BH3-Domain-Only Proteins are Key Regulators of p75NTR-Mediated Apoptosis

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Abstract: The p75 neurotrophin receptor (p75NTR) is a member of the Tumor Necrosis Factor Receptor (TNFR) superfamily. Similar to TNFR members, p75NTR has been reported to mediate programmed cell death (PCD) in a variety of systems, including during development and in response to central nervous system (CNS) injury. However, in contrast to TNFR members, which typically signal via Caspase 8 to elicit cell death, studies conducted in our laboratory have demonstrated that p75NTR induces cell death via an intrinsic mitochondrial death cascade involving c-Jun N-terminal kinase (JNK) activation, mitochondrial cytochrome *c* release and activation of Caspases 9, 3 and 6.

In an effort to determine the mechanism by which JNK communicates with the mitochondria, we examined downstream targets of JNK including the BH3-domain-only proteins. BH3-domain-only proteins are members of the Bcl-2 family of proteins, which have been dubbed mitochondrial gate-keepers, referring to their ability to regulate release of mitochondrial proteins such as cytochrome *c* in response to apoptotic stimuli. Our studies revealed that p75NTR overexpression does not transcriptionally upregulate BH3-domain-only proteins, rather, it results in phosphorylation of BH3 proteins, Bad and Bim_{EL} at Serines 128 and 65, respectively, in a JNK-dependent manner. Furthermore, through loss of function studies employing RNA interference constructs targeting either Bad or Bim, as well as Bad S128A- or Bim_{EL} S65A dominant negative constructs, we demonstrated that Bad is critical for p75NTR-mediated apoptosis, while Bim_{EL} may contribute but is less critical. Together, these studies reveal key roles for BH3-domain-only proteins in p75NTR-mediated apoptosis.

Les protéines à domaine BH3 unique sont des éléments clefs de la voie apoptotique induite par p75NTR

Résumé : Le récepteur aux neurotrophines p75 (p75NTR) appartient à la superfamille des récepteurs au TNF (TNFR). De la même façon que les autres membres de cette superfamille, p75NTR peut provoquer la mort cellulaire programmée (ou apoptose) dans nombre de circonstances dans le système nerveux central, en particulier au cours du développement ou suite à des blessures. Cependant, contrairement aux autres membres de la superfamille des TNFR, qui induisent l'apoptose en activant une voie de signalisation impliquant la Caspase 8 (ou voie apoptotique extrinsèque), des études menées au laboratoire ont montré que p75NTR induit l'apoptose via l'activation d'une voie de signalisation mitochondriale (ou voie apoptotique intrinsèque) qui implique l'activation de la kinase Jun (JNK) et aboutit à la libération du cytochrome c mitochondrial et à l'activation des Caspases 9, 3 et 6.

Dans le but de déterminer le mécanisme par lequel JNK communique avec la mitochondrie, nous avons recherché quelles étaient les cibles de JNK parmi les protéines à domaine BH3 unique. En effet, les protéines à domaine BH3 unique sont des membres de la famille Bcl2, aussi appelées «gardiens mitochondriaux» puisqu'elles sont capables de réguler la libération de protéines mitochondriales telles que le cytochrome c en réponse à des signaux apoptotiques. Les résultats de notre étude ont montré que la surexpression de p75NTR n'active pas la transcription des protéines à domaine BH3 unique mais provoque la phosphorylation de deux d'entre elles, Bad et Bim_{EL}, sur les sérines 128 et 65 respectivement, et ce de façon JNK-dépendante. En outre, des études de perte de fonction, menées grâce à l'utilisation d'ARNi ciblant Bad et Bim, ainsi que JNK, et des études reposant sur l'utilisation d'un dominant négatif de Bim, Bim_{EL} S65A, nous

ont permis d'établir que Bad est crucial pour le déclenchement de l'apoptose induite par p75NTR, tandis que Bim_{EL} semble également être impliqué mais dans une moindre mesure. Ensemble, ces résultats révèlent l'importance des protéines à domaine BH3 unique dans l'induction de l'apoptose par p75NTR.

Contribution of Authors

Chapter 2: BH3-Domain-Only Proteins are Key Regulators of p75NTR-Mediated Apoptosis

I. Asha L. Bhakar, Jenny L. Howell, Christine E. Paul, Amir H. Salehi, Esther B. E. Becker, Farid Said, Azad Bonni and Philip A. Barker. (2003) **Apoptosis Induced by p75NTR Overexpression Requires Jun Kinase-dependent Phosphorylation of Bad**. *J. Neurosci.* 23(36):11373-81.

As second author of the above indicated manuscript, I performed transient transfections and adenoviral infections of PC12 cells followed by either cell lysis or immunocytochemistry in order to determine the JNK-dependency of Bad oligomerization, as well as the requirement for Bad in p75NTR apoptotic paradigms (Figures 8 *C* and 9, respectively, Appendix). As well, I validated the Bad RNAi construct demonstrating a dose-dependent reduction in endogenous Bad in PC12 cells (Supplementary Fig. 1, Appendix). Furthermore, I treated PC12 cells with either staurosporine or p75NTR adenovirus, followed by fixation, in order to assess the incidence of cleaved caspase 3 positive cells with TUNEL positive cells (Supplementary Fig.2, Appendix).

II. Esther B. E. Becker, Jenny L. Howell, Yuki Kodama, Philip A. Barker, and Azad Bonni. **JNK-dependent Phosphorylation and Activation of Bim_{EL} Mediates $p75^{NTR}$-Induced Apoptosis.** In Press *J. Neurosci.*

As second author of the above indicated manuscript, I performed adenoviral infections of PC12 cells followed by cell lysis and western analysis in order to determine if p75NTR overexpression induces Bim phosphorylation at Serine 65 and if this phosphorylation is JNK-dependent (Figure 5 *A*, Appendix). In addition, I performed transient transfections and adenoviral infections, followed by fixation and immunocytochemistry, or lysis and subsequent western analysi, in order to determine the requirement for Bim in p75NTR-induced cell death, and more specifically the role for JNK-mediated Bim Serine 65 phosphorylation in p75NTR apoptotic systems (Figure 5 *C*, Appendix). I also validated Bim RNAi demonstrating a dose-dependent reduction in endogenous Bim levels in PC12 cells (Figure 5 *B*, Appendix).

III. I performed all experiments for the Paclitaxel studies.

List of Scientific Abbreviations

AIF	Apoptosis Inducing Factor
ANT	Adenine Nucleotide Exchanger
AP-1	Activating Protein-1
Apaf-1	Apoptotic Protease Activating Factor-1
ATP	Adenosine Triphosphate
BDNF	Brain-derived Neurotrophic Factor
BH	Bcl-2 Homology
CARD	Caspase Activation and Recruitment Domain
CNS	Central Nervous System
CRD	Cysteine-rich Domain
CRIB	Cdc42/Rac1 Interactive Binding
DAG	Diacylglycerol
DD	Death Domain
DED	Death Effector Domain
DISC	Death-inducing Signaling Complex
ECD	Extracellular domain
ER	Endoplasmic Reticulum
FADD	Fas-associated Death Domain
Gab-1	Grb-associated binder-1
GDP	Guanosine Diphosphate
GFP	Green Fluorescent Protein
GPI	Glycosyl Phosphotidylinositol
GTP	Guanosine Triphosphate
HEK	Human Embryonic Kidney
IAP	Inhibitor of Apoptosis
ICAD	Inhibitor of Caspase Activated DNase
ICD	Intracellular Domain
IgG	Immunoglobulin
IL-3	Interleukin-3
IMM	Inner Mitochondrial Membrane
IP_3	Inositol triphosphate
IRS	Insulin Receptor Substrate
JIP	JNK Interacting Protein
JM	Juxtamembrane
JNK	c-Jun N-terminal Kinase
KCl	Potassium Chloride
kDa	kilodalton
LINGO-1	LRR and Ig domain-containing, Nogo Receptor interacting protein
M6PR	Mannose-6-phosphate Receptor
MAG	Myelin-associated Glycoprotein
MAGE	Melanoma Antigen
MAPK(K)	Mitogen-activated Protein Kinase (Kinase)
MLK	Mixed Lineage Kinase

mNGF	Mature Nerve Growth Factor
NE	Neurotensin
NF-κB	Nuclear factor-kappa B
NGF	Nerve Growth Factor
NgR	Nogo Receptor
NRAGE	Neurotrophin Receptor-interacting MAGE Homolog
NRIF	Neurotrophin Receptor-interacting Factor
NT	Neurotrophin
OMgp	Oligodendrocyte-myelin Glycoprotein
OMM	Outer Mitochondrial Membrane
p75NTR	p75 Neurotrophin Receptor
PC12	Pheochromocytoma Cell line 12
PCD	Programmed Cell Death
PDK1	Phosphoinositide-dependent Kinase-1
PDZ	Present in PSD95, Dlg, and ZO-1
PI3-K	Phosphatidylinositol 3-Kinase
PIP2	Phosphoinositol 4,5-bisphosphate
PKA	Protein Kinase A
PKB	Protein Kinase B
PKC	Protein Kinase C
PLAD	Pre-Ligand Assembly Domain
PLC	Phospholipase C
PM	Plasma Membrane
PNS	Peripheral Nervous System
proNGF	Pro-Nerve Growth Factor
PSD	Post-Synaptic Density
PT(P)	Permeability Transition (Pore)
RhoGDI	Rho GDP Dissociation Inhibitor
RIP	Receptor-interacting Protein
ROCK	Rho Kinase
RTK	Receptor Tyrosine Kinase
SDS/PAGE	Sodium Dodecyl Sulfate/Polyacrylamide Gel Electrophoresis
Smac	Second Mitochondria-derived Activator of Caspase
TFD	Trophic Factor Deprivation
TNFR	Tumor Necrosis Factor Receptor
TRADD	TNFR-associated Death Domain
TRAF	TNFR-associated Factor
TRAIL	TNF-related Apoptosis-inducing Ligand
Trk	Tropomyosin-related Kinase
TUNEL	Terminal deoxynucleotidyl tranferase biotin-dUTP Nick End Labeling
VDAC	Voltage Dependent Anion Channel
Vps10p	Vacuolar Protein Sorting 10 Protein

Chapter 1

Introduction and Literature Review

Introduction

Seminal studies performed in the 1940s by Rita Levi-Montalcini and Victor Hamburger were propelled by the intriguing observation that cellular death is a naturally occurring phenomenon during embryonic development. Together they demonstrated that approximately 50% of motor neurons are destined to die during embryonic development and that loss or support of neurons could be determined by removal or addition of target tissue, respectively (Hamburger and Levi-Montalcini, 1949). This fostered the 'neurotrophic factor hypothesis,' a concept proposing that target-derived trophic factors are produced in limiting quantities resulting in selective support or elimination of innervating neurons. It is now well established that excess neurons are produced and eliminated during development in both the central and peripheral nervous systems (CNS, PNS).

In collaboration with Stanley Cohen, Levi-Montalcini proceeded to isolate the first target-derived trophic factor, nerve growth factor (NGF), providing the first direct support for the neurotrophic hypothesis (Levi-Montalcini, 1966). An array of trophic factors have since been discovered, including the well characterized neurotrophins (NTs), which have been shown to bind two classes of receptors: the survival-mediating Tropomyosin-related kinase (Trk) receptors, and the p75 neurotrophin receptor (p75NTR).

While neurotrophin signaling via Trk receptors remains an unequivocal pro-survival path, a defined p75NTR signaling schema has remained elusive. Studies to date have identified seemingly paradoxical roles for p75NTR ranging from survival to apoptosis (Carter *et al.*, 1996; Bhakar *et al.*, 1999; Casaccia-Bonnefil *et al.*, 1996; Frade

and Barde, 1999). Intensive efforts to elucidate p75NTR-dependent apoptotic signaling mechanisms have revealed requirement of a c-Jun N-terminal kinase (JNK)-dependent mitochondrial death pathway involving cytochrome *c* release and activation of Caspases 9, 3 and 6 (Bhakar *et al.*, 2003). However, the link between JNK activation and mitochondrial cytochrome *c* release remained unclear. This thesis will discuss the research conducted during my Master's studies in order to ascertain the mechanism by which JNK communicates with the mitochondria in p75NTR apoptotic paradigms, specifically via the actions of the BH3-domain-only proteins, Bad and Bim.

The Neurotrophins

The mammalian class of neurotrophins comprises NGF, brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and neurotrophin 4/5 (NT-4/5) (Snider, 1994). NTs are synthesized initially as 30-35kDa glycosylated precursors that are subject to calcium-dependent Furin and Convertase 1 and 2-mediated cleavage during transit through the secretory path. Cleavage at a conserved dibasic site releases a biologically active, 13-15kDa mature neurotrophin. Post-proteolytic processing, NTs exist as non-covalently associated homodimers that are secreted either via a constitutive secretory pathway, as is observed for NGF and NT-3, or via a regulated secretory pathway, as reported for BDNF (Mowla *et al.*, 1999).

Although originally characterized as target-derived trophic factors, the NTs have since been shown to mediate diverse biological functions including cell survival and differentiation, axonal outgrowth, synaptic plasticity, and apoptosis (Purves *et al.*, 1988; Oppenheim, 1991; Chao, 1992; Barbacid, 1994; Dechant and Barde, 1997; Poo, 2001).

Interestingly, NTs are upregulated in the nervous system following many forms of injury. Sciatic nerve transection and kindling-induced seizure in rats induce upregulation of mature NGF (mNGF) (Heumann *et al.*, 1987; Bengzon *et al.*, 1992), while upregulation of proNGF has been reported in Alzheimer's patients, and following spinal cord injury in rats (Fahnestock *et al.*, 2001; Beattie *et al.*, 2002). The consequence of NT upregulation post-trauma remains obscure and a subject of intense debate. Two contrasting theories have been put forth: one proposing that NT upregulation post-injury reflects the nervous system's attempt to salvage damaged neurons, and an opposing theory, which suggests that upregulated NTs may in fact be acting via apoptotic signaling cascades to eliminate damaged neurons. The observation that proNGF is upregulated post-injury in conjunction with its recent identification as an apoptotic p75NTR ligand, may help to clarify this issue (Lee *et al.*, 2001).

The Trk Receptor Tyrosine Kinases

Mature, homodimeric neurotrophins bind two classes of receptors, the Trk receptor tyrosine kinases, and the p75NTR. As members of a larger receptor tyrosine kinase family, Trk receptors are Type 1 transmembrane proteins that exhibit a distinct neuronal distribution (Martin-Zanca *et al.*, 1989). The Trk receptors include TrkA, TrkB and TrkC, which in the presence of p75NTR bind a cognate neurotrophin with high affinity ($K_d \sim 10^{-11}$M). Specifically, NGF associates with TrkA, while BDNF and NT-4/5 associate with TrkB and NT-3 associates with TrkC (Kaplan et al., 1991b; Klein et al. 1991b; Lambelle et al., 1991). Notably, in the absence of p75NTR, high concentrations of NT3 can activate TrkA or TrkB (Segal, 2003).

The extracellular domains (ECDs) of the Trk receptors are characterized by the presence of two cysteine-rich domains flanking a leucine-rich repeat, followed by two immunoglobulin (IgG)-like domains, the latter of which mediates NT binding (Urfer *et al.*, 1995, 1998). Ligand binding leads to receptor dimerization and subsequent activation via trans-phosphorylation of the associated Trk's activation loop (Jing *et al.*, 1992; Cunningham *et al.*, 1997). Two additional trans-phosphorylation events at tyrosine residues (Y490 and Y785 for TrkA) in the juxtamembrane (JM) domain and in the carboxy- terminus generate docking sites for signaling adaptors that lead to the activation of Ras, phosphotidylinositol 3-kinase (PI-3K) and phospholipase C γ (PLC) signaling pathways. (Kaplan and Miller, 2000; Roux and Barker, 2002).

