
fax: (514)398-1319

**From :** "amir salehi" <amirsalehi@hotmail.com>
**To :** abhakar@hotmail.com
**Subject :** Re:
**Date :** Thu, 21 Aug 2003 15:03:46 -0400

I ACCEPT.

Amir H Salehi; 21st Aug. 2003.

>From: "Asha Bhakar"
>To: amirsalehi@hotmail.com, christine.paul@mail.mcgill.ca, howell_jenny86@hotmail.com, phil.barker@mcgill.ca, azad_bonni@hms.harvard.edu, asfarid@hotmail.com
>Date: Thu, 21 Aug 2003 06:45:22 +0000
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>August 20, 2003
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>Montreal, Quebec, H3A 2B4
>Canada
>abhakar@hotmail.com
>(514)490-1516
>fax: (514)398-1319
>

**From :** "Esther B. E. Becker" <esther_pluger@student.hms.harvard.edu>
**To :** "Asha Bhakar" <abhakar@hotmail.com>
**Subject :** Re: copyright permission
**Date :** Thu, 21 Aug 2003 16:18:46 -0400

Dear Asha,

I ACCEPT.

Good luck with your thesis.

- Esther

>August 20, 2003
>
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>Re: Copyright waivers for McGill University from Co-authors
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>Montreal, Quebec, H3A 2B4
>Canada
>abhakar@hotmail.com
>(514)490-1516
>fax: (514)398-1319
>
>_____
>The new MSN 8: advanced junk mail protection and 2 months FREE*
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.....
Esther B. E. Becker, M.Sc.
Harvard Medical School
Department of Pathology/Bonni Lab
Armenise Building, Room 439
200 Longwood Ave
Boston, MA 02115, USA
Phone: +1 617-432-4105
Fax : +1 617-432-4101

**From :** Azad Bonni <azad_bonni@hms.harvard.edu>
**To :** "Asha Bhakar" <abhakar@hotmail.com>
**Subject :** Re:
**Date :** Sat, 23 Aug 2003 12:58:32 -0500

I accept

August 20, 2003

Re: Copyright waivers for McGill University from Co-authors

To All Co-authors,

I am writing to obtain your authorization to reprint the manuscript: Bhakar AL, Howell JL, Paul CE, Salehi AH, Becker EBE, Said F, Bonni A and Barker PA. "Apoptosis Induced by p75NTR Requires Jun Kinase-dependent Phosphorylation of Bad", in my Ph.D. thesis. Please email back "I accept" with this text inserted in the email if you are in agreement.

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abhakar@hotmail.com
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fax: (514)398-1319



**********
Azad Bonni, M.D., Ph.D.
Assistant Professor,
Department of Pathology,
Harvard Medical School
200 Longwood Ave
Boston, MA 02115

Tel: 617-432-4104
Fax: 617-432-4101
Email: azad_bonni@hms.harvard.edu
**********

**From :** Phil Barker <phil.barker@mcgill.ca>
**To :** Asha Bhakar <abhakar@hotmail.com>
**Subject :** Re: copyright permission for p75 paper
**Date :** Tue, 26 Aug 2003 10:37:33 -0400

No problem - Phil

Asha Bhakar wrote:

> August 20, 2003
>
> Re: Copyright waivers for McGill University from Co-authors
>
> To All Co-authors,
>
> I am writing to obtain your authorization to reprint the manuscript: Bhakar AL, Howell JL, Paul CE, Salehi AH, Becker EBE, Said F, Bonni A and Barker PA. "Apoptosis Induced by p75NTR Requires Jun Kinase-dependent Phosphorylation of Bad", in my Ph.D. thesis.
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> Montreal, Quebec, H3A 2B4
> Canada
> abhakar@hotmail.com
> (514)490-1516
> fax: (514)398-1319
>
> _____
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==========
Philip A Barker, PhD
Associate Professor
Montreal Neurological Institute
McGill University
3801 University Avenue
Montreal, Quebec, Canada, H3A 2B4
Ph: 514-398-3064
Fax: 514-398-5214
Email: phil.barker@mcgill.ca
==========

**From :** Farid Arab Said <farid.as@aegera.com>
**To :** 'Asha Bhakar' <abhakar@hotmail.com>
**Subject :** RE: waiver
**Date :** Thu, 20 Nov 2003 13:35:42 -0500

Hi Asha,

I accept.

Thanks.

DR. Farid ARAB SAID

Research Scientist

810, Chemin du Golf, Ile-Des-Soeurs,

(QC) H3E 1A8 Canada

Tel: 514-288-5532 Ext:229

Fax: 514-288-9280

e-mail: farid.as@aegera.com

-----Original Message-----
**From:** Asha Bhakar [mailto:abhakar@hotmail.com]
**Sent:** Thursday, November 20, 2003 1:19 PM
**To:** farid.as@aegera.com
**Subject:** waiver

Hi Farid, Can you answer this asap, thank you, asha

**From :** cpaul3 <cpaul3@po-box.mcgill.ca>
**To :** Asha Bhakar <abhakar@hotmail.com>
**Subject :** RE: copyright permission for p75 paper
**Date :** Tue, 26 Aug 2003 10:06:10 -0400

I accept

>===== Original Message From Asha Bhakar <abhakar@hotmail.com>
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>(514)490-1516
>fax: (514)398-1319
>
>