In brief, Y490 serves as a docking site for two adaptor proteins, Shc and FRS-2 (Dikic *et al.,* 1995; Meakin *et al.*, 1999). The competition between Shc and FRS-2 establishes either a transient or sustained signal, which ultimately confers a proliferation or differentiation response, respectively. Although both pathways converge on a canonical Raf/MAPK kinase (MEK)/Mitogen activated protein kinase (MAPK) cascade, the subtleties in the recruitment of different adaptors such as Crk, as occurs for FRS-2, and activation of different G-proteins (Ras for Shc signaling versus Rap1 for FRS-2 signaling), as well as activation of distinct Rafs (Shc activates c-Raf while FRS-2 activates B-Raf), are critical for dictating the appropriate cellular response.

The PI3-K/Akt signaling pathway is regarded by many in the field as the most critical survival-promoting pathway in neurons (Kaplan and Miller, 2000). PI3-K is activated dually, directly through Ras association or through Trk recruitment of Shc-Grb2 complexes, which in turn associate with insulin receptor substrates (IRS)-1 and -2

and Grb-associated binder-1 (Gab-1) (Holgado-Madruga *et al.*, 1997). Gab-1 subsequently activates PI3-K leading to activation of phosphoinositide-dependent kinase-1 (PDK1), which upon activation, phosphorylates downstream Protein Kinase B (PKB), also termed Akt. Akt regulates various cell survival-regulating targets and is responsible for 'silencing' the pro-apoptotic BH3-domain-only protein Bad by inducing it's sequestration with adaptor protein 14-3-3 (Vanhaesebroeck and Alessi, 2000, Datta *et al.*, 1997).

Finally, association of PLCγ with Y785 of TrkA serves to regulate intracellular calcium levels and activity of protein kinase c (PKC) via cleavage of phosphoinositol 4, 5-bisphosphate (PIP2). Cleavage of PIP2 leads to production of diacylglycerol (DAG) and inositol triphosphate (IP_3) (Vetter *et al.*, 1991). While IP_3 increases cytosolic calcium levels via association with mitochondrial IP_3 receptors, DAG serves to activate PKC, which is involved in NT-mediated NT release and in synaptic plasticity (Canossa *et al.*, 1997; Bibel and Barde, 2000).

While Trk signaling via Ras, PI3-K and PLCγ pathways remain well defined, the same cannot be said for signaling schemas mediated by the p75NTR. The remainder of this chapter will now address aspects of the p75NTR.

The p75 Neurotrophin Receptor

An Introduction to p75NTR: A member of the TNFR superfamily

The Type 1 transmembrane p75NTR is a member of the Tumor Necrosis Factor Receptor (TNFR) superfamily. As the original member of the TNFR superfamily, p75NTR was initially cloned as the NGF receptor (Johnson *et al.*, 1986). A number of

TNFR members have since been identified, including TNFR1 and 2, Fas receptor (FasR/CD95) and CD40. To date, there are approximately 29 TNFR members (Dempsey *et al.*, 2003).

Structurally, TNFR members are characterized by the presence of a cysteine-rich ECD. Four cysteine-rich domains (CRDs 1-4) comprise p75NTR's ECD, of which CRD3 is thought to mediate neurotrophin binding (Yan and Chao, 1991; Baldwin *et al.*, 1992). In contrast to the Trk receptors which demonstrate high-affinity binding to cognate NTs, all NTs exhibit equal, low-affinity binding to p75NTR ($K_d \sim 10^{-9}M$) (Rodriquez *et al.*, 1990, 1992). Similarly, an invertebrate ligand cysteine-rich neurotrophic factor (CRNF) also exhibits nanomolar affinity for p75NTR (Fainzilber *et al.*, 1996). The recognition however that mature NTs poorly activate p75NTR signaling pathways provided the impetus to search for other potential physiological ligands and has since lead to the identification of proNGF as a bonafide p75NTR ligand ($K_d \sim 10^{-10}M$) (Lee *et al.*, 2001).

Despite the structural homology present within the ECDs of TNFR members, p75NTR binds dimeric, unrelated ligands while members of the TNFR superfamily bind trimeric, related, Type II transmembrane ligands. Indeed, this appears consistent with p75NTR's tendency to dimerize rather than trimerize, as is observed for other TNFR members. While in Fas, TNFR1 and CD40, CRD1 mediates ligand-independent receptor trimerization, the role of CRD1 in p75NTR dimerization remains uncertain (Chan *et al.*, 2000; Siegel *et al.*, 2000).

The disparate nature of p75NTR, with regard to the TNFR superfamily, is further exemplified at the level of ligand-induced changes in receptor aggregation. It has been established that TNFRs reside in the plasma membrane (PM) juxtaposed in a trimeric

complex via CRD1, in what is termed a Pre-Ligand Assembly Domain (PLAD). Upon binding of related, trimeric ligand, a conformational change is induced resulting in the separation of the receptor's aggregated cytoplasmic tails and subsequent recruitment of various signaling adaptors, all while maintaining extracellular contacts. In contrast, recent co-crystallization of NGF with the ectodomain of p75NTR provides evidence that association of dimeric NGF disrupts existing p75NTR dimers due to an imposed allosteric change which renders NGF's second p75NTR-binding site defective (He and Garcia, 2004). Notably, although dimeric NGF associates asymmetrically with a single p75NTR molecule, the TrkA binding site within the NGF core remains intact, supporting the possibility of the association of a tripartite complex involving NGF, TrkA and p75NTR (He and Garcia, 2004).

Interestingly, none of the TNFR members exhibit intrinsic catalytic activity, rather signals initiated by ligand-receptor association are transduced via the recruitment of adaptor molecules to the receptor's intracellular domain (ICD). p75NTR's ICD harbors a number of potential adaptor association motifs, including its highly conserved JM domain, a potential TNFR-associated factor (TRAF) binding site which possesses a motif similar to the TRAF6 consensus sequences found in CD40, a carboxy-terminal Type II death domain (DD), as well as an extreme carboxy-terminal PDZ (present in PSD95, Dlg, and ZO-1) binding motif (Large *et al.*, 1989; Pullen *et al.*, 1999; Liepinsh *et al.*, 1997; Sheng and Sala, 2001).

Signaling adaptors identified to date include neurotrophin receptor-interacting factors (NRIF) -1 and -2, which interact with the JM region and DD of p75NTR (Casademunt *et al.*, 1999; Benzel *et al.*, 2000). Structural homology with Zac1, a tumor

suppressor gene that regulates apoptosis and induces cell-cycle arrest, suggests that NRIF1/2 may regulate p75NTR-dependent apoptosis and/or cell-cycle arrest (Spengler *et al.*, 1997). Neurotrophin-receptor-interacting MAGE homolog (NRAGE), a MAGE family member identified in a yeast-two-hybrid screen, associates with p75NTR via its JM domain. Studies conducted by Salehi and colleagues identified roles for NRAGE in cell cycle exit as well as in NGF-mediated p75NTR-dependent PCD (Salehi *et al.*, 2000). Intriguingly, NRAGE competes with TrkA for binding to p75NTR suggesting that recruitment of NRAGE may terminate TrkA-mediated survival signals (Salehi *et al.*, 2000).

NADE, also identified in a yeast-two-hybrid screen, associates with p75NTR's DD in a NGF-dependent fashion and has been shown to mediate apoptosis in both mammalian cell lines and primary cultures (Mukai *et al.*, 2000; Park *et al.*, 2000b). Other identified cytosolic adaptors include TRAF 4 and 6, which promote survival, although some studies suggest that all TRAFs associate with p75NTR (Krajewska *et al.*, 1998; Khursigara *et al.*, 1999; Ye *et al.*, 1999b). Finally, helix 5 of p75NTR's DD, which exhibits homology to the GTPase activation domains in the wasp venom peptide mastoparan, has been shown to associate with Rho GDP dissociation inhibitor (RhoGDI), leading to activation of RhoA and subsequent neurite outgrowth inhibition (Yamashita and Tohyama, 2003).

Evidence of p75NTR Function

I. *The Paradox: Roles in Survival and Programmed Cell Death (PCD)*

The precise role of the p75NTR remains ambiguous and seemingly paradoxical as emerging studies implicate p75NTR in roles of regulation of neurite outgrowth inhibition to regulation of both survival and apoptosis (Yamashita and Tohyama, 2003; Carter *et al.*, 1996; Bhakar *et al.,* 1999; Casaccia-Bonnefil *et al.*, 1996; Frade and Barde, 1999).

Evidence in support of neurotrophin-mediated p75NTR-dependent apoptosis during development and in response to injury however, is substantial. *In vitro* studies performed by Chao and colleagues revealed that application of NGF to postnatal rat oligodendrocytes resulted in a sustained increase of intracellular ceramide and JNK activity, leading to apoptosis (Casaccia-Bonnefil *et al.,* 1996). *In vivo* studies employing transgenic mice expressing p75NTR's intracellular domain demonstrated profound reductions in numbers of sympathetic and peripheral sensory neurons (Majdan *et al.,* 1997). Pilocarpine-induced epileptic seizures in rats revealed significant correlation between transcriptional and translational upregulation of p75NTR within neurons of the hippocampus, piriform cortex and entorhinal cortex, and TUNEL-positive neurons, supporting a role for p75NTR in injury-induced apoptosis within the CNS (Roux *et al.,* 1999). Studies employing NGF and p75NTR function blocking antibodies revealed a requirement for both in apoptosis of retinal ganglion cells during development of the chick retina, and subsequent studies examining $p75NTR^{ExonIII-/-}$ mice revealed roles for p75NTR and NGF in the developing mouse retina and spinal cord (Frade *et al.,* 1996; 1999).

However, despite compelling *in vivo* evidence demonstrating the functional potency of p75NTR in PCD, only recently has a physiological apoptotic ligand been identified. Lee and colleagues demonstrated that proNGF binds to p75NTR with higher affinity than mNGF, while exhibiting only weak affinity for TrkA, and consistent with this observation, proNGF induced apoptosis in p75NTR-expressing smooth muscle cells more potently than mNGF. In light of this evidence, one can conclude that regulation of cell survival may therefore depend upon the ratio of proNTs to mNTs. Furthermore, the observation that proNTs comprise a large portion of the secreted NT pool and are upregulated in response to injury concomitant with p75NTR, supports the link between this ligand-receptor complex and injury-induced apoptosis (Lee *et al.*, 2001b, Beattie *et al.*, 2002).

Ironically, p75NTR has also been implicated in survival. Analogous to other TNFR members, which elicit either survival or death through nuclear factor-kappa B (NF-κB) activation or DD interactions, respectively, p75NTR has been reported to activate NF-κB in a number of cell types (Dempsey *et al.*, 2003; Carter *et al.*, 1996; Yoon *et* al., 1998; Bhakar *et al.*, 1999; Gentry *et al.*, 2000). Although direct p75NTR-mediated NF-κB activation appears modest relative to TNF-mediated activation, p75NTR enhances TNF-mediated NF-κB activation and direct p75NTR-mediated NF-κB activation can be enhanced following cellular stress (Carter *et al.*, 1996; Bhakar *et al.*, 1999). Finally, low levels of p75NTR have been reported to promote survival through the activation of PI3-K and Akt in a TrkA-independent manner (Roux *et al.*, 2001).

II. *p75NTR is a Trk Co-Receptor*

The apparent paradox of p75NTR's role in both survival and PCD may be attributed in part to p75NTR's ability to signal autonomously or function as a Trk co-receptor, whereby p75NTR augments Trk's affinity for its preferred ligand. Specifically, p75NTR enhances the affinity of TrkA for NGF, while decreasing affinity for NT-3. Similarly, p75NTR differentially modulates interactions of TrkB with BDNF and NT-4/5 (Bibel *et al.,* 1999). The recent co-crystallization of NGF with p75NTR's ectodomain supports the notion of the existence of a tripartite complex involving NGF, TrkA and p75NTR. He and Garcia clearly demonstrated that homodimeric NGF associates asymmetrically with a single p75NTR molecule while maintaining the structural conformation of NGF's TrkA binding site, suggesting that NGF may simultaneously occupy p75NTR and TrkA, permitting p75NTR augmentation of TrkA's ligand specificity (He and Garcia, 2004). Interestingly, the observation that NRAGE prevents TrkA association with p75NTR in order to elicit death, suggests that autonomous signaling via p75NTR initiates PCD, while action as a Trk co-receptor mediates survival, likely through suppression of p75NTR signaling (Yoon *et al.,* 1998; Salehi *et al.*, 2000). Notably however, many of the studies reporting p75NTR-mediated survival are independent of Trks suggesting that autonomous p75NTR signaling may also dictate cell survival (Cheng and Mattson, 1991; Shimohama *et al.*, 1993).

III. *Sortilin: A Required Co-Receptor for proNGF-Induced p75NTR-Mediated PCD*

The past few years have experienced a surge in the identification of novel p75NTR co-receptors. Although each new addition seemingly improves our

understanding of p75NTR function, each discovery yields new and perplexing questions. Such is the case for Sortilin, a member of the vacuolar protein sorting 10 protein (Vps10p) domain receptor family, recently identified as a required co-receptor for proNGF-induced p75NTR-dependent cell death (Nykjaer *et al.*, 2004). Nykjaer and colleagues revealed that proNGF associates with Sortilin directly via its prodomain and that Sortilin association with p75NTR is enhanced in the presence of proNGF. Furthermore, endocytosis of proNGF is permitted only in the presence of both receptors and proNGF selectively induces sympathetic neuronal death through p75NTR and not TrkA (Nykjaer *et al.*, 2004).

Although originally identified as a neurotensin (NE) receptor (Zsürger *et al.*, 1994), Sortilin has primarily been characterized as a sorting molecule involved in endoplasmic reticulum (ER)-golgi trafficking. Consistent with this role, Sortilin exhibits homology with established sorting receptors, Vps10p and mannose-6-phosphate receptor (M6PR) (Petersen *et al.*, 1997). Expressed initially as a 100kDa precursor, Sortilin is subject to furin-mediated cleavage in late Golgi compartments, which releases an amino-terminal propeptide, generating a 95kDa Type 1 glycoprotein. Processing is critical for Sortilin function since in its precursor form, the prodomain has been demonstrated to bind a resident Vps10p-like domain, sterically occluding ligand binding (Petersen *et al.*, 1999). Moreover, Sortilin remains unable to bind ligand post-cleavage until it reaches compartments for acid-assisted dissociation and clearance of the propeptide. Once activated, Sortilin may associate with ligands in the trans-Golgi network (TGN), in secretory vesicles or on the PM.

Interestingly, only 5% of Sortilin is expressed at the PM, however; exposure to secreted factors such as NE and insulin have been reported to induce translocation from an intracellular vesicular pool to the PM (Mazella *et al.,* 1998; Chabry *et al.,* 1993; Morris *et al.,* 1998). Sortilin recruitment to the PM has been hypothesized to scavenge excess NE and terminate NE signaling through subsequent endocytosis and lysosomal-mediated degradation (Petersen *et al.,* 1999). Studies examining mechanisms of Sortilin endocytosis have revealed a requirement for a C-terminal sequence 'YSVL,' which conforms to the classical YXXϕ motif that signals rapid endocytosis through coated pits of several other proteins including M6PR (Nielsen *et al.,* 2001). Furthermore, these studies revealed that following endocytosis, Sortilin travels to the TGN bypassing late endosomes, displaying only minor recycling to the PM. Intriguingly, recent investigations examining the kinetics and route of ligand-induced internalization of the p75NTR demonstrated clathrin-mediated endocytosis of the p75NTR-ligand complex, bypass of late endosomes and accumulation within non-acidified intracellular vesicles. Accordingly, p75NTR exhibited slow recycling to the PM. Collectively, the observed parallels in Sortilin and p75NTR endocytic properties provide additional evidence in support of the establishment of a Sortilin-p75NTR receptor complex (Bronfman *et al.,* 2003).

The identification of Sortilin as a required co-receptor for proNGF-induced p75NTR-dependent apoptosis has not surprisingly raised a number of interesting questions. Since Sortilin functions as a trafficking protein in the secretory pathway, one might posit that Sortilin facilitates enhanced secretion of proNGF post-injury. Alternatively, Sortilin in complex with proNGF may translocate to the PM where they

can then associate with p75NTR to form an apoptotic signaling complex, or rather proNGF itself may act as an extracellular cue to trigger PM insertion of intracellular Sortilin receptors, ultimately facilitating Sortilin-p75NTR association and subsequent ligand binding. The mechanism by which a proNGF-Sortilin-p75NTR complex is formed will undoubtedly be a subject of intense interest as it could provide a potential therapeutic target through which we can combat neurodegenerative disease, which is often characterized by aberrant PCD, and/or trauma-induced PCD.

IV. *A p75NTR, NogoR and LINGO-1 Complex Negatively Regulates Neurite Outgrowth*

Inhibition of neurite outgrowth in the CNS remains a major challenge in axon regeneration. Much of the axon inhibitory activity in the CNS has been associated with myelin-derived proteins, including Nogo66, myelin-associated glycoprotein (MAG), and oligodendrocyte-myelin glycoprotein (OMgp), all of which exert their effects through the Nogo receptor (NgR) to activate RhoA (Fournier *et al.*, 2001; Liu *et al.*, 2002; Kottis *et al.*, 2002). RhoA is a small GTPase that regulates actin polymerization in a state-dependent manner, such that in its active GTP-bound state, RhoA stabilizes the actin cytoskeleton thereby preventing growth cone collapse and neurite outgrowth (Schmidt and Hall, 2002). Recent studies demonstrating p75NTR-dependent RhoA modulation in response to MAG, despite the fact that MAG does not directly associate with p75NTR, raised the possibility that MAG may be signaling via a p75NTR co-receptor (Yamashita *et al.*, 2002). This observation in conjunction with the fact that glycosyl phosphotidylinositol (GPI)-linked NgR lacked an intracellular domain, thus suggesting requirement of an additional transmembrane protein with signaling capabilities, lead to

the identification of p75NTR as a NgR co-receptor (Wong *et al.*, 2002; Wang *et al.*, 2002a).

Consistent with a role in axonal outgrowth inhibition, $p75NTR^{ExonIII-/-}$ mice were unresponsive to myelin or myelin-derived proteins and truncated p75NTR lacking its ICD attenuated myelin-induced outgrowth inhibition when overexpressed in primary neurons (Wang *et al.*, 2002a). Furthermore, recent studies have shown that MAG or Nogo66 treatment promotes p75NTR association with RhoGDI, an inhibitor which sequesters RhoA. Relief of RhoA inhibition permits RhoA activation through exchange of GDP for GTP, and subsequent inhibition of outgrowth which is thought to proceed through pathways which suppress Rho kinase (ROCK) and that act upon the actin cytoskeleton (McKerracher and Winton, 2002). Notably, these effects are hampered by addition of NT, which inhibits RhoA activity (Yamashita and Tohyama, 2003). Intriguingly, p75NTR has also been implicated as a positive modulator of PNS myelination, a role that appears at odds with its role in the CNS (Cosgaya *et al.*, 2002).

Recently however, failure to reconstitute outgrowth inhibition *in vitro* using NgR and p75NTR together has lead to the identification of an additional co-receptor, LINGO-1 (LRR and Ig domain-containing, Nogo Receptor interacting protein). Together, NgR in association with p75NTR and LINGO-1 form a ternary complex capable of mediating axonal outgrowth inhibition (Mi *et al.*, 2004).

Mechanisms of Neuronal Apoptosis

I. *Pro-apoptotic TNFRs Activate an Extrinsic Death Cascade*

Neuronal apoptosis proceeds via two classical death mechanisms, an extrinsic pathway that is characterized by activation of a caspase cascade, and an intrinsic pathway that is characterized by organelle dysfunction (Dempsey *et al.*, 2003). Typically, the extrinsic pathway is employed by DD-containing TNFR members such as TNFR1 and Fas receptor (FasR). Despite observed similarities in TNFR family receptor activation and structure however, it is interesting to note that receptor recruitment of different cytosolic adaptors dictates unique cellular outcomes.

The apoptotic pathways activated by TNFR superfamily receptors are exemplified by the FasR. Binding of trimeric Fas ligand induces a conformational change in the FasR complex, leading to association of the DD-containing adaptor Fas Associated Death Domain (FADD), via DD-DD interactions. Procaspase 8, which possesses two carboxy-terminal death effector domains (DEDs), subsequently associates with the DED of FADD, resulting in the assembly of a death-inducing signaling complex (DISC) (Kischkel *et al.*, 1995; Boldin *et al.*, 1996; Muzio *et al.*, 1996). Aggregation within the DISC promotes cleavage of Caspase 8's prodomain rendering Caspase 8 active in what is termed a proximity model of caspase activation (Yang *et al.*, 1998). Upon activation, Caspase 8 acts as an executioner caspase to activate downstream effector caspases, Caspase-3, -6 and -7. Effector caspases mediate proteolytic cleavage of various cellular substrates including inhibitor of caspase activated DNase (ICAD). Subsequent release of DNases leads to DNA fragmentation, a hallmark of apoptotic death.

In contrast to FasR, TNFR1 has the ability to initiate either survival or death signals. In fact, TNFR1 most often induces the transcription and activation of inflammatory genes. This property reflects TNFR1's ability to recruit TNFR associated death domain (TRADD), also via DD-DD interactions, which in turn recruits TRAF2 and receptor-interacting protein (RIP). The assembled complex activates NF-κB and JNK signaling pathways, which initiate inflammatory rather than apoptotic responses (Hsu *et al.*, 1996). TRADD can also recruit FADD however, leading to the initiation of a Caspase 8-dependent apoptotic pathway. Thus, the resulting cellular outcome reflects a delicate balance between the two competing receptor complexes, a balance which in turn is regulated through levels of cFLIP. NF-κB transcriptionally upregulates cFLIP, which in turn serves to inhibit the assembled Caspase-8-FADD complex. Through this mode of regulation TNF-mediated apoptosis only occurs when NF-κB activation and subsequent cFLIP transcription are insufficient (Danial and Korsmeyer, 2004).

II. *Death Induced Via Trophic Factor Deprivation Activates an Intrinsic Death Cascade*

The intrinsic death pathway, which is characterized by organelle dysfunction such as that at the level of the ER or mitochondria, has been demonstrated primarily in trophic factor withdrawal death paradigms. Early studies conducted by Eugene Johnson's group employing a model of NGF deprivation in sympathetic neurons have elegantly demonstrated induction of an intrinsic apoptotic cascade requiring transcription and translation, JNK activation and subsequent activation of its target, the activating protein (AP)-1 transcription factor c-Jun, as well as mitochondrial cytochrome *c* release and activation of Caspase 9 (Martin *et al.*, 1988; Deshmukh *et al.* 1996; Deshmukh and

Johnson, 1998; Martinou *et al.*, 1999; Putcha *et al.*, 1999; Eilers *et al.*, 2001; Bruckner *et al.*, 2001; Harding *et al.*, 2001).

Upstream signaling events following NGF withdrawal appear to involve activation of GTPases Cdc42 and Rac1, which associate via a Cdc42/Rac1 interactive binding (CRIB) motif found in MAPKKKs such as MEKK1-MEKK4 and mixed lineage kinases (MLK)-2 and -3 (Bazenet *et al.*, 1998; Hirai *et al.*, 1996; Tibbles *et al.*, 1996; Sakuma *et al.*, 1997). Activation of MAPKKKs leads to initiation of a MAPK cascade in which MAPKKKs phosphorylate and activate MAPKKs such as MKK4/7, which in turn phosphorylate and activate MAPKs such as JNK, which in turn target AP-1 transcription factors such as c-Jun, ATF-2 and Elk-1 (Xu *et al.*, 2000; Sakuma *et al.*, 1997; Bruckner *et al.*, 2001).

Recent studies have revealed that following NGF deprivation in sympathetic neurons or potassium chloride (KCl) deprivation in cerebellar granule neurons, that c-Jun-mediates transcription of the BH3-domain-only proteins Bim_{EL} and Dp5/Hrk (Harris and Johnson, 2001; Whitfield *et al.*, 2001). The BH3-domain-only proteins have been the subject of intense investigation since they are thought to regulate mitochondrial membrane integrity, therefore indirectly regulating release of intermembrane space proteins including cytochrome *c*, apoptosis inducing factor (AIF), second mitochondria-derived activator of caspase (Smac/Diablo) and endonuclease G (Liu *et al.*, 1996a; Susin *et al.*, 1999; Du *et al.*, 2000; Verhagen *et al.*, 2000; Li *et al.*, 2001).

Cytochrome *c* release represents a critical step in the mitochondrial death path since upon its release it drives oligomerization of apoptotic protease activating factor-1 (Apaf-1), which in turn associates with Procaspase 9 via its caspase activation and

recruitment domain (CARD) (Zou *et al.*, 1997). Collectively, these proteins assemble a functional apoptosome that upon ATP hydrolysis activates Caspase 9 through cleavage of its prodomain (Shiozaki *et al.*, 2002). As an executioner caspase, Caspase 9 may then proceed to activate downstream effector caspases, Caspase-3, -6, and -7.

III. *The Extrinsic and Intrinsic Death Pathways are not Mutually Exclusive*

It is important to note however, that the extrinsic and intrinsic cell death pathways are not mutually exclusive. Until recently the only known mechanism of cross-talk was via a BH3-domain-only protein Bid. When subject to Caspase-8 mediated cleavage the resulting truncated product tBid was shown to translocate to the mitochondria where it induces cytochrome *c* release (Li *et al.*, 1998; Luo *et al.*, 1998). Recent investigations however, have revealed that mitochondrial events are required for apoptosis induced by TNF-related apoptosis-inducing ligand (TRAIL), which associates with members of the TNFR superfamily known as TRAIL receptors. Specifically, TRAIL-induced apoptosis requires Bax-dependent mitochondrial release of Smac/Diablo, but not cytochrome *c* (Deng *et al.*, 2002). Studies by the same group have also revealed that a JNK-dependent pathway is required for TNFα-induced apoptosis. The authors of the study show that JNK mediates a Caspase-8-independent cleavage of Bid to generate jBid. jBid in turn translocates to the mitochondria to induce preferential release of Smac/DIABLO, which in turn disrupts TRAF2-c-inhibitor of apoptosis (IAP)-1 complexes that function to prevent Caspase 8 activation (Deng *et al.*, 2003).

IV. *Mechanisms of p75NTR-Dependent Apoptosis*

Efforts in our lab have sought to determine the mechanism by which p75NTR elicits an apoptotic response. Does p75NTR induce death via an extrinsic pathway similar to other members of the TNFR superfamily or does p75NTR initiate an intrinsic, mitochondrial death response as observed in trophic factor deprivation paradigms? Accumulating evidence indicates quite convincingly that p75NTR-mediated cell death proceeds via an intrinsic, mitochondrial death cascade. Furthermore, studies suggest that p75NTR may in fact facilitate death induced through trophic factor deprivation. This is evidenced in sympathetic neurons derived from $p75NTR^{ExonIII-/-}$ mice which exhibit a significant delay in death following NGF deprivation, as compared to control littermates (Bamji *et al.*, 1998). Moreover, reductions in p75NTR expression in differentiated pheochromocytoma (PC12) cells and sensory neurons render these cells less sensitive to trophic factor withdrawal-mediated death (Barrett and Georgiou, 1996; Barrett and Bartlett, 1994).

Consistent with these observations, many of the signaling effectors activated in response to trophic factor withdrawal are also observed in p75NTR death paradigms. Oligodendrocytes undergoing p75NTR-dependent apoptosis demonstrate both Rac and JNK activation (Harrington *et al.*, 2002), as observed in sympathetic neurons subject to NGF withdrawal. p75NTR-mediated JNK activation and activation of its downstream target c-Jun has been observed in a number of systems, including oligodendrocytes, sympathetic neurons, hippocampal neurons, cortical neurons and PC12 cells (Casaccia-Bonnefil *et al.*, 1996; Yoon *et al.*, 1998; Aloyz *et al.*, 1998; Bamji *et al.*, 1998; Friedman, 2000; Troy *et al.*, 2002; Bhakar *et al.*, 2003; Roux *et al.*, 2001). Despite reports of c-Jun

activation however, Cre-lox conditional c-Jun knockouts suggest that while c-Jun is essential for sympathetic neuronal death induced by NGF withdrawal, it is not required for p75NTR-mediated death (Palmada *et al.*, 2002). Data from this same group also suggests that p75NTR-mediated death requires protein synthesis and although no evidence to date indicates a requirement for transcription, studies from our lab indicate that p75NTR also phosphorylates the AP-1 transcription factor ATF-2 (Bhakar, unpublished).

p75NTR activation has also been shown to lead to mitochondrial cytochrome *c* release, again analogous to modes of TFD-induced death. Similarly, expression of NRAGE, a p75NTR interactor that induces JNK-dependent death, also mediates cytochrome *c* release (Salehi *et al.*, 2002; Bhakar *et al.*, 2003). Furthermore, a number of studies have also demonstrated p75NTR-mediated activation of Caspase-9, -3 and -6, (Gu *et al.*, 1999; Wang *et al.*, 2001; Troy *et al.*, 2002; Bhakar *et al.*, 2003).

Finally, evidence to suggest that p75NTR does not elicit death in a manner analogous to other TNFR members include the observation that p75NTR does not associate with adaptor molecules FADD and TRADD, likely due to structural differences in the orientation of α-helices contained within p75NTR's DD, and consistent with failure to bind FADD or TRADD, p75NTR does not activate Caspase-8 (Wang *et al.*, 2001; Barker, unpublished results).

Prelude to Chapter 2

Numerous lines of evidence support a role for p75NTR in apoptosis both during development and in response to injury (Casaccia-Bonnefil *et al.*, 1996; Frade and Barde, 1999; Roux *et al.*, 1999; Beattie *et al.*, 2002). Significant progress has been made in understanding the signaling cascade that defines p75NTR-mediated cell death including recent work from our laboratory which demonstrates in multiple cell lines and in primary neurons that p75NTR activates a mitochondrial, intrinsic death pathway requiring JNK activation, mitochondrial cytochrome *c* release and activation of Caspase-9, -3 and -6 (Bhakar *et al.*, 2003). Despite this progress, key aspects of the pathway remain elusive, specifically the mechanism by which JNK communicates with the mitochondria to mediate cytochrome *c* release. The observation that JNK phosphorylates and activates AP-1 transcription factors c-Jun and ATF-2, of which the former has been shown to mediate transcription of the BH3-domain-only proteins Bim_{EL} and Dp5/Hrk in trophic factor withdrawal paradigms, suggests that p75NTR may also elicit death through transcriptional upregulation of this class of proteins (Harris and Johnson, 2001; Whitfield *et al.*, 2001).