# RESEARCH COMPLIANCE CERTIFICATES

4. RESEARCH PERSONNEL: (attach additional sheets if preferred)

| Name | Department | Check appropriate classification | | | | Fellow |
|---|---|---|---|---|---|---|
| | | Investigator | Technician & Research Assistant | Student | | |
| | | | | Undergraduate | Graduate | |
| **Dr. Phil Barker** | **Neurol and Neurosurg** | X | | | | |
| **Dr. Sylvie LaBoissier** | **Neurol and Neurosurg** | | X | | | |
| **Dr. Matt Grapes** | **Neurol and Neurosurg** | | | | | X |
| **Ms. Laura Lee Tannis** | **Neurol and Neurosurg** | | X | | | |

5. EMERGENCY: Person(s) designated to handle emergencies

Name: **Dr. Phil Barker** Phone No: work: **398-3064** home: **830-3243**

Name: **Ms. Laura Lee Tannis** Phone No: work: **398-3212** home: **289-8638**

6. Briefly describe:

i) the biohazardous material involved (e.g. bacteria, viruses, human tissues) & designated biosafety risk group

- **Tissue culture waste**
- **Non-pathogenic bacterial waste**
- **Broken glass/sharps**
- **Organic solvents**

ii) the procedures involving biohazards

**Cell lines will be used to express recombinant DNA fragments produced in vitro by standard molecular biology techniques. The constructs tested represent a new class of proteins that show no ability to transform cells.**

**Some of these proteins will be produced as recombinant adenovirus using bacteria and a mammalian packaging line (293 cells) – virus is replication incompetent outside packaging lines such as 293. Recombinant virus will be used to infect cell lines and primary cells maintained in vitro.**

- **Tissue culture will be performed in an approved laminar flow hood located in a room dedicated to this purpose.**
- **Personnel working in this area will be suitably trained in sterile techniques.**

- **Biohazardous waste will be disposed of separately from regular garbage. Cell and bacterial culture waste is placed in biohazard autoclave bags and autoclaved prior to disposal; liquid waste is neutralized with 0.1% Roccal or sodium hypochlorite solution (5.25% bleach diluted 1:10)**
- **Containers/equipment leaving the lab will be decontaminated with 1% bleach of 70% ethanol.**
- **Working areas will be regularly wiped with 70% ethanol**
- **Chloroform and phenol are disposed of as toxic waste**
- **Sharps are disposed in impermeable sealed plastic containers; glass in sealed cardboard boxes.**
- **Organic/caustic chemicals are stored in a reinforced cabinet and used in a fume hood.**

iii) the protocol for decontaminating spills:

**Spills will be decontaminated by:**

- **Allowing aerosols to settle**
- **Covering spill with paper towel and then applying 1% bleach from the perhery inwards**
- **After a 30 minute incubation period in the applied bleach, the paper towel will be disposed of in a biohazard bin and sunsequently autoclaved.**
- **Spills onto clothing will be decontaminated by autoclaving.**

7. Does the protocol present conditions (e.g. handling of large volumes or high concentrations of pathogens) which could increase the hazards of the infectious agent(s)? **NO**

8. Do the specific procedures to be employed involving genetically engineered organisms have a history of safe use? **YES**

9. What precautions are being taken to reduce production of infectious droplets and aerosols?
**Biosafety cabinets are used**

10. List the biological safety cabinets to be used.

| Building | Room No. | Manufacturer | Model No. | Serial No. | Date Certified |
|---|---|---|---|---|---|
| **MNI Molson Pavillion** | **MP032** | **NuAire** | **NU440-400** | **67060** | **8/30/99** |
| | | | **NU440-400** | **67062** | **8/31/99** |
| | | | **NU440-400** | **67064** | **9/01/99** |
| | | | | | |

b) **Will the project involve breeding animals?** NO☐ YES ☒
**Will the project involve the generation of genetically altered animals?** NO ☐ YES ☒
**Will field studies be conducted?** NO ☒ YES ☐

**c) Description of Animals**

| | Sp/strain 1 | Sp/strain 2 | Sp/strain 3 | Sp/strain 4 | Sp/strain 5 | Sp/strain 6 |
|---|---|---|---|---|---|---|
| **Species** | rabbit | rat | mouse | mouse | mouse | mouse |
| **Supplier/Source** | Charles River | Charles River | Charles River | Charles River | Charles River | inhouse colony |
| **Strain** | New Zealand White | Sprague Dawley | Balb/c X C3H | Balb/c | SV129 | NFkB transgenic (CD1 and C-57 bkgrd) |
| **Sex** | NA | female | both (breeders) | both | female (oocyte donors) | both |
| **Age/Wt** | > 4 months | > 2 months | all | > 3 months | > 3 months | all |
| **# To be purchased** | 10 | 50 | none | 50 | 10 | none |
| **# Produced by in-house breeding** | NA | NA | 600 | NA | NA | all |
| **# Other (e.g.field studies)** | NA | NA | NA | NA | NA | NA |
| **#needed at one time** | 2 | 1 | 100 | 10 | 2 | 50 |
| **# per cage** | 1 | 2 | 5 | 5 | 2 | 5 |
| | | | | | | |
| | **Sp/strain 1** | **Sp/strain 2** | **Sp/strain 3** | **Sp/strain 4** | **Sp/strain 5** | **Sp/strain 6** |
| **Species** | mouse | mouse | mouse | | | |
| **Supplier/Source** | inhouse colony | inhouse colony | inhouse colony | | | |
| **Strain** | p75 (exon3) NTR-/- (C57bl6xBalb /c) | p75 ICD transgenic (C57 bkgrd) | Traf-6 (c57xBlb/c) | | | |
| **Sex** | both | both | both | | | |
| **Age/Wt** | all | all | all | | | |
| **# To be purchased** | none | none | none | | | |
| **# Produced by in-house breeding** | all | all | all | | | |
| **# Other (e.g.field studies)** | NA | NA | NA | | | |
| **#needed at one time** | 50 | 50 | 50 | | | |
| **# per cage** | 5 | 5 | 5 | | | |
| **TOTAL# /YEAR** | **100** | **100** | **100** | | | |
| | | | | | | |

**Quality Control Assurance: To prevent introduction of infectious diseases into animal facilities, a health status report or veterinary inspection certificate may be required prior to receiving animals from all non-commercial sources or from commercial sources whose animal health status is unknown or questionable. Quarantine and further testing may be required for these animals.**

## 7. Justification of Animal Usage

**a) Please justify the number of animals requested for each species described in the table 6c above, BASED ON THE EXPERIMENTAL OBJECTIVES OF THE PROJECT. Include information on experimental and control groups, # per roup, and failure rates. Also justify in terms of statistical requirements, product yield, etc. For breeding, specify how many adults are used, number of offspring produced, and how many offspring are used in experimental procedures. The numbers of animals are for one year only, not the length of funding. Use the table below when applicable (space will expand as needed).**

1. We expect to raise a total of 5 polyclonal antibodies on this protocol. We use 2 rabbits per antigen hence a total of 10 animals.
2. We produce one culture per week from embryonic rat tissue. We will therefore require 1 timed pregnant female each week to produce these cultures, a total of 50 rats.
3. We expect to produce transgenic mice (Balb/c x C3H) during the course of this protocol – we typically breed litters from 10 founder females for overlapping expression patterns, with an average litter size of 10 animals per F1 line (total = about 100) before determining which lines to focus our studies on. Thereafter, we will use 1 litter per week for a period of 1 year (600 mice), while maintaining a viable colony of about 100 mice.
4. We expect to go through 5 rounds of ascites production, using 10 mice (balb/c) per round, hence a total of 50 animals.
5. We will need 10 SV129 oocyte donors per year for the generation of transgenic animals.
6. To maintain as assured supply of pregnant animals of the correct genotype and to generate cultures of primary neurons and fibroblasts from transgenic mice, we will maintain 50 animal per colony. With aging, maintaining colonies of this size will require 100 animals per year for each mouse colony described above.

| Test Agents or Procedures e.g. 2 Drugs | # of Animals and Species Per Group e.g. 6 rats | Dosage and/or Route Administration e.g. .03, .05 mg/kg – IM, IP (4 variables) | # of endpoints e.g. 1, 7, 10 days (3 variables) | Other variables (i.e. sex, weight, genotypes,etc.) e.g. Male, Female groups (2 variables) | Total number of animals per year e.g. 2 x 6 x 4 x 3 x 2 = 288 |
|---|---|---|---|---|---|
| | | | | | |

*** For the above table, enter the first agent/procedure, press 'enter', then the 2nd agent... complete the first column, then the 2nd, then the 3rd...**

**b) Please justify the need for live animals versus alternate methods (e.g. tissue culture, computer simulation).**
It is crucial that we understand the function of signaling receptors such as p75NTR in their native cellular context. While we will use continuous, established cell lines for much of the work described in my grant, we must validate the results of these studies using primary neurons, either in culture (ie. derived directly from embryos) or in situ (within transgenic animals).

**c) Describe the characteristics of the animal species selected that justifies its use in the proposed study (consider characteristics such as body size, species, strain, data from previous studies or unique anatomic/physiological features)**

Rabbits are the preferred species for small scale antibody production, given their docile characteristics and blood volume. Mice are the model species around which most animal transgenic technology has been developed, because of their breeding capacity, the extensive genetic information already available and their relatively low cost. We have experience in transgenic mice from our previous studies and they are clearly the preferred species for the preferred studies. Rats are used to derive primary neurons because neurons from rats tend to survive more readily in culture than those derived from other species.

## 8. Animal Husbandry and Care

**a) Special cages** NO ☒ YES ☐ **Specify:**

**Special diet** NO ☒ YES ☐ **Specify:**

**Special handling** NO ☒ YES ☐ **Specify:**

**b) Is there any component to the proposed procedures which will result in immunosuppression or decreased immune function (e.g. stress, radiation, steroids, chemotherapeutics, genetic modification of the immune system)?**

NO ☒ YES ☐ **Specify:**

c)
**Multiple institution facility housing:** NO ☒ YES ☐

| | | | | |
|---|---|---|---|---|
| **Indicate all facilities where animals will be housed:** | **Building:** | McIntyre Medical Building (Rabbits)<br>M.G.H. Animal Facility(transgenics -J.P.Julien)<br>R.V.C. Animal Facility(transgenics -A. Peterson)<br>M.N.I. Animal Facility (rats and mice) | **Room No:** | 865 |
| **Indicate area(s) where animal use procedures will be conducted:** | **Building:** | M.N.I .- C.N.S. | **Room No:** | MP038 |

**If animal housing and animal use are in different locations, briefly describe procedures for transporting animals:**
Rabbits at the Royal Victoria animals quarters will not be .
Mice and rats will be placed in transport cages, wrapped in green plastics bags and brought down to MP038 for experiments. Animals will be used immediately and therefore will not be housed for any period of time in the lab.