However, recent studies in which neurons were deprived of trophic support or subjected to stress have reported post-translational modifications of the BH3-domain-only proteins, Bad and Bim (Konishi *et al.*, 2002; Donovan *et al.*, 2002; Lei and Davis, 2003; Putcha *et al.*, 2003). In light of these evidences, I sought to determine the involvement of BH3-domain-only proteins, Bad and Bim, in p75NTR-mediated apoptosis and more specifically, the role of post-translational modifications induced through

p75NTR overexpression. These investigations which formed the basis of my Master's research are discussed in Chapter 2.

Chapter 2

BH3-Domain-Only Proteins are Key Regulators of p75NTR-Mediated Apoptosis

An Introduction to the Bcl-2 Protein Family

BH3-domain-only proteins are members of a larger class of proteins termed the Bcl-2 family. The Bcl-2 proteins constitute a critical intracellular checkpoint in the intrinsic death pathway due to their ability to regulate the integrity of organelles such as the ER and mitochondria. The Bcl-2 family is characterized by three subsets of proteins, defined in part by the homology shared within conserved Bcl-2 homology (BH) domains. Anti-apoptotic members such as Bcl-2, Bcl-X_L, Bcl-W and MCL-1 display conservation throughout all four of the BH domains (BH1-4) (Boise *et al.*, 1993; Gibson *et al.*, 1996; Kozopas *et al.*, 1993). The "multidomain" pro-apoptotic members Bax and Bak possess BH1-3 domains and are sequestered either in the cytosol or at the outer mitochondrial membrane (OMM), respectively, through association with anti-apoptotic Bcl-2 members. Activation through release exposes their hydrophobic α-helical BH3-domain, which serves to target them to the ER or OMM where they can oligomerize and compromise organelle integrity (Wei *et al.*, 2001; Scorrano *et al.*, 2003). Finally, the pro-apoptotic BH3-domain-only proteins, which as the name suggests, share homology only in this nine amino acid segment with other members of the Bcl-2 family. To date, 10 mammalian BH3-domain-only proteins have been identified including Bad, Bik/Nbk, Bid, Bim/Bod, Bmf, Dp5/Hrk, NOXA and PUMA and one *C. elegans* BH3-only protein, EGL-1 (Puthalakath and Strasser, 2002).

I. *The Pro-survival Bcl-2 Members*

The Bcl-2 family of proteins exhibit a finely-tuned level of regulation, such that slight alterations in the balance of respective members dictate a particular cellular

outcome. Pro-survival Bcl-2-like members including Bcl-2 and Bcl-X_L protect cells against a broad range of insults and are essential for cell survival (Veis *et al.*, 1993). As suggested, these cytosolic proteins localize to the OMM, as well as to the ER and nuclear envelope, where they function to inhibit the multi-domain pro-apoptotic Bax and Bak proteins from multimerizing and compromising organelle integrity (Lithgow *et al.*, 1994; Krajewski *et al.*, 1994; O'Reilly *et al.*, 2001). In the case of the OMM, this prevents mitochondrial cytochrome *c* release and therefore subsequent assembly of a functional apoptosome involving Apaf-1 and Caspase-9 (Green and Reed, 1998). In regards to the ER, this may prevent rises in intracellular calcium, which at sufficiently high levels trigger an apoptotic response (McCormick *et al.*, 1997; Hacki *et al.*, 2000).

II. *The Pro-Apoptotic, Multi-Domain Proteins Bak and Bax*

Bak and Bax together constitute a critical segway to mitochondrial permeability, as evidenced by failure to release cytochrome *c* in Bak, Bax doubly deficient cells (Zong *et al.*, 2001; Cheng *et al.*, 2001; Wei *et al.*, 2001). Similarly, reports suggest that Bak and Bax localize to the ER where they compromise ER integrity, leading to calcium release and death (Nutt *et al.*, 2001). Activation of Bax and Bak involves exposure of their hydrophobic BH3-domains (Wei *et al.*, 2000; Antonsson *et al.*, 2001). Structural analysis reveals that in its monomeric, inactive form, Bax's hydrophobic pocket, formed by BH1, 2 and 3 domains, harbors its carboxy-terminal α9 helix, which is essential for mitochondrial targeting. Following activation, which may occur either through release from anti-apoptotic Bcl-2 members or direct activation by BH3-domain-only proteins, a

conformational change occurs displacing the carboxy-terminal α9 helix, thus facilitating mitochondrial targeting (Suzuki *et al.*, 2000).

The mechanism by which Bak and Bax compromise organelle integrity remains speculative and a number of hypotheses have been put forth. One model supports that oligomerized Bax or Bak may form pores capable of releasing cytochrome *c*. This theory is based primarily on the structural similarities apparent between the amphipathic BH3-α-helix, possessed in both multi-domain Bax and Bak proteins as well as BH3-domain-only proteins, and the pore-forming helices of bacterial toxins (Muchmore *et al.*, 1996). Evidence in favor of this model is the observation that recombinant Bax forms homo-oligomeric pores in liposomes harboring cytochrome *c*, leading to cytochrome *c* release. Alternatively, Bax and Bak have been proposed to interact with resident mitochondrial proteins such as voltage dependent anion channel (VDAC) or adenine nucleotide exchanger (ANT), which have been proposed to release cytochrome *c* directly or trigger the mitochondrial permeability transition (PT) (Shimizu *et al.*, 1999; Marzo *et al.*, 1998). The PT pore (PTP), of which VDAC and ANT are proposed components, is a high-conductance, non-selective inner mitochondrial membrane (IMM) channel whose opening increases IMM permeability to solutes up to 1500 Da, without necessarily inducing mitochondrial swelling or depolarization (Bernardi, 1999; Huser *et al.*, 1998). The existence of the PTP remains highly controversial however, since under some experimental conditions Bax was noted to induce PT, while in others it was not (Marzo *et al.*, 1998; Narita *et al.*, 1998; Pastorino *et al.*, 1998; Jurgensmeier *et al.*, 1998; Eskes *et al.*, 1998). Furthermore, oligomeric Bax appears to release cytochrome *c* in the absence of a PT, again suggesting that PT may not be required for intrinsic death pathways

(Gogvadze *et al.*, 2001). Although these discrepancies may reflect differences in experimental design, much remains to be resolved surrounding the role of Bax and Bak in the formation of the PT. Finally, global mechanisms of OMM permeabilization including altered membrane curvature and lipid pores are also being investigated.

III. *The Pro-Apoptotic BH3-Domain-Only Proteins*

The BH3-domain-only proteins function as upstream sentinels that selectively respond to proximal death cues. Alternative hypotheses have also been proposed for the mechanism by which BH3-domain-only proteins exert their biological functions. One model suggests that BH3-domain-only proteins autonomously induce cytochrome *c* release, without the involvement of Bax or Bak, but rather the involvement of lipids such as cardiolipin which are present in both the OMM and IMM (Kuwana *et al.*, 2002). Alternatively, BH3-domain-only proteins are proposed to interact and inhibit Bcl-2 family members, a model for which there is substantial evidence (Kelekar *et al.*, 1997; O'Connor *et al.*, 1998; Chang *et al.*, 1999; Puthalakath *et al.*, 2001). BH3-domain-only proteins have also been reported to directly activate Bax and Bak, thereby mediating Bax-Bak oligomerization and cytochrome *c* release. Data supporting this model regards the BH3-domain-only protein Bid. As discussed previously, Caspase-8 mediated cleavage of Bid generates tBid, a pro-apoptotic peptide which initiates apoptosis by activating mitochondrial Bax and Bak (Li *et al.*, 1998; Luo *et al.*, 1998). It is important to recall however that in Bax's monomeric, inactive state, its carboxy-terminal α9 helix lies within a hydrophobic pocket formed by BH1-3 domains. If BH3-domain-only proteins are to function by inserting their BH3-peptide into the groove formed by BH1-2 domains,

one must consider whether the tBid-Bax association is more favorable than the hydrophobic interactions stabilizing the α9 helix. Only if the tBid-Bax association is more energetically favorable would this permit disengagement of the peptide and subsequent mitochondrial targeting (Letai *et al.*, 2002; Suzuki *et al.*, 2000). Interestingly, Bid has been also reported to interact with anti-apoptotic Bcl-2 members. Finally, BH3-domain-only proteins have been reported to directly interact with ANT and VDAC to induce mitochondrial dysfunction and cytochrome *c* release (Zamzami *et al.*, 2000; Sugiyama *et al.*, 2002).

The actions of BH3-domain-only proteins are evident in both extrinsic and intrinsic death pathways and are regulated through transcriptional and post-translational means. Transcriptional regulation has been reported for the BH3-domain-only proteins Bim_{EL}, Dp5/Hrk, NOXA and PUMA. As indicated previously, Bim and Dp5/Hrk are transcriptionally upregulated in response to trophic factor withdrawal, while UV-irradiation has been reported to mediate p53-dependent transcription of NOXA and PUMA (Harris and Johnson, 2001; Whitfield *et al.*, 2001; Oda *et al.*, 2000; Nakano and Vousden, 2001). Studies using chemical inhibitors have also demonstrated that transcriptional activation of Dp5/Hrk requires JNK activation (Harris and Johnson, 2001; Bozyczko-Coyne *et al.*, 2001). What is interesting about transcriptional regulation of the *bim* gene is that it encodes three different transcripts, Bim_{EL}, Bim_L and Bim_S, yet the Bim_{EL} isoform is selectively upregulated in response to trophic factor deprivation, perhaps suggesting another point of regulation at the level of Bim mRNA.

Interestingly, Bim is also regulated through post-translational modification. Under normal circumstances, Bim_L and Bim_{EL} are tethered to the microtubular dynein motor

complex via association with dynein light chain LC8 (Puthalakath *et al.*, 1999). A consensus sequence present in both Bim_L and Bim_{EL}, but absent in Bim_S mediates this association, thereby suppressing Bim's apoptotic activity. The absence of this motif in Bim_S renders this isoform highly apoptotic. Intriguingly this isoform has not been identified in cells, suggesting that it may only be produced in instances when a rapid, efficient death response is required (O'Reilly *et al.*, 2000). Two independent studies have demonstrated JNK-mediated phosphorylation of Bim results in its release from the dynein motor complex, thereby permitting it to exert its pro-apoptotic effects. Specifically, trophic factor deprivation in sympathetic neurons results in JNK-mediated phosphorylation of Bim_{EL} at Serine 65 and subsequent death, while UV-irradiation leads to phosphorylation of Bim_L, release from the dynein motor complex and subsequent apoptosis (Putcha *et al.*, 2003; Lei and Davis, 2003). The latter study also demonstrates JNK-mediated phosphorylation of the BH3-domain-only protein Bmf, mediating its release from the myosin V motor complex and induction of death (Lei and Davis, 2003).

Other BH3-domain-only proteins subject to post-translational regulation include the protein Bad. Pro-survival signaling cascades such as those mediated by Interleukin-3 (IL-3) have been shown to mediate PI3-K/Akt-dependent and RSK1-dependent phosphorylation of Bad at residues Serine-136 and -112, respectively (Zha *et al.*, 1996; del Peso *et al.*, 1997; Datta *et al.*, 1997). These residues lie within a consensus motif that promotes Bad association with the adaptor protein 14-3-3, thereby suppressing Bad's apoptotic activity (Datta *et al.*, 2000). Both Protein Kinase A (PKA) and RSK1-mediated phosphorylation of Bad at Serine 155 also have been shown to prevent Bad association with anti-apoptotic Bcl-X_L, thus facilitating survival (Tan *et al.*, 2000; Zhou *et al.*, 2000;

Hirai and Wang, 2001). This line of evidence is consistent with a model in which BH3-domain-only proteins act to sequester anti-apoptotic molecules thereby, preventing them from inhibiting the pro-apoptotic, multi-domain proteins, Bax and Bak.

Recent studies have emerged however, demonstrating an additional phosphorylation of Bad at Serine-128, which lies within the 14-3-3 consensus motif. Bonni and colleagues report Cdc2-mediated phosphorylation of Bad at Serine-128 in response to KCl deprivation in cerebellar granule neurons and JNK-mediated Bad phosphorylation in response to overexpression of the upstream MAPKKK, MEKK1 (Konishi *et al.*, 2002; Donovan *et al.*, 2002). In both instances, the consequence of Bad phosphorylation at Ser128 was dissociation from 14-3-3 and subsequent death.

Finally, it is evident from these studies that subcellular localization imposes a critical form of regulation on the BH3-domain-only proteins. Cells have adopted multiple mechanisms in which to render BH3 proteins inactive. Specifically, Bad is inhibited at within the cytosol in proximity to the mitochondria and ER through association with adaptor protein 14-3-3; Bim and Bmf are tethered to the dynein motor complex and myosin V motor complex, respectively, through conserved light chain motifs; Bik, which primarily localizes to the ER, is inhibited through association of anti-apoptotic Bcl-2 proteins and Bid is also inhibited in the cytosol through association with Bcl-2 proteins. Notably, disinhibition mediated through cellular signaling facilitates release and translocation to mitochondrial and ER membranes, where the BH3-domain-only proteins can exert their pro-apoptotic functions.

Rationale and Statement of Hypothesis

In light of the similarities observed between trophic factor deprivation paradigms and death induced through the p75NTR, we hypothesized that the link between activated JNK and mitochondrial cytochrome *c* release lie in the regulation of the BH3-domain-only proteins. Through means of reverse transcriptase polymerase chain reaction (RT-PCR), studies conducted in our lab indicated that p75NTR overexpression did not lead to transcriptional upregulation of the BH3-domain-only proteins Bim, Bmf, Dp5/Hrk, Bik/ PUMA and NOXA. However, the recent studies demonstrating JNK-dependent phosphorylation of Bad and Bim in both stress and trophic factor deprivation models, suggested that p75NTR may also mediate death through post-translational modification of these proteins. Thus, I hypothesized that p75NTR overexpression would induce Bad and Bim phosphorylation at Serine-128 and -65, respectively, and that these post-translational modifications would be critical for execution of a p75NTR intrinsic death pathway.

Material and Methods

Materials. Phospho-Serine 65 Bim antibody was obtained from Dr. Azad Bonni (Harvard Medical School, Boston, MA). To generate the phospho65-Bim antibody, a phosphopeptide of the sequence CLAPPApSPGPFATR (Tufts Synthesis Facility, Tufts Medical School, Boston, MA), was synthesized and coupled to keyhole limpit hemocyanin using the Imject Maleimide Activated mcKLH Kit (Pierce). The antigen was injected into New Zealand White rabbits (Covance Research Products), from which serum was collected approximately every three weeks. Serum was affinity-purified by subsequently passing it on an Immunopure immobilized protein A column (Pierce) and an agarose-iodoacetyl column (Pierce), to which a synthetic peptide of the sequence CLAPPASPGPFATR (Tufts Synthesis Facility) was coupled. The final eluate was desalted and concentrated using Amicon Ultra centrifugal filter devices (Millipore).

Phospho-Serine 128 Bad antibody, also obtained from Dr. Azad Bonni (Harvard Medical School, Boston, MA) was prepared as described above, directed against phosphopeptide C-EGMEEELpSPFRGRS conjugated to keyhole limpit cyanin.