## 9. Standard Operating Procedures (SOPs)

**Complete this section if you plan to use any of the UACC SOPs listed below. IT IS UACC POLICY THAT THESE SOPS BE USED WHEN APPLICABLE. Any proposed variation of the SOPs must be described and justified. The Standard Operating Procedures can be found at the UACC website at www.mcgill.ca/fgsr/rgo/animal/ . The completed and signed SOP form must be attached to the protocol.**

**Check all SOPs that will be used:**

| | | | |
|---|---|---|---|
| **Blood Collection (UACC#1 )** | ☐ | **Production of Monoclonal Antibodies (UACC#7 )** | ☒ |
| **Anaesthesia (rodents) (UACC#2 )** | ☐ | **Production of Polyclonal Antibodies(UACC#8 )** | ☒ |
| **Analgesia (rodents/larger species) (UACC#3 )** | ☐ | **Collection of Amphibian Oocytes (UACC#9 )** | ☐ |
| **Breeding (transgenics/knockouts) (UACC#4 )** | ☒ | **Rodent Surgery (UACC#10 )** | ☐ |
| **Transgenic Generation (UACC#5 )** | ☒ | **Neonatal Rodent Anaesthesia and Euthanasia (UACC#11)** | ☐ |
| **Knockout/in Generation (UACC#6 )** | ☐ | | ☐ |

## 10. Description of Procedures

a) **FOR EACH EXPERIMENTAL GROUP, DESCRIBE ALL PROCEDURES AND TECHNIQUES IN THE ORDER IN WHICH THEY WILL BE PERFORMED - surgical procedures, immunizations, behavioural tests, immobilization and restraint, food/water deprivation, requirements for post-operative care, sample collection, substance administration, special monitoring, etc. IF A PROCEDURE IS COVERED BY AN SOP, WRITE "AS PER SOP", NO FURTHER DETAIL IS REQUIRED. Appendix 2 of the Guidelines provides a sample list of points that should be addressed in this section.**

Polyclonal antibodies will be produced as per the McGill University Faculty of Medicine Standard Protocol for Generation of Polyclonal Antibodies in Rabbits (attached).

For ascites production, we will follow the procedures within the McGill University Faculty Of Medicine Standard Protocol for Generation Of Monoclonal Antibodies In Rats And Mice (attached).

We will produce primary cultures of peripheral and central neurons from normal and transgenic embryonic mice and from normal embryonic rats. For this, neonatal mice will be sacrificed by decapitation and superior cervical ganglia, hippocampi, or brain cortices will be dissected, dissociated and grown in tissue culture incubators.

Transgenic mice produced for these studies will be produced at the Transgenic Facility at the MGH (under the supervision of JP Julien; protocol #2310) or at the Royal Victoria Hospital (under the supervision of A Peterson; protocol #3596) and

the transported to the MNI under aseptic conditions for continued housing and analysis. Tail samples are collected by staff at the MNI Animal Facility under halothane anaesthesia on animals which are weaned and at least 6 weeks old. No more than 1 cm of tail will be removed from any animal over its lifetime. DNA will be extracted from tails using standard techniques and then genotyped by polymerase chain reaction. We have already characterized mice with more severe gene defects than the ones planned for these studies and have observed no evidence of physical or behavioral abnormalities. We anticipate a milder, more targeted phenotype in the new mice we will create (using the dopamine-b-hydroxylase promoter to drive expression of p75NTR in selected neuronal populations) but will monitor new strains for signs of stress and behavioral dysfunction. We maintain rigorous records of lineage and of any behavioral or physical impairment.

**b) Field Studies – Provide all relevant details. Procedures to be conducted (e.g. surgery, blood collection, tagging etc.) should be described above.**

**Method of capture/restraint, duration of captivity, potential injury/mortality, monitoring frequency:**
N/A

**Transportation and /or housing of animals in the field:**
N/A

**Special handling required:**
N/A

**Capture of non-target species, potential injury/mortality:**
N/A

**Will captured animals be released at or near the capture site** YES ☐ NO ☐
**If not, specify if they will be relocated to other locations and/or populations.**
N/A

**Describe any potential ecological disruption this study may cause:**
N/A

**It is the responsibility of the investigator to obtain all necessary permits for work with wildlife. Copies of these permits must be forwarded to the Research Ethics Officer (Animal Studies) when they are obtained.**

**c) Pre-Anaesthetic/Anaesthetic/Analgesic Agents: List all drugs that will be used to minimize pain, distress or discomfort. Table will expand as needed. (*complete 1st column pressing 'enter' after each species, then 2nd column...)**

| Species | Agent | Dosage (mg/kg) | Total volume(ml) per administration | Route | Frequency |
|---|---|---|---|---|---|
| Mouse | halothane | | | inhalation | once, during tailing of mice > 6 weeks of age for genotyping |

**d) Administration of non-anaesthetic substances: List all non-anaesthetic agents under study in the experimental component of the protocol, including but not limited to drugs, infectious agents, viruses (table will expand as needed). (*complete 1st column pressing 'enter' after each species, then 2nd column...)**

| Species | Agent | Dosage (mg/kg) | Total volume (ml) per administration | Route | Frequency |
|---|---|---|---|---|---|
| | | | | | |

**e) Endpoints : 1) Experimental – for each experimental group indicate survival time .**
**2) Clinical - describe the conditions, complications, and criteria (e.g. >20% wt.loss, tumour size, vocalizing, lack of grooming) that would lead to euthanasia of an animal before the expected completion of the experiment (specify per species and project if multiple projects involved).**

As per SOP

**Specify person(s) who will be responsible for animal monitoring and post-operative care**
**Name:** Kathleen Dickosn **Phone#:** 398-3212

**f) Method of Euthanasia – According to CCAC guidelines, justification must be provided for use of any physical method of euthanasia without prior use of anaesthesia (justify here):**

In order to avoid any interference of anaesthetic with the molecular biology of the neuronal proteins which we are investigating, rats and mice are not anaesthetized prior to sacrifice.

**Specify Species**

| | |
|---|---|
| | ☐ **anaesthetic overdose, list agent/dose/route:** |
| Rabbit | ☒ **exsanguination with anaesthesia, list agent/dose/route:** ketamine/xylazine (30-50mg/kg; 5 mg/kg) |
| Rat (embryo) | ☒ **decapitation without anaesthesia** ☐ **decapitation with anaesthesia,list agent/dose/route:** |
| Mouse | ☒ **cervical dislocation** |
| Rat (adult) | ☒ **$CO_2$ chamber** |
| | ☐ **other (specify)** |
| | ☐ **not applicable (explain)** |

**11. Category of Invasiveness:** B ☐ C ☐ D ☒ E ☐

**Categories of Invasiveness (from the CCAC *Categories of Invasiveness in Animal Experiments*). Please refer to this document for a more detailed description of categories.**

**Category A: Studies or experiments on most invertebrates or no entire living material.**

**Category B: Studies or experiments causing little or no discomfort or stress.** ***These might include holding animals captive, injection, percutaneous blood sampling, accepted euthanasia for tissue harvest, acute non-survival experiments in which the animals are completely anaesthetized.***

**Category C: Studies or experiments involving minor stress or pain of short duration.** ***These might include cannulation or catheterizations of blood vessels or body cavities under anaesthesia, minor surgery under anaesthesia, such as biopsy; short periods of restraint, overnight food and/or water deprivation which exceed periods of abstinence in nature; behavioural experiments on conscious animals that involve short-term stressful restraint.***

**Category D: Studies or experiments that involve moderate to severe distress or discomfort.** ***These might include major surgery under anaesthesia with subsequent recovery, prolonged (several hours or more) periods of physical restraint; induction of behavioural stresses, immunization with complete Freund's adjuvant, application of noxious stimuli, procedures that produce pain, production of transgenics (in accordance with University policy).***

**Category E: Procedures that involve inflicting severe pain, near, at or above the pain threshold of unanaesthetized, conscious animals.** ***Not confined to but may include exposure to noxious stimuli or agents whose effects are unknown; exposure to drugs or chemicals at levels that (may) markedly impair physiological systems and which cause death, severe pain or extreme distress or physical trauma on unanaesthetized animals.*** **According to University policy, E level studies are not permitted.**

**12. Potential Hazards to Personnel and Animals It is the responsibility of the investigator to obtain the necessary Biohazard and/or Radiation Safety permits before this protocol is submitted for review. A copy of these certificates must be attached, if applicable.**

**No hazardous materials will be used in this study:** ☐

**a) Indicate which of the following will be used in animals:**

☐ **Toxic chemicals** ☐ **Radioisotopes** ☐ **Carcinogens** ☐ **Infectious agents** ☒ **Transplantable tumours**

**b) Complete the following table for each agent to be used (use additional page as required).**

| **Agent** | B cell hybridomas | | |
|---|---|---|---|
| **Dosage** | 1 million cells/animal | | |
| **Route of administration** | injection | | |
| **Frequency of administration** | once per animal | | |
| **Duration of administration** | once | | |
| **Number of animals involved** | 10 per experiment (50 per year) | | |
| **Survival time after administration** | up to 4 weeks | | |

**c) After administration the animals will be housed in:**

☒ **the animal care facility**

☐ **laboratory under supervision of laboratory personnel**

**Please note that cages must be appropriately labeled at all times.**

**d) Describe potential health risk (s) to humans or animals:**
None

**e) Describe measures that will be used to reduce risk to the environment and all project and animal facility personnel:**

**13. Reviewer's Modifications (to be completed by ACC only): The Animal Care Committee has made the following modification(s) to this protocol during the review process. Please make these changes to your copy. You must comply with the recommended changes as a condition of approval.**

**McGILL UNIVERSITY**
**UNIVERSITY ANIMAL CARE COMMITTEE**

Standard Operating Procedure #UACC-4 October 2001 form version

# TRANSGENIC OR KNOCKOUT/IN BREEDING

## 1. *INTRODUCTION*

Standard Operating Procedures (SOPs) provide a detailed description of commonly used procedures. SOPs offer investigators an alternative to writing detailed procedures on their protocol forms. Any deviation from the approved procedures must be clearly described and justified in the Animal Use Protocol form. Approval of the protocol indicates approval of the deviation from the SOP for that project only.