The p75NTR antibody αP1 was directed against the p75NTR intracellular domain (Roux *et al.*, 1999). Phospho-Thr^{183}/Tyr^{185} JNK (catalog #9255), cleaved caspase-3 (Asp175, catalog #9661), phospho-Ser^{63} c-Jun (catalog #9261), c-jun (catalog #9162), phospho-$Thr^{69/71}$ ATF-2 (catalog #9225) and ATF-2 (catalog #9226) -specific antibodies were obtained from Cell Signaling Technology (Beverly, MA). JNK-1 antibody (C-17, catalog #sc-474), IκBα (catalog #sc-371), Bcl-X_L (catalog #sc-634) and Bad C-20 (catalog #sc-943) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Bcl-2 antibody was purchased form BD Transduction Laboratories (catalog #B46620-050). β-

Galactosidase (LacZ) antibody was purchased from Promega (Madison, WI) (catalog #23781). Actin antibody was obtained from ICN Biochemicals (Costa Mesa, CA) (Cat. #69100). Bim antibody was purchased from Calbiochem (La Jolla, CA) (catalog #20200). Horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). Immunoreactive bands were detected using enhanced chemiluminescence purchased from PerkinElmer Life Sciences (Emeryville, CA). Paclitaxel was purchased from Calbiochem (LaJolla, CA) (catalog #580556).

Plasmids and recombinant adenovirus. Preparation of recombinant adenovirus expressing β-galactosidase (AdLacZ), full-length p75NTR (Adp75NTR), the Flag-tagged JNK-binding domain of JNK interacting protein (JIP1) (AdJBD) have been previously described (Roux *et al.*, 2002). All adenoviruses were amplified in 293A cells and purified on sucrose gradients, as previously described (Roux *et al.*, 2002). Viruses were titered by optical density and using the tissue culture infection dose 50 (TCID) assay in 293A cells. Titers are expressed in terms of plaque-forming units.

All Bad and Bim dominant negative and RNA interference constructs were obtained from Dr. Azad Bonni (Harvard Medical School, Boston, MA). The Bad dominant-negative plasmid consisting of green fluorescent protein (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 by targeting the sequence GGGAGCATCGTTCAGCAGCAGC in rat BAD, as previously described (Gaudilliere *et al.*, 2002). Full-length Bim_{EL} cDNA was cloned by RT-PCR from RNA isolated from rat cerebellar granule neurons. Bim_{EL} cDNA was then subcloned into the polylinker of the bacterial GST gene fusion vector pGEX-4T-3

(Amersham Pharmacia Biotech) and into the polylinker of the mammalian expression vector pcDNA3 (Invitrogen) with an NH_2-terminal HA-tag. Point mutations to Ser-65 were made using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The U6/BS-Bim and Cdk2 RNA interference vectors were designed as described (Gaudilliere *et al.*, 2002). The sense strand of the encoded hairpin RNA was designed to specifically target the 20-nucleotide region GGTATTTCTCTTTTGACACAG in rat Bim RNA. The targeted region in Bim RNA showed no significant homology with any other gene by BLAST.

Cell culture, transient transfection and infection. Human glioma (U343 and U373) cell lines were provided by Dr. Roland Del Maestro (McGill University, Montreal, Quebec, Canada) and maintained in 5% CO_2 at 37°C in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBC) (Hyclone), 2mM L-glutamine and 100μg/ml penicillin-streptomycin. The rat pheochromocytoma cell line (PC12) was maintained in 10% CO_2 at 37°C in DMEM supplemented with 6% horse serum, 6% bovine calf serum (BCS), 2mM L-glutamine, and 100μg/ml penicillin-streptomycin, and the PC12rtTA cell line, which was purchased from Clontech, was maintained in 10% CO_2 at 37°C in DMEM supplemented with 6% horse serum, 6% bovine calf serum (BCS), 2mM L-glutamine,100μg/ml penicillin-streptomycin and 100μg/ml G418. Cells were plated 18-24 hours prior to transfection or treatment and were harvested or fixed according to treatment. PC12 and PC12rtTA cell lines were plated onto poly-L-lysine coated plates and transfected using Lipofectamine2000 as directed by the manufacturer (Invitrogen). For Bad oligomer studies, human glioma and PC12rtTA cell lines were infected and/or treated with Paclitaxel 24 hours post-plating.

Glioma cells were plated in 6-well dishes at a density of 200,000 cells/well while PC12rtTA cells were plated at a density of 900,000 cells/well. For Bad and Bim loss-of-function studies, PC12 and PC12rtTA cell lines were infected 48 hours post-transfection.

Western Analysis. Cells were lysed in radioimmunoprecipitation assay buffer (10mM Tris, pH 8.0, 150mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1μg/ml aprotinin, 1μg/ml leupeptin, 1μg/ml pepstatin, 1mM sodium orthovanadate, and 1mM phenylmethylsulfonyl fluoride) and analyzed for protein content using the BCA assay (Pierce, Rockford, IL). 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 (10mM Tris, pH 8.0, 150mM NaCl, and 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 blocking solution, Tris-buffered saline-Tween supplemented with 5% (w/v) dried skim milk powder or Tris-buffered saline-Tween supplemented with 5% (w/v) BSA for phospho-specific antibodies, with the exception of phospho-Ser65Bim, which was prepared in TBS containing 0.025% Tween-20 supplemented with 5% (w/v) dried skim milk powder. Immunoreactive bands were detected by chemiluminescence (Perkin-Elmer Life Sciences), according to the manufacturer's instructions.

Cleaved Caspase-3Activation Assay. PC12 or PC12rtTA cells were plated onto poly-L-lysine coated 6-well dishes at a density of 500,000 or 900,000 cells/well, respectively. 24 hours post-plating, cells were transfected using Lipofectamine2000 (Invitrogen),

according to the manufacturer's protocol, alone with GFP or GFP-BadDN, or co-transfected with GFP and either U6-Blue Script, Bad RNAi, Bim RNAi, Cdk2 RNAi, pcDNA3, Bim WT, or Bim S65A dominant negative in a 1:7 ratio. 48 hours post-transfection, cells were either left uninfected or infected with either LacZ or p75NTR adenovirus at 100 MOI. 24 hours post-infection, cells were fixed in 4% paraformaldehyde in PBS. Cells were blocked in TBS supplemented with 5% donkey serum and 0.3% Triton X-100 for 30 minutes at room temperature after which they were incubated with antibodies directed against cleaved caspase-3 in TBS supplemented with 2% donkey serum and 0.3% Triton X-100, for 18 hours at 4°C. Secondary antibodies (donkey anti-rabbit conjugated Cy3) and Hoescht 33248 were applied for 2hr at 4°C. GFP-positive cells were scored for the presence of activated caspase 3 by a blinded observer, with 150-300 cells counted per condition. Experiments with BadDN and Bad RNAi were repeated three times, while experiments with Bim S65A and Bim RNAi were repeated twice. Composite data were analyzed for statistical significance by ANOVA (Tukey's honestly significant difference (HSD) multiple comparison).

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 4 h of a 28 hour treatment. 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 nonspecific absorbances were read at 570 and 690 nm, respectively. In each experiment, each data point was performed in triplicate or quadruplicate and each experiment was carried out twice.

Death Assay. Analysis of cell death was performed by the LDH assay using the cytotoxicity detection kit (Roche, catalogue no. 1644793) according to the manufacturer's instructions. Twenty-four hours after treatment, cell cultures were centrifuged at 500 × *g*, and the supernatant was added to an equal volume of LDH reaction mixture supplied by the manufacturer (Roche). After 15-30 min of incubation, specific and nonspecific absorbances were read at 490 ($A_{490\,nm}$) and 690 ($A_{690\,nm}$), respectively. Values reported were calculated using the following formula: ($A_{490\,nm} - A_{690\,nm}$). Each data point was performed in triplicate or quadruplicate and each experiment was carried out independently twice.

Results

Despite a recent surge in the identification of novel p75NTR ligands and co-receptors, the events leading to p75NTR activation and initiation of an apoptotic cascade *in vivo* remain complex. Previous studies from our laboratory have shown that overexpression of recombinant adenovirus encoding full-length p75NTR reliably induces apoptosis in the absence of added ligand in multiple cell lines and in primary neurons (Roux *et al.*, 2001; Bhakar *et al.*, 2003). Thus, we employed this approach for the purpose of determining the mechanism by which JNK communicates with the mitochondria to mediate cytochrome *c* release.

p75NTR does not Transcriptionally Upregulate BH3-Domain-Only Proteins

BH3-domain-only proteins are classically termed "mitochondrial gate-keepers," referring to their ability to transduce proximal death cues into alterations of mitochondrial permeability, facilitating cytochrome *c* release. They are regulated through both transcriptional and post-translational means, leading to either their activation or sequestration. Evidence of transcriptional regulation comes from trophic factor deprivation (TFD) studies in sympathetic and cerebellar granule neurons, which have both reported transcriptional upregulation of the BH3-domain-only proteins Dp5/Hrk and Bim (Harris and Johnson, 2001; Whitfield *et al.*, 2001). In light of the similarities between the apoptotic cascades initiated in response to either TFD or p75NTR expression, previous studies from our lab assessed the effects of p75NTR overexpression on mRNA levels of BH3-domain-only proteins Bim, Bmf, Hrk, Bik, Puma, and Noxa. Briefly, cortical neurons remained untreated or were infected with adenovirus expressing

either control β-galactosidase (LacZ) or p75NTR, after which mRNA levels were assessed by RT-PCR (Figure 7, Appendix - Bhakar). Although mRNA corresponding to each of the members was evident in mock, LacZ-infected and p75NTR-infected cells, p75NTR-dependent increases were not observed. Thus, p75NTR does not transcriptionally upregulate the BH3-domain-only proteins in order to execute an intrinsic death pathway.

Bad is a Critical Mediator of p75NTR-Induced Apoptosis

BH3-domain-only proteins are also subject to post-translational modifications. Specifically, the BH3-domain-only protein Bad is phosphorylated at Ser112 and -136 through pro-survival cascades such as PI3-K/Akt and MAPK (Zha *et al.*, 1996; Datta *et al.*, 1997). These events serve to tether Bad to the adaptor protein 14-3-3, thereby preventing Bad from exerting its pro-apoptotic actions. However, recent reports have identified a novel site at Ser128 that is phosphorylated in response stress or TFD, leading to 14-3-3 dissociation and cell death (Donovan *et al.*, 2002; Konishi *et al.*, 2002). In light of these reports, we assessed Bad Ser128 phosphostatus following p75NTR infection. U373 and PC12 cells remained untreated or were infected with adenovirus encoding either LacZ or p75NTR (Figure 8 *A* and *B*, respectively, Appendix - Bhakar). Subsequent immunoblot analysis revealed that while cells expressing p75NTR adenovirus showed phospho-Ser128 Bad immunoreactivity, mock cells or cells expressing control LacZ adenovirus did not. Notably, in addition to monomeric Bad (~25kDa), an additional immunoreactive band was evident at ~75kDa. This band was detected with total Bad antibodies directed against N- and C-terminals (Bad N19 and C20, respectively),

suggesting that the band reflects a stable, oligomeric complex comprising phospho-Ser128 Bad molecules.

In order to determine if the formation of oligomeric Bad was JNK-dependent, we co-infected PC12 cells with p75NTR adenovirus in the absence or presence of AdJBD, adenovirus which when overexpressed acts as a dominant negative JNK suppressor through expression of the JNK binding domain of the scaffold protein Jip-1. Figure 8 *C* shows a p75NTR-dependent oligomerization of Bad, which is abrogated upon co-infection with AdJBD, indicating that Bad oligomerization is JNK-dependent (Appendix – Bhakar).

We next assessed the physiological significance of p75NTR-mediated Bad phosphorylation at Ser128 using an immunocytochemical approach that assayed Cleaved Caspase-3 immunoreactivity. Briefly, PC12 cells were transfected with either GFP alone or a GFP-conjugated dominant-negative Bad serine 128 mutant allele cDNA (DN-Bad) (Konishi *et al.*, 2002), after which they were infected with control or p75NTR adenovirus. Cells were subsequently immunostained and scored for cleaved Caspase-3 immunoreactivity (Figure 9, Appendix - Bhakar). Control and LacZ-infected cells exhibited a basal level of caspase-3 immunoreactivity of ~10%, while p75NTR overexpression lead to a four-fold increase in cleaved caspase-3 immunoreactive cells. This increase however, was prevented in cells expressing the dominant negative Bad S128A peptide. Thus, expression of the Bad S128A peptide conferred protection against p75NTR-mediated apoptosis, suggesting that p75NTR-mediated phosphorylation of Bad at Ser128 is critical for death. To confirm that p75NTR-mediated cell death requires Bad, endogenous levels of Bad were reduced through RNA interference prior to infection. In

order to validate the efficacy of a RNA interference construct targeted against Bad, PC12 cells were initially transfected with either control vector alone (U6-BS) or Bad RNAi and subjected to western analysis (Supplementary Figure 1, Appendix). Immunoblot analysis revealed a dose-dependent reduction in endogenous Bad levels in lanes expressing Bad RNAi (lanes 4-6), but not control vector (lanes 1-3). Following validation, cells were co-transfected with GFP and Bad RNAi and then infected with either control or p75NTR adenovirus. Figure 9 shows that p75NTR-induced apoptosis is inhibited through reduction of endogenous Bad levels, as evident from reduced cleaved Caspase-3 immunoreactivity, indicating that Bad is required for p75NTR-mediated apoptosis.

Finally, in order to confirm that cleaved Caspase-3 immunoreactivity serves as an accurate surrogate for apoptosing cells, we treated PC12 cells with staurosporine (10μg/ml), a kinase inhibitor well documented to induce apoptosis (Kabir *et al.*, 2002), or infected them with p75NTR at 100 MOI for 24 hours, after which they were fixed and immunostained for cleaved Caspase-3 and TUNEL (Supplementary Figure 2, Appendix). Cells were subsequently scored for coincidence of cleaved Caspase-3 and TUNEL immunoreactivity. Supplementary Figure 2 shows that cells treated with staurosporine exhibited ~70% coincidence between cleaved Caspase-3 and TUNEL immunoreactive cells, demonstrating that cleaved Caspase-3 immunoreactivity serves as a valid surrogate for apoptosing cells. Similarly, cells infected with p7NTR exhibited ~75% coincidence between cleaved Caspase-3 and TUNEL immunoreactivity.

Paclitaxel: A Chemotherapeutic Drug that Induces Bad Oligomerization

Paclitaxel (Taxol) has achieved enormous success as a chemotherapeutic agent used for the treatment of ovarian, breast, head, neck and lung carcinomas (Zong *et al.*, 2004). Taxol binds reversibly to tubulin along the surface of microtubules were it increases the rate at which tubulin monomers polymerize, stabilizing the microtubule cytoskeleton against depolymerization, resulting in cell cycle arrest at the G2/M transition, and subsequent cell death (Arnal and Wade, 1995; Ling *et al.*, 1998; Torres and Horwitz, 1998). Despite its success, drug resistance to Taxol has been observed (Yu *et al.*, 1998; Kavallaris *et al.*, 1999). Multiple lines of evidence suggest that susceptibility versus resistance to Taxol treatment reflects, at least in part, differences in levels of Bcl-2 family members (Gazitt *et al.*, 1998; Siegel *et al.*, 1998; Liu *et al.*, 1998). Studies have shown that Taxol resistance is rendered through upregulated levels of Bcl-2 and Bcl-X_L. Furthermore key regulatory sites have been identified in the loop region of Bcl-2, (Ser70 and Ser87) that are phosphorylated in response to Taxol treatment (Basu *et al.*, 1997; Haldar *et al.*, 1998). Although the functional significance of these events have not completely been elucidated, it is hypothesized that phosphorylation inactivates the anti-apoptotic activity of Bcl-2, therefore facilitating Taxol-induced apoptosis (Haldar *et al.*, 1995; Srivastava *et al.*, 1999). Consistent with this, deletion of the loop harboring Ser70 and Ser87 residues enhances Bcl-2's anti-apoptotic activity (Srivastava *et al.*, 1999). Finally, a number of studies indicate that Taxol-induced Bcl-2 phosphorylation and inactivation proceed via a Ras-JNK signaling pathway (Lee *et al.*, 1998; Wang *et al.*, 1998; Yamamoto *et al.*, 1999).