A signed SOP (pages up to signature) must be attached to the Animal Use Protocol form. The relevant SOP number must be referred to in the Procedures section.

## 2. *INFORMATION REQUIRED*

| |
|---|
| 2.1 **Species:** Mouse<br>**Background strain:** C3H<br>**DNA construct injected: Dopamice B Hydroxylase promoter driving expression of the p75 neurotrophin receptor intracellular domain.**<br>**Gene locus removed:** |
| 2.2 **Supplier:** Please include a complete address, contact person, phone, fax and email information. Note that a Certificate of health must be available to FACC personnel PRIOR to animal arrival.<br>1. Commercial:<br>2. Academic: In House |
| 2.3 **Phenotype:** Include any trait included in a published article or reported to you by the originating investigator that may affect either the breeding, physical ability of the animal to move, eat or drink, or result in a decreased lifespan.<br>1. Heterozygotes: related strains show ptosis, loss of peripheral sympathetic and sensory neurons, loss of CNS neurons. No behavioral phenotype has been noted.<br>2. Homozygotes: will not be produced |
| 2.4 **Distress:** Detail plans to monitor and alleviate distress.<br>**Animals observed every 24 hours for signs of difficulty in feeding/breathing once born. One week after birth, monitor every 72 hours for signs of peripheral neuropathy.** |
| 2.5 **Endpoints:** Describe endpoints to be used in monitoring distress. These should include physical |

No anaesthesia is required for the following methods (ear tag, tail snip for DNA analysis).
For tail snipping, place the tail between 2 blocks of ice (vasoconstriction and some mild analgesia). Use a fresh scalpel blade to transect tail at a 90 degree angle. Following the amputation, compress tip using manual pressure for 30-45 seconds to stop hemorrhaging.
For a single rat lifetime:
Maximum length of tail obtained : 1.0 cm
Recommended number of transections: 2

**2. Rats older than 2 weeks of age:**

Anaesthesia is required. Same procedure as above. Analgesia usually not required however, based on individual needs.

N.B. If hemorrhaging persists despite manual compression, apply a silver nitrate stick to the tip of the tail.

McGILL UNIVERSITY
UNIVERSITY ANIMAL CARE COMMITTEE

Standard Operating Procedure #UACC-4 October 2001 form version

# TRANSGENIC OR KNOCKOUT/IN BREEDING

## 1. *INTRODUCTION*

Standard Operating Procedures (SOPs) provide a detailed description of commonly used procedures. SOPs offer investigators an alternative to writing detailed procedures on their protocol forms. Any deviation from the approved procedures must be clearly described and justified in the Animal Use Protocol form. Approval of the protocol indicates approval of the deviation from the SOP for that project only.

A signed SOP (pages up to signature) must be attached to the Animal Use Protocol form. The relevant SOP number must be referred to in the Procedures section.

## 2. *INFORMATION REQUIRED*

| |
|---|
| 2.1 **Species:** Mice<br>**Background strain:** C57Bl6; C57Bl6xSV129; C57Bl6; CD1<br>**DNA construct injected:**<br>**Gene locus removed:** NFkB; p75 exon3 null; p75-ICD; Traf-6 |
| 2.2 **Supplier:** Please include a complete address, contact person, phone, fax and email information. Note that a Certificate of health must be available to FACC personnel PRIOR to animal arrival.<br><br>1. Commercial:<br><br>2. Academic: In house colonies |
| 2.3 **Phenotype:** Include any trait included in a published article or reported to you by the originating investigator that may affect either the breeding, physical ability of the animal to move, eat or drink, or result in a decreased lifespan.<br><br>1. Heterozygotes: NFkB, p75-intracellular domain, and Traf-6 mice are normal.<br><br>2. Homozygotes: Normal |
| 2.4 **Distress:** Detail plans to monitor and alleviate distress.<br><br>**Animals are observed every 72 hours for signs of peripheral neuropathy.** |
| 2.5 **Endpoints:** Describe endpoints to be used in monitoring distress. These should include physical and behavioural traits.<br><br>Skin ulcerations |

amputation, compress tip using manual pressure for 30-45 seconds to stop hemorrhaging.
For a single rat lifetime:
Maximum length of tail obtained : 1.0 cm
Recommended number of transections: 2

**2. Rats older than 2 weeks of age:**

Anaesthesia is required. Same procedure as above. Analgesia usually not required however, based on individual needs.

N.B. If hemorrhaging persists despite manual compression, apply a silver nitrate stick to the tip of the tail.

# MCGILL UNIVERSITY FACULTY OF MEDICINE

## STANDARD PROTOCOL FOR GENERATION OF POLYCLONAL ANTIBODIES IN RABBITS

1. **Animal handling:** All procedures, including immunization and blood collection are performed by animal health technicians (AHT) or by other persons determined by the Facility Animal Care Committee to be competent by training or experience.

   Indicate here who will be handling the animals: Animal care technicians at the Royal Victoria Hospital.

2. **Antigen used:** Describe the antigen(s) used / maximum of two (2) rabbits per antigen

   GST-fusion protein containing fragments of NRAGE and MAGE gene products

   **Immunogen resuspended in:** (describe: SDS-PAGE suspension, PBS, detergent content, others)
   The immunogen will be resuspended in PBS (150 ul total volume).

3. **Serum sampling prior to immunization:** At DAY 0, a pre-immune sample will be collected from each animal by drawing a 10 ml sample (or less) from the central artery of the ear.

4. **Immunization:** The following emulsion will be prepared:

   a solution of NRAGE GST-fusion protein in PBS (150ul) will be dissolved in
   (quantity(ies) and product(s)

   150 ul of Titermax-Gold Others
   (qty) (specify, e.g. Ribi, Titermax, others)

   Injection sites will be shaved and swabbed with 70% alcohol prior to injection. Injection sites are inspected by the principal investigator or a suitable designate at least three times per week after each injection. Any lesions that develop are reported immediately to the AHT or veterinarian. The resulting emulsion will be injected either sub-cutaneously (s.c.) at four sites in the back region (0.25 ml at each site) or intramuscularly, 1 ml per rabbit, 0.5 ml per rear leg, in the caudal thigh muscle.

# CURRICULUM VITAE

# Asha BHAKAR

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Fluent in English and French

## EMPLOYMENT

*January 1996 to present*

**Ph.D Student**, Department of Neurology & Neurosurgery
Montreal Neurological Institute, McGill University, Canada
Project Area: Molecular Neurosciences

- Numerous projects using different approaches to reveal themechanisms of survival and death in nervous tissue.
- Patent application and licencing agreement.
- Direct supervision of undergraduate and graduate students.

*January 1999 to present*

**Scientific Consultant**, Drug Discovery Unit
Aegera Therapeutics Inc., Montréal, Canada

- Technical and analytical consultancy contract to help implement novel screening technologies.

*June 1995 to Aug 1995*

**Research Assistant**, Department of Biochemistry
McGill University, Canada (Dr M. Tremblay)

- Promoter analysis and deletion mutant generation for a murine receptor type protein phosphatase, NU-3.

*January 1995 to May 1995*

**Research Assistant**, Departments of Biochemistry & Experimental Medicine McGill Cancer Center, McGill University, Canada (Dr M. Zannis-Hadjopoulos, Director)

- Structure and sequence analysis of *ors12*, a mammalian autonomously replicating sequence.

*May 1994 to August 1994*

**Research Assistant**, Department of Experimental Medicine
Meakins-Chrisite Laboratories, McGill University, Canada (Dr W. Powell)

- Chemotactic analysis of 5-oxo-eicosanoid metabolites on human neutrophils.
- Publication.

## EDUCATION

| | |
|---|---|
| *January 1996 to present* | **Ph.D. Neurological Sciences,** McGill University<br>Supervisor: Dr Philip Barker<br>Title - Signaling pathways involved in the regulation of neuronal survival and apoptosis. |
| *September 1991 to December 1995* | **B.Sc. Honours Biochemistry,** McGill University |

## AWARDS

| | | |
|---|---|---|
| *2002 to 2003* | Standard Life Dissertation Fellowship | |
| *1997 to present* | Doctoral Research Award from the Medical Research Council of Canada (5 year tenure). | |
| *1996 to 1997* | Doctoral Studentship from the Rick Hansen Man in Motion Foundation. | |
| *1991 to 1995* | Undergraduate Awards: | Canada Science Scholarship<br>J.W. McConnell Award<br>Balfour Scholarship |

## INTERESTS AND ACHIEVEMENTS

During the summer of 1991, received full scholarship for and toured with National Youth Orchestra of Canada. As a founding member of the Assiniboine String Quartet, competed at the finals of the Canadian National Music Festival (Aug 1991). Member of the McGill Symphony Orchestra, 1992-1993. Founder of the Mont-Royal String Quartet, 2002-2003.

Enjoy squash, travel, hockey, and gourmet food.

## REFERENCES

Dr Philip Barker, Montreal Neurological Institute, Montreal, Quebec, (514) 398-3064

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Dr Timothy Kennedy, Montreal Neurological Institute, Montreal, Quebec, (514) 398-7136 timothy.kennedy@mcgill.ca

Dr Peter McPherson, Montreal Neurological Institute, Montreal, Quebec, (514) 398-7355

peter.mcpherson@mcgill.ca

## RESEARCH RECORD

### (a) Publications

**Bhakar AL**, Howell JL, Paul CP, Salehi AH, Becker EBE, Said F, Bonni A, and Barker PA. Apoptosis Induced by p75NTR Requires Jun Kinase-dependent Phosphorylation of Bad. Submitted to J Neurosci. Aug 2003.

Vaillancourt F*, Grapes MGR*, **Bhakar AL**, Dorval G, Roux PP, and Barker PA. TRAF4 Is A p75 Neurotrophin Receptor Binding Protein That Alters Intracellular Trafficking Of The Receptor. Submitted to the J Biol Chem 2003.

Hussain NK, Montarop Y, **Bhakar AL**, Metzler M, Ferguson SS, Hayden MR, McPherson P, and Kay BK. A Role for ENTH/ANTH Domains in Tubulin Binding and Implications for their Role in Neurite Outgrowth. Accepted in J Biol Chem. 2003.