In addition to negative regulation of anti-apoptotic Bcl-2 members, Taxol also induces death through the transcriptional regulation of BH3-domain-only proteins such as Bim (Sunters *et al.*, 2003). Studies in nine different breast cancer lines revealed an upregulation of Bim, both at the level of the mRNA and protein. Furthermore, siRNA specific for Bim reduced Taxol-induced death in these lines. Interestingly, Taxol has also been reported to induce phosphorylation of Bad at Ser112 and Ser136 through activation of PI3-K/Akt and MAPK cascades (Mabuchi *et al.*, 2002). Surprisingly, inhibition of these signaling pathways sensitized cells to Taxol-induced apoptosis suggesting that initial Taxol-mediated Bad phosphorylation may represent the cell's attempt to prevent onset of an apoptotic cascade by inducing sequestration of Bad molecules via phosphorylation within the 14-3-3 consensus motif (Mabuchi *et al.*, 2002). Of particular interest to our investigations however, was a report of Taxol-induced Bad oligomerization (Bonni, personal communication). The observation that both p75NTR- and Taxol-mediated death paradigms induce JNK activation and Bad oligomerization, prompted us to employ Taxol as a surrogate to examine properties of the Bad oligomer. In order to do so, we utilized U343 and U373 human glioma cell lines, since both lines are susceptible to p75NTR-mediated apoptosis, although U343s to a lesser extent than U373s, and because p75NTR overexpression induced Bad oligomerization in U373s (Bhakar *et al.*, 2003).

Initial studies involved treatment of U343s and U373s with Taxol for 24 hours at varying doses in order to assess dose-dependent effects. Cells were subsequently lysed and analyzed by immunoblot. Figure 1 *A* shows Taxol-induced JNK activation at doses of 0.1μM and 10μM and this correlated with Bcl-2 and Bcl-X_L phosphorylation, consistent

with other reports of Taxol-induced post-translational modification of these proteins. Intriguingly, multiple phospho-Ser128 Bad immunoreactive bands became evident that were not present in mock cells or cells treated with low doses of Taxol (0.001μM). These included bands corresponding to molecular weights of monomeric Bad (~25kDa), and oligomeric species of approximately 150kDa. Furthermore, electromobility shifts were observed in pre-existing protein complexes of 50kDa and approximately 100kDa. Curiously however, many of the observed Taxol-induced phospho-Ser128 Bad immunoreactive bands were not evident in immunoblots probed with total Bad antibody (BadC20). Rather, electromobility shifts were evident only for species migrating at 25kDa, consistent with phosphorylation of monomeric Bad, and bands migrating at 70kDa. The latter molecular weight species was of particular interest since our investigations involving p75NTR overexpression induced formation of oligomeric complexes of ~75kDa in U373 cells. However, the fact that this species was present in untreated cells and was not detectable by phospho-Ser128 Bad antibodies raised the possibility that the Taxol-induced higher molecular weight complexes may not be the same as those induced through p75NTR overexpression.

Interestingly, U343 cells were highly resistant to Taxol-induced apoptosis. Lysates showed no cleaved caspase-3 immunoreactivity (data not shown) and analysis by LDH and MTT revealed no alterations in death or survival, respectively, in response to Taxol treatment, as compared to human embryonic kidney (HEK) 293T cells (Figure 1 *B*). In contrast, U373s were susceptible to Taxol-induced death as evident by robust cleaved caspase-3 immunoreactivity (Figure 2). Despite the observed susceptibility, Taxol induced similar responses in U373 cells as it did in U343s, notably JNK activation

and activation of its downstream target c-Jun, as well as Bcl-X_L phosphorylation. Furthermore, phospho-Ser128 Bad immunoreactivity was evident at 50kDa, 100kDa and 150kDa, while total Bad immunoreactivity was evident only for species of 25kDa and ~70kDa. Despite Taxol's similar mode of action in both U343 and U373 cells, the observation again that Bad oligomers of ~70kDa were evident in mock conditions and were not detected by phospho-Ser128 Bad antibodies suggested that these higher molecular species weight may not be the same as those induced through p75NTR overexpression.

As a means to ascertain whether the oligomers induced through Taxol treatment were the same as those induced through p75NTR overexpression, we examined whether Taxol treatment in the presence of p75NTR could potentiate Bad oligomer formation. To do so, U343 cells remained untreated or were treated with Taxol alone or in the presence of control or p75NTR adenovirus. Cells were also treated independently with LacZ or p75NTR to assess viral-specific effects. Immunoblot analysis revealed that Taxol induced JNK activation, as well as both Bcl-X_L and Bcl-2 phosphorylation (Figure 3). A Taxol-induced phospho-Ser128 Bad immunoreactive band was evident at 150kDa that was not present in control lanes, and electromobility shifts at 25 and 100kDa were evident, as seen previously. Total Bad immunoblots showed electromobility shifts in monomeric Bad and a 70kDa species, also consistent with initial studies. However, Taxol treatment in conjunction with p75NTR infection did not potentiate formation of the 150kDa phospho-Ser128 Bad immunoreactive species, nor did it potentiate the electromobility shift of the 70kDa species observed in BadC20 immunoblots. Furthermore, although phosphorylated monomeric Bad was evident in U343s treated with p75NTR adenovirus

alone, p75NTR overexpression did not induce oligomer formation at any molecular weight as assessed by both phospho-Ser128 Bad or BadC20 immunoblot analysis. This result was somewhat surprising in light of previous work that demonstrated p75NTR-dependent Bad oligomerization in U373 cells. However, absence of this phenomenon in U343 cells highlights key differences in responsiveness to apoptogenic factors between these two cell lines. Thus, from these data, we concluded that the oligomers formed through Taxol treatment and p75NTR overexpression are distinct and consequently, Taxol cannot serve as a surrogate to analyze p75NTR-dependent Bad oligomerization in future studies. We consequently returned our efforts to examination of other BH3-domain-only proteins and their involvement in p75NTR-mediated apoptosis.

p75NTR-Mediated Apoptosis Requires JNK-dependent Phosphorylation of Bim_{EL}

The BH3-domain-only protein Bim is regulated through both transcriptional and post-translational mechanisms. Despite reports of transcriptional upregulation of Bim in TFD paradigms, our investigations indicated that p75NTR does not transcriptionally upregulate BH3-domain-only proteins in order to elicit a death response, raising the possibility that p75NTR may regulate Bim through post-translational means. Normally, the pro-apoptotic actions of Bim are suppressed through its association with the dynein motor complex. Recently however, two independent studies have demonstrated JNK-mediated phosphorylation of Bim that results in its release from the dynein motor complex and subsequent death. Trophic factor deprivation in sympathetic neurons has been reported to induce JNK-dependent phosphorylation of Bim_{EL} at Ser65, while UV-irradiation in transfected HEK 293T cells has been reported to induce JNK-dependent

phosphorylation of Bim_L (Putcha *et al.*, 2003; Lei and Davis, 2003). In an effort to determine if Bim is regulated in a p75NTR-dependent manner, we assessed if p75NTR overexpression correlated with phosphorylation of Bim_{EL} at Ser65 and if this event was JNK-dependent. As Figure 4 *A* shows, PC12 cells infected with control virus showed little immunoreactivity to phospho-Ser65 Bim antibodies (lanes 1-2). In contrast, cells co-infected with p75NTR and LacZ control virus exhibited robust Bim_{EL} Ser65 phosphorylation (lane 3). This effect was abrogated through co-infection of AdJBD indicating that p75NTR mediates JNK-dependent phosphorylation of Bim_{EL} at Ser65. Confirmation of p75NTR-dependent Bim phosphorylation was also obtained from total Bim immunoblots, in which cells co-infected with p75NTR and control virus (lane 3) exhibited reduced electrophoretic mobility as compared to cells co-infected with p75NTR and AdJBD (lane 4) (also Figure 5 *A*, Appendix - Becker).

In order to address the physiological significance of p75NTR-mediated Bim phosphorylation, we assessed cleaved Caspase-3 immunoreactivity in the single cell assay used previously to assess the requirement of Bad in p75NTR cell death. Two approaches were employed, use of a dominant negative Bim harboring a Ser65 to Ala mutation as well as Bim RNA interference. The constructs were validated initially using biochemical means to demonstrate efficacy and specificity of their actions.

In order to validate the Bim RNAi cDNA, PC12 cells were mock transfected or transfected with a dose of U6-BS control vector, Bim RNAi, or an unrelated Cdk2 RNAi to demonstrate that observed reductions in Bim levels did not reflect the expression of any RNAi construct. Figure 4 *B* shows clearly that endogenous Bim levels were not affected by expression of either U6-BS control vector or Cdk2 RNAi, rather increasing

quantities of Bim RNAi vector correlated with a dose-dependent reduction in Bim levels (also Figure 5 *B*, Appendix - Becker). Furthermore, immunoblots of IκBα, Erk1/2 and Actin confirmed equal protein loading and demonstrated that Bim RNAi does not non-specifically reduce levels of other proteins.

Both Bim RNAi and Bim S65A dominant negative constructs were subsequently validated in a biochemical experiment in which PC12rtTA cells were transfected with GFP alone or co-transfected with GFP and either U6-BS control vector, Bim RNAi, Cdk2 RNAi, pcDNA3 control vector, Bim WT or Bim S65A, after which cells were infected with LacZ control or p75NTR adenovirus. Cells were subsequently lysed, protein assayed and subjected to western analysis to assess p75NTR-mediated phosphorylation of Bim_{EL}, and ATF-2. Through this experiment we wanted to confirm that Bim S65A did not affect downstream activation of other identified p75NTR targets such as ATF-2, thus demonstrating specificity of its action. Figure 5 *A* shows a p75NTR-dependent phosphorylation of both endogenous Bim and Bim WT that is not evident in Bim S65A-transfected lanes (lanes 8-13). Bim WT phosphorylation was also evident in LacZ-infected lanes suggesting that overexpression alone may lead to stress-induced phosphorylation. Immunoblotting with total Bim revealed electromobility shifts indicative of phosphorylation in p75NTR-infected lanes that were not evident in control virus-infected lanes. Importantly, P-ATF-2 immunoreactivity was evident in all p75NTR-expressing lanes and was not affected by expression of Bim S65A, indicating that expression of dominant negative Bim S65A does not affect activation of other p75NTR targets (lane 10). Furthermore, expression of Bim RNAi did not affect P-ATF-2 immunoreactivity, nor did it affect total levels of p75NTR (lanes 6, 12).

Subsequent immunocytochemical analysis revealed that expression of Bim RNAi and Bim S65A conferred significant protection against p75NTR-dependent Caspase-3 activation, although to a lesser extent than DN-Bad- or Bad RNAi-expressing cells (Figure 5 *B*; also Figure 5 *C*, Appendix - Becker). Expression of Bim RNAi and Bim S65A constructs reduced cleaved caspase-3 immunoreactivity by approximately 50% indicating that although Bim contributes to p75NTR-mediated cell death, it may not represent JNK's primary target.

Chapter 3

Discussion and Closing Remarks

Discussion

Previous work in our lab has shown that contrary to other members of the TNFR superfamily, p75NTR induces an intrinsic death pathway that requires JNK activation, mitochondrial cytochrome *c* release and subsequent activation of Caspase-9, -3 and -6 (Bhakar *et al.*, 2003). However, the mechanism by which JNK communicates with the mitochondria to mediate cytochrome *c* release remained elusive. Here we show that p75NTR-dependent cell death is not accompanied by transcriptional upregulation of the BH3-domain-only proteins. Rather, p75NTR overexpression induces JNK-dependent phosphorylation of Bad and Bim_{EL}, both of which are critical for execution of a p75NTR-dependent death pathway.

p75NTR Induces Transcription-Independent Death via Bad and Bim_{EL}

It is now well established that JNK can facilitate apoptosis through both transcriptional-dependent and –independent means. JNK has long been known to activate AP-1 transcription factors such as c-Jun, which have in turn been shown to transcriptionally upregulate BH3-domain only proteins such as Bim and Dp5/Hrk (Harris and Johnson, 2001; Whitfield *et al.*, 2001). Previous work in our lab assessed mRNA levels of Bim, Bmf, Hrk, Bik, Puma, and Noxa in primary neurons following infection either with control or p75NTR adenovirus. Our examinations showed that although mRNAs of all BH3-only proteins examined were present in untreated, LacZ-infected and p75NTR-infected neurons, p75NTR-dependent increases were not observed, indicating that p75NTR does not transcriptionally upregulate BH3-domain-only proteins.

Alternatively, studies have demonstrated that JNK can induce death through direct activation of the cell death machinery. Specifically, reports have revealed JNK-mediated phosphorylation of BH3-domain-only proteins Bad, Bim and Bmf following cell stress (Donovan *et al.*, 2002; Putcha *et al.*, 2003; Lei and Davis, 2003). Consistent with these reports, we reveal that p75NTR induces the phosphorylation of Bad at Ser128 and interestingly, the formation of a JNK-dependent higher molecular weight Bad species. Furthermore, we show that p75NTR induces phosphorylation of Bim at Ser65, also in a JNK-dependent manner.

Serine 128 lies within a consensus motif that serves to tether Bad to the adaptor protein, 14-3-3, thereby suppressing Bad's pro-apoptotic actions. Trophic factor signaling through kinases such as Akt promote Bad-14-3-3 association; however, upon exposure to cellular stress, this association is disrupted through JNK-mediated phosphorylation of Ser128, leading to death (Donovan *et al.*, 2002). Similarly, Serine 65 lies within a dynein light chain consensus motif that serves to tether Bim to microtubules, thereby preventing Bim from exerting its pro-apoptotic effects. Stress-induced JNK-mediated phosphorylation of Ser65 has also been reported to facilitate Bim release and subsequent cell death (Lei and Davis, 2003). The observation that p75NTR induces phosphorylation of Bad and Bim_{EL} at Ser128 and Ser65, respectively, therefore suggests that p75NTR antagonizes trophic factor-dependent mechanisms that suppress the cell death machinery. Collectively, these actions would facilitate execution of an intrinsic death pathway.

Through loss of function studies we confirmed this rationale by demonstrating that both Bad and Bim_{EL} are critical mediators of p75NTR-dependent cell death. Our studies assessing the role of Bad in p75NTR-mediated apoptosis reveal that expression of

either Bad RNAi or dominant negative Bad confers significant protection from p75NTR-induced death. In fact, cleaved Caspase-3 immunoreactivity is reduced to control levels in cells expressing these constructs indicating that Bad is a critical mediator of p75NTR-dependent apoptosis. Furthermore, we reveal that expression of either Bim RNAi or dominant negative Bim_{EL} S65A also confers significant protection against p75NTR-induced cell death, however, to a lesser degree than Bad RNAi or dominant negative Bad constructs. In contrast to Bad loss of function studies in which we demonstrate complete protection from p75NTR-induced death, expression of Bim RNAi or dominant negative Bim_{EL} S65A reduces cleaved Caspase-3 immunoreactivity by only 50%.