**Bhakar AL**, Tannis LL, Zeindler C, Russo MP, Jobin C, Park DS, McPherson S, and Barker PA. Constitutive Nuclear Factor-kB Activity Is Required For Central Neuron Survival. J Neurosci. 2002 Oct 1;22(19):8466-75.

Roux PP, **Bhakar AL**, Kennedy TE, Barker PA. The p75 Neurotrophin Receptor Activates Akt (Protein Kinase B) Through A Phosphatidylinositol 3-Kinase-Dependent Pathway. J Biol Chem. 2001 Jun 22;276(25):23097-104.

Salehi AH, Roux PP, Kubu CJ, Zeindler C, **Bhakar A**, Tannis LL, Verdi JM, Barker PA. NRAGE, A Novel MAGE Protein, Interacts With The p75 Neurotrophin Receptor And Facilitates Nerve Growth Factor-Dependent Apoptosis. Neuron. 2000 Aug;27(2):279-88.

**Bhakar AL**, Roux PP, Lachance C, Kryl D, Zeindler C, Barker PA. The p75 Neurotrophin Receptor (p75NTR) Alters Tumor Necrosis Factor-Mediated NF-KappaB Activity Under Physiological Conditions, But Direct p75NTR-Mediated NF-KappaB Activation Requires Cell Stress. J Biol Chem. 1999 Jul 23;274(30):21443-9.

Ladiwala U, Lachance C, Simoneau SJ, **Bhakar A**, Barker PA, Antel JP. p75 Neurotrophin Receptor Expression On Adult Human Oligodendrocytes: Signaling Without Cell Death In Response To NGF. J Neurosci. 1998 Feb 15;18(4):1297-304.

Majdan M, Lachance C, Gloster A, Aloyz R, Zeindler C, Bamji S, **Bhakar A**, Belliveau D, Fawcett J, Miller FD, Barker PA. Transgenic Mice Expressing The Intracellular Domain Of The p75 Neurotrophin Receptor Undergo Neuronal Apoptosis. J Neurosci. 1997 Sep 15;17(18):6988-98.

Powell WS, MacLeod RJ, Gravel S, Gravelle F, **Bhakar A**. Metabolism and Biologic Effects of 5-Oxoeicosanoids on Human Neutrophils. J Immunol. 1996 Jan 1;156(1):336-42.

## (b) Patents and Contracts

PS McPherson, AR Ramjan, PA Barker, and **A Bhakar**. Regulation of JNK Activity by Modulation of the Interaction Between the Endocytic Protein Endophilin and the Germinal Center Kinase Like Kinase (GLK) **Patent pending** 60/286049 «US».

**A Bhakar**, S McPherson, and P Barker. Co-inventor of a transgenic mouse. **Licencing agreement** (Biopharmaceutical Company) «US».

## (c) Abstracts

**Bhakar AL**, Salehi AH, Paul CP, Howell JL, and Barker PA. Signaling Mechanisms Initiated by the p75NTR Neurotrophin Receptor, an Atypical TNFR Superfamily Member. Keystone Symposia. Molecular Mechanisms of Apoptosis. **Banff AB, 2003**.

**Bhakar AL**, Tannis LL, Zeindler C, Russo MP, Jobin C, Park DS, MacPherson S, and Barker PA. Constitutive Nuclear Factor-kB Activity is Required for Central Neuron Survival. Keystone Symposia. NF-kappaB: From Bench to Bedside. **Keystone CO, 2002**.

Roux PP, **Bhakar AL**, Kennedy TE, and Barker PA. The p75 Neurotrophin Receptor (p75NTR) Activates Akt (PKB) Through a PI3-Kinase Dependent Pathway. Annual Meeting of the Society for Neuroscience, **New Orleans LA, 2000**.

Roux PP, **Bhakar AL**, Kennedy TE, and Barker PA. The p75 Neurotrophin Receptor (p75NTR) Activates Akt (PKB) Through a PI3-Kinase Dependent Pathway in Neurons and in Non-Neuronal Cells. NGF and Related Molecules Conference, **Montreal QC, 2000**.

Salehi AH, Roux PP, Kubu CJ, Zeindler C, **Bhakar A**, Tannis LL, Verdi JM, and Barker PA. Nrage, a Novel Mage Protein, Interacts with the p75 Neurotrophin Receptor and Facilitates Nerve Growth Factor Dependent Apoptosis. NGF and Related Molecules Conference, **Montreal QC, 2000**.

**Bhakar AL**, Zeindler C, MacPherson S, Tannis LL, and Barker PA. NF-kB Transcriptional Activity in Intact Brain and Primary Neurons: Analysis of a Transgenic Reporter Strain. Annual Meeting of the Society for Neuroscience, **Miami FL, 1999**.

**Bhakar AL**, Roux PP, and Barker PA. The Low Affinity Neurotrophin Receptor, p75, Does Not Activate NF-kB in Several Immortal Cell Lines. Annual Meeting of the Society for Neuroscience, **New Orleans LA, 1997**.

MacPhee IJ, **Bhakar AL**, Barker PA. Brain-Derived Neurotrophic Factor Binding to the p75 Neurotrophin Receptor Reduces TrkA Signaling While Increasing Serine Phosphorylation in the TrkA Intracellular Domain. Canadian Neuroscience Network: Rick Hansen Man In Motion Meeting, **Vancouver BC, 1997**.