The apparent difference in level of contribution to the p75NTR apoptotic pathway between Bad and Bim can be rationalized in a number of ways. First, one could argue that the loss of function constructs employed to assess Bim's role in p75NTR apoptotic paradigms were not specific or efficacious. However we believe this is not the case since prior to use, all of the indicated constructs were validated biochemically to ensure efficacy and specificity of their actions. We recognize that effects displayed through western analysis may not appear to mimic immunocytochemical data; however, this discordance reflects critical differences between the approaches. Transfection efficiency of the PC12 and PC12rtTA cell lines is notably poor and therefore, western analysis effects will be dampened through the pooling of both non-transfected and transfected cells. This is in contrast to immunocytochemical data, which reflects only transfected cells. Consequently, the fact that western analysis revealed reductions in endogenous Bim through expression of Bim RNAi, and absence of phosphorylation of exogenous Bim_{EL} S65A in response to p75NTR infection while phosphorylation of another p75NTR target

ATF-2 was evident, we conclude that our constructs are both efficacious and specific. Thus, the observed differences in the level of contribution between Bad and Bim_{EL} accurately reflect what is occurring in our p75NTR apoptotic paradigm.

Going forth, the data supports two possible models. The simplest model is one in which Bad represents JNK's primary target, while Bim_{EL} serves as a secondary target. In this model, Bad would function as the primary death effector inhibiting anti-apoptotic Bcl-2 members at the level of the mitochondria thereby facilitating Bax-Bak oligomerization and subsequent cytochrome *c* release. As a secondary death effector, Bim_{EL} activation would only occur in response to chronic p75NTR signaling or signaling associated with severe trauma, when the actions of Bad are insufficient to promote mitochondrial cytochrome *c* release. Thus, in this model, Bim_{EL} would serve to compliment the actions of Bad, but only in extreme circumstances when Bad activity is inadequate. Alternatively, one could rationalize a model in which Bad and Bim_{EL} cooperate to compromise mitochondrial integrity; however, the activities of Bim_{EL} are dependent on the actions of Bad. In this model, both Bad and Bim_{EL} represent primary JNK targets. Thus, following p75NTR-mediated JNK activation, Bad and Bim_{EL} would be released from 14-3-3 and the dynein motor complex, respectively, in a JNK-dependent manner. Upon release however, anti-apoptotic Bcl-2 or Bcl-X_L sequester Bim_{EL} in a final attempt to rescue the cell from Bim's pro-apoptotic actions. Only upon Bad association with Bcl-2 or Bcl-X_L and a subsequent conformational change in these proteins is Bim_{EL} released, allowing Bim_{EL} to directly activate Bax and Bak at the level of the mitochondria, facilitating cytochrome *c* release and death.

We support the latter model proposing cooperative action between Bad and Bim_{EL}, based upon two complementary lines of evidence. The first line of evidence is in regards to studies showing that Bcl-2 and Bcl-X_L sequester BH3-domain-only proteins such as tBid and Bim, thereby preventing activation of Bax and Bak (Cheng *et al.*, 2001). Anti-apoptotic Bcl-2 proteins were long thought to exert their effects solely through the sequestration of multi-domain, pro-apoptotic Bax and Bak. Consequently, these studies are important because they reveal a novel mechanism by which anti-apoptotic Bcl-2 members function and yet another mode of negative regulation affecting the pro-apoptotic BH3-domain-only proteins. Notably, these studies demonstrated that Bcl-X_L could also sequester Bad. The second line of evidence involves studies in which Stanley Korsmeyer and colleagues analyzed the individual BH3 peptides within all members of the BH3-domain-only class of proteins. From these investigations, Korsmeyer classed the BH3-only proteins into two distinct subclasses: a Bid-like subset, typified by tBid and Bim that directly activate pro-apoptotic Bax and Bak, as well as a Bad-like subset, which occupy the pockets of Bcl-2 members, thus sensitizing the cell to the actions of other activating BH3-only proteins (Letai *et al.*, 2002). These studies demonstrated that the BH3 peptide derived from Bim was capable of inducing oligomerization of Bax and Bak and subsequent cytochrome *c* release in a manner analogous to tBid. In contrast, BH3 peptides derived from Bad were unable to activate Bax and Bak, but rather bound to Bcl-2's hydrophobic pocket, displacing bound Bim, thus restoring Bim's ability to mediate cytochrome *c* release. This mode of action supports a model in which Bad sensitizes the mitochondria to the pro-apoptotic actions of BH3-only proteins such as Bim and tBid by inducing their release from anti-apoptotic Bcl-2 and Bcl-X_L proteins.

We must consider however, the energetic requirements associated with a model in which Bim_{EL} directly activates Bak and Bak, following Bad-dependent release from Bcl-2 or Bcl-X_L. As mentioned previously, Bax's carboxy-terminal α9 helix lies within a hydrophobic pocket formed by its BH1-3 domains in its inactive, monomeric state. If Bim_{EL} is to activate Bax by inserting its BH3-peptide into the groove formed by BH1-2 domains, one must consider whether the Bim_{EL}-Bax association is more favorable than the hydrophobic interactions stabilizing Bax's α9 helix. Only if this association is more energetically favorable would this permit disengagement of the peptide and subsequent mitochondrial targeting. Reports by Korsmeyer and colleagues suggest that this is possible since exogenous BH3 peptide derived from Bim induced oligomerization of Bax; however, these investigations were performed on purified mitochondria with concentrations of peptide that may not reflect physiological levels (Letai *et al.*, 2001). Thus, if we are to accept a model in which Bad induces release of Bim_{EL} from Bcl-2 or Bcl-X_L, allowing Bim_{EL} to directly activate Bax and Bak, we are accepting that Bim_{EL}-Bax association constitutes a lower energy state as compared to Bax in its monomeric form. Future studies comparing BH3 peptides derived from Bim versus α9 helical peptides derived from Bax and their ability to induce Bax oligomerization will hopefully clarify this issue.

Finally, a model in which Bad sensitizes the mitochondria to the actions of Bim_{EL}, by facilitating Bim_{EL}'s release from Bcl-2 or Bcl-X_L, suggests that through Bcl-2 or Bcl-X_L overexpression, mitochondrial integrity will be maintained and subsequent initiation of a caspase cascade will be thwarted, thereby rescuing the cell.

p75NTR Induces the Formation of Higher Molecular Weight Bad Complexes

Our investigations into the roles of BH3-domain-only proteins in p75NTR apoptotic paradigms yielded an intriguing phenomenon: the appearance of a JNK-dependent higher molecular weight complex that was immunoreactive with phospho-Ser128 Bad, Bad C20 and Bad N19 antibodies. This suggested that the observed immunoreactive species may indeed reflect a stable, oligomeric Bad complex.

In an attempt to gain insight into the properties of this higher molecular weight complex, we investigated the chemotherapeutic drug Paclitaxel as a potential surrogate for p75NTR-induced Bad oligomerization. These investigations were based upon personal communications reporting that oligomeric Bad reactive species had been observed in Paclitaxel-treated cells. Our initial assessment of U343 and U373 glioma cell lines, both of which are p75NTR-sensitive, revealed that Taxol treatment induced the appearance of phospho-Ser128 Bad immunoreactive species at 25kDa and 150kDa, the former corresponding to phosphorylated, monomeric Bad, and the latter potentially corresponding to an oligomer comprising phosphorylated Ser128 Bad molecules. Since Bad has been shown to exert its pro-apoptotic effects through sequestration of Bcl-X_L, it is also possible that this higher molecular weight complex comprises stable aggregates of phosphorylated Bad-Bcl-X_L (Zha *et al.*, 1997; Tan *et al.*, 2000; Hirai *et al.*, 2001). Absence of a corresponding band on Bcl-X_L immunoblots however suggests either that this is not the case, or perhaps the band is not detectable because of aggregation-induced blockade of Bcl-X_L's immunoreactive epitope.

Taxol treatment also altered the electrophoretic mobilities of pre-existing species at 50kDa and 100kDa. Whether these immunoreactive bands represent pre-oligomerized

Bad molecules or just cross-reactive species remains unclear, particularly since studies to date have not reported the existence of stable (i.e. sodium dodecyl sulfate (SDS)-resistant), higher-order complexes comprising either Bad alone or in complex with other proteins in the absence of apoptotic stimuli. Notably, Bad-14-3-3 complexes, which are induced through trophic factor signaling, are not SDS-resistant.

Curiously, none of the observed phospho-Ser128 Bad immunoreactive species, with the exception of monomeric Bad, were detected in total Bad immunoblots. Rather, electromobility shifts were evident only for species migrating at 25kDa, again consistent with phosphorylation of monomeric Bad, and bands migrating at 70kDa. Of all the Taxol-induced Bad immunoreactive species, this particular band most closely resembled, at least in molecular weight, that which was induced through p75NTR overexpression in U373 and PC12rtTA cells. However, the observation that this band was present in mock treated cells and was not detectable by phospho-Ser128 Bad antibodies suggested that it was distinct from the 75kDa band induced through p75NTR overexpression. Subsequent studies in which U343 cells were treated with Taxol alone or in the presence of either control or p75NTR adenovirus revealed that co-treatment with p75NTR failed to potentiate Taxol-induced formation of the 150kDa species or modification of the 70kDa species. Furthermore, although p75NTR expression alone induced phosphorylation of monomeric Bad, it did not induce Bad oligomerization nor did it alter the phosphostatus of any higher molecular weight Bad immunoreactive species. This result was unexpected considering we had previously shown that p75NTR induces Bad oligomerization in U373 cells. However, these cells had also shown differences in their susceptibility to Taxol-induced apoptosis. Both LDH and MTT analysis of U343 cells had shown resistance to

Taxol treatment at concentrations in which 293T cells were susceptible. Immunoblot analysis of U343 and U373 lysates had also revealed that while U373s showed robust cleaved caspase-3 immunoreactivity in response to Taxol treatment, U343 lysates did not. Furthermore, although both cell lines are p75NTR-responsive, U343 cells are much less susceptible to p75NTR insult as compared to U373 cells as evidenced by MTT analysis (Bhakar *et al.*, 2003). Whether differences in susceptibility reflect the fact that U373s harbor a defective p53 gene remains unclear. Nevertheless, it was evident from our data that Taxol-induced alterations in Bad were distinct from those induced through p75NTR overexpression. Consequently, we concluded that Taxol could not serve as a surrogate for analysis of p75NTR-induced Bad oligomers.

Although we did not pursue characterization of the Bad oligomer, our initial studies suggested that the 75kDa species may harbor Bad molecules complexed to Bcl-2, as evidenced by immunoblot analysis that revealed a Bcl-2 immunoreactive band migrating at ~75kDa. Although this data is consistent for a role in which Bad inhibits anti-apoptotic Bcl-2 members, only through mass spectrometric analysis of this complex will we obtain pertinent information as to its composition, thereby providing insight into the mode by which these oligomers function to elicit a p75NTR-dependent death response. Furthermore, although Taxol could not be used in order to improve our understanding of the p75NTR-induced Bad oligomer, we have demonstrated a novel Taxol-dependent regulation of Bad at Ser128.

The p75NTR Overexpression Paradigm: Relevance in the Big Picture

In this and previous studies, we have relied upon overexpression of an adenovirus encoding full length p75NTR to delineate the mechanism(s) by which p75NTR mediates PCD. Until the recent identification of proNGF as a high-affinity, pro-apoptotic p75NTR ligand, studies have employed mature neurotrophins, which are notoriously poor in their ability to activate p75NTR apoptotic signaling cascades. Consequently, we adopted this gain-of-function approach out of necessity since it reliably induces p75NTR-dependent cell death that cannot be achieved through the application of mature neurotrophins. However, with the recent surge in the identification of novel p75NTR co-receptors and ligands, we recognize that these novel binding partners will undoubtedly modulate p75NTR activity and likely the mechanism by which it signals. This brings into question then: how does p75NTR signal in the absence of ligand in our overexpression paradigm and does it accurately reflect what occurs *in vivo*?

The recent co-crystallization of NGF in complex with the ectodomain of p75NTR has provided valuable insight into the proximal membrane events regulating p75NTR receptor complex composition, and ultimately p75NTR's mode of action. It is well established that members of the TNFR superfamily reside in the PM juxtaposed in a trimeric PLAD. Upon binding of related, trimeric ligand, a conformational change is induced resulting in the separation of the receptor's aggregated cytoplasmic tails and subsequent recruitment of signaling adaptors, all while maintaining extracellular contacts. In contrast, p75NTR exists as a dimer in the PM awaiting association of dimeric ligand. It has now been shown that ligand association disrupts the existing p75NTR dimer due to an imposed allosteric change which renders NGF's second p75NTR-binding site

defective (He and Garcia, 2004). Notably however, the TrkA binding site within the NGF core remains intact, supporting the possibility of the association of a tripartite complex involving NGF, TrkA and p75NTR (He and Garcia, 2004). In light of this data, it is easy to conceive that proNGF would behave in a manner analogous to mNGF, driving disruption of existing p75NTR dimers through its mature portion, while associating with Sortilin via its prodomain. Furthermore, it therefore seems plausible that all neurotrophins signal via monomeric p75NTR complexed with an appropriate co-receptor: mature NGF in complex with TrkA and p75NTR to mediate survival and proNGF in complex with Sortilin and p75NTR to elicit death.

There is also evidence to suggest that p75NTR's ability to signal is regulated through sequestration to specialized lipid microdomains within the PM, termed lipid rafts. In fact, Higuchi and colleagues demonstrate that NGF added to cells lacking TrkA, binds to p75NTR activating cAMP and its downstream kinase, PKA. PKA subsequently phosphorylates p75NTR's juxtamembrane linker region, driving p75NTR into lipid rafts (Higuchi *et al.*, 2003). Interestingly, another study performed in PC12 cells and sympathetic neurons, both of which contain TrkA, failed to report NGF induced phosphorylation of p75NTR. However, the disparity between these results likely reflects p75NTR's ability to function as a TrkA co-receptor rather than autonomously (Taniuchi *et al.*, 1996). Since proNGF has been demonstrated to show preferential high-affinity binding to p75NTR, and little or no binding to TrkA, it is reasonable to assume that proNGF association with p75NTR could also drive p75NTR into a lipid raft. However, the distinction between the model proposed by Higuchi and colleagues, which shows dimeric p75NTR in lipid rafts, is that proNGF association would lead to disruption of

existing dimeric p75NTR, consistent with recent crystallization data, driving monomeric p75NTR into lipid raft domains. In line with this model, in order for proNGF to initiate a death cascade, it is imperative that Sortilin also be present in lipid rafts in association with p75NTR. However, whether Sortilin exists in lipid rafts or can be driven to lipid rafts remains unclear. Investigations to date have not reported such a phenomenon because Sortilin is predominantly intracellular and consequently, studies assessing neurotensin- or insulin-induced PM translocation have only examined subcellular localization through crude methods. Research into this phenomenon will undoubtedly provide crucial insight into the mechanisms by which p75NTR signaling is regulated.

Finally, the observation that p75NTR also acts as a co-receptor to NgR, which also resides in lipid rafts, raises the possibility that myelin or myelin-derived inhibitory molecules may also disrupt dimeric p75NTR, driving it into lipid rafts where it can associate with NgR. Although MAG, Nogo66 and OMgp have not been reported to bind p75NTR directly, it is possible that this process is driven by a yet undefined ligand. Collectively, these data support a model in which lipid rafts serve as critical microdomains that organize impinging extracellular cues into distinct signaling cascades through the sequestration of appropriate receptors.

In light of this model, we propose that in our system overexpression of p75NTR drives it into lipid rafts in association with Sortilin. High receptor density facilitates recruitment of appropriate signaling adaptors such as Rac and initiation of an apoptotic cascade in the absence of exogenous ligand. Furthermore, since p75NTR is upregulated dramatically following neuronal trauma and this is correlated with death *in vivo*, we believe that our system mimics what occurs *in* vivo following injury and consequently,

our findings are relevant. However, with the identification of proNGF as a high-affinity, pro-apoptotic p75NTR ligand, we recognize that the days of overexpression paradigms are at an end and the next crucial step will be to assess the effects of proneurotrophins directly on p75NTR signaling events.

Closing Remarks

p75NTR continues to elude us; with each new discovery that seemingly improves our understanding of this enigmatic receptor, we are confronted with new and perplexing questions. While its role as an apoptotic receptor in nervous system development and injury has gained acceptance, the mechanism by which p75NTR elicits cell death is only beginning to emerge. With the recent discovery of proNGF as a high-affinity p75NTR ligand and Sortilin as a required co-receptor, we are finally beginning to understand the membrane events regulating formation of a p75NTR apoptotic receptor complex (Lee *et al.*, 2001; Nykjaer *et al.*, 2004).

Through our efforts to define the mechanism by which p75NTR elicits death, we have previously shown that p75NTR activates an intrinsic death pathway requiring JNK activation, mitochondrial cytochrome *c* release and activation of Caspase-9, -3 and -6 (Bhakar *et al.*, 2003). The present investigations sought to determine the mechanism by which JNK communicates with the mitochondria to mediate cytochrome *c* release. Through our analysis of the BH3-domain-only proteins, our lab has shown that in contrast to trophic factor deprivation paradigms, p75NTR does not transcriptionally upregulate BH3-only proteins. Consistent with this we and others have shown that the AP-1 transcription factor c-Jun, which regulates transcription of BH3-only proteins, is not a primary p75NTR target and is not required for p75NTR-mediated cell death (Palmada *et al.*, 2002). Rather, we show that p75NTR induces JNK-dependent phosphorylation of Bad and Bim_{EL} at Ser128 and Ser65, respectively, and that these events are critical for p75NTR-mediated cell death. Future analysis of neurons derived from Bad -/- and Bim-/- mice will hopefully lend *in vivo* credence to these data.

It is imperative to recognize however, that only through analysis of p75NTR null animals will we gain an accurate perspective into the *in vivo* workings of p75NTR. Of the two p75NTR null mice that have been generated, $p75NTR^{ExonIII-/-}$ and $p75NTR^{ExonIV-/-}$, both have been shown to harbor p75NTR products, making it difficult to draw inferences about p75NTR function (von Schack *et al.*, 2001; Paul *et al.*, 2004). However, we must overcome the technical challenges associated with generating complete p75NTR defective mice because only through generation of such lines will we enhance our knowledge of the physiological functions of the p75NTR.

Figure Legends

Figure 1. Taxol induces post-translational modification and oligomerization of Bad in U343 cells. *A.* U343 cells were treated with Taxol for 24 hours at the indicated concentrations; lysates were protein assayed and equal amounts of protein were immunoblotted to assess JNK activation, total JNK levels, Bad phosphostatus, total Bad levels, as well as alterations in Bcl-X_L and Bcl-2 electrophoretic mobilities. *B.* U343 (grey bars) and HEK 293T (black bars) cells were treated with an increasing dose of Taxol for 24 hours, after which cells were analyzed by MTT and LDH to assess survival and death, respectively. Triton X-treated cells served as a control for apoptotic cells. Conditions were performed in quadruplicate.

Figure 2. Taxol induces post-translational modification and oligomerization of Bad in U373 cells. U373 cells were treated with Taxol for 24 hours at the indicated concentrations; lysates were protein assayed and equal amounts of protein were immunoblotted to assess JNK activation, total JNK levels, c-Jun activation, total c-Jun levels, Bad phosphostatus, total Bad levels, alterations in Bcl-X_L electrophoretic mobility, and cleavage of caspase-3 as a marker for apoptosis.

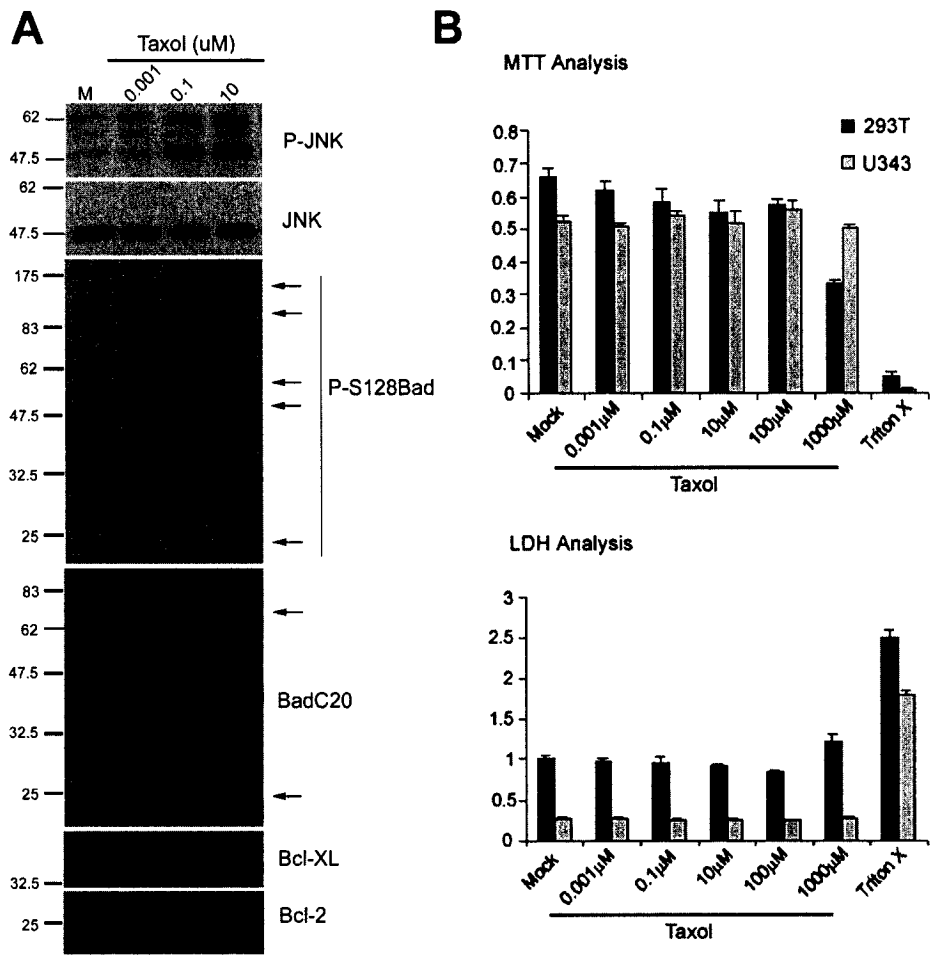
Figure 3. p75NTR does not potentiate Taxol-induced Bad oligomerization in U343 cells. U343 cells remained untreated or were treated with 10μM Taxol (T) alone or in the presence of LacZ (LZ) or p75NTR adenovirus (100MOI), or were treated alone with LacZ or p75NTR adenovirus (100MOI) for 24 hours. Cells were subsequently lysed and subjected to western analysis to assess JNK activation, total JNK levels, Bad

phosphostatus, total Bad levels, alterations in Bcl-X_L and Bcl-2 electrophoretic mobilities, levels of actin, β-galactosidase and p75NTR.

Figure 4. p75NTR induces JNK-dependent phosphorylation of Bim_{EL}. *A.* PC12 cells were infected with 100 MOI of LacZ control or p75NTR adenovirus together with 5 MOI of either LacZ control virus (L) or AdJBD (J). Lysates were immunoblotted with phospho-Ser65 Bim or total Bim antibody. *B.* PC12 cells were transfected with increasing amounts of U6 control vector, Bim RNAi or Cdk2 RNAi. Lysates were immunoblotted for total Bim levels, and IκBα, Erk1/2 and Actin to demonstrate equal protein loading.

Figure 5. JNK-Mediated Phosphorylation of Bim_{EL} at Ser65 is Critical for p75NTR-Dependent Apoptosis. *A.* PC12rtTA cells were transfected with GFP alone or GFP together with U6 control vector, Bim RNAi, Cdk2 RNAi, pcDNA3 control vector, Bim_{EL} WT or Bim_{EL} S65A, after which cells were infected with LacZ or p75NTR. 24 hours post-infection, cell lysates were immunoblotted to assess the specificity of Bim RNAi and Bim_{EL} S65A constructs. *B.* PC12rtTAs were treated as described in *C* up until adenoviral infection, after which cells were fixed and immunostained for cleaved Caspase-3 immunoreactivity. Transfected cells were scored by a blind observer (n = 150 cells/condition). '*' indicates a difference of $p<0.05$ between p75NTR-infected, pcDNA3-transfected cells (Bar 8) and BimS65A-transfected cells (Bar 10). '**' indicates a difference of $p<0.001$ between p75NTR-infected, pcDNA3-transfected cells (Bar 8) and mock or LacZ-infected cells (Bars 1-7), and p75NTR-infected, U6-transfected (Bar 11) and BimRNAi-transfected cells (Bar 12). Statistical differences indicated by ANOVA.

Figure 1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Taxol (uM)
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0.001
0.1
10
62
47.5
P-JNK
JNK
175
83
P-S128Bad
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25
BadC20
Bcl-XL
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B
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U343
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10μM
100μM
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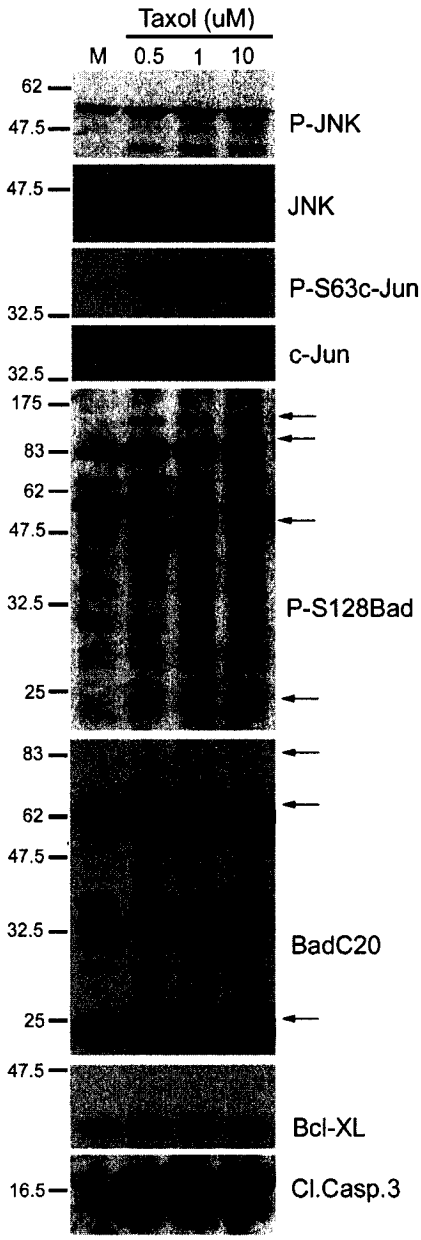
Triton X
Taxol
LDH Analysis

Figure 2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32.5
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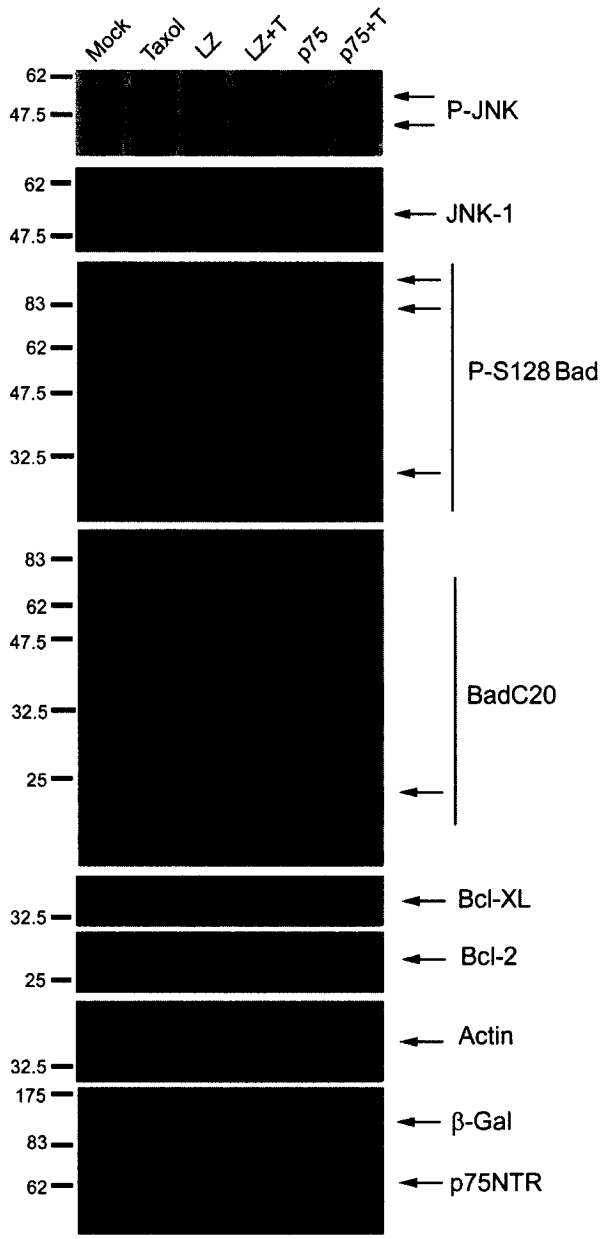
Figure 3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P-S128 Bad
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175
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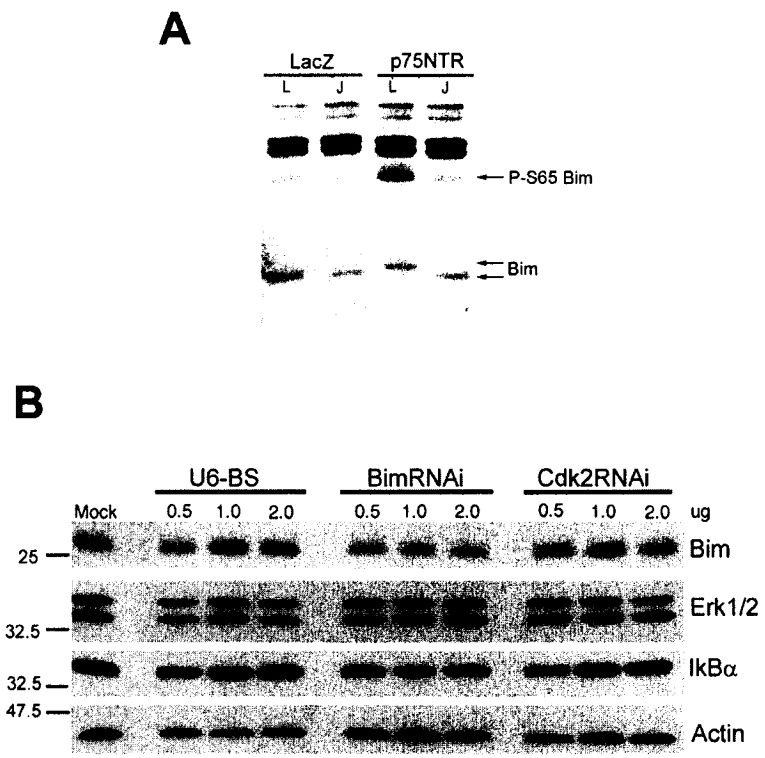
62
β-Gal
p75NTR

Figure 4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1.0
2.0
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1.0
2.0
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32.5
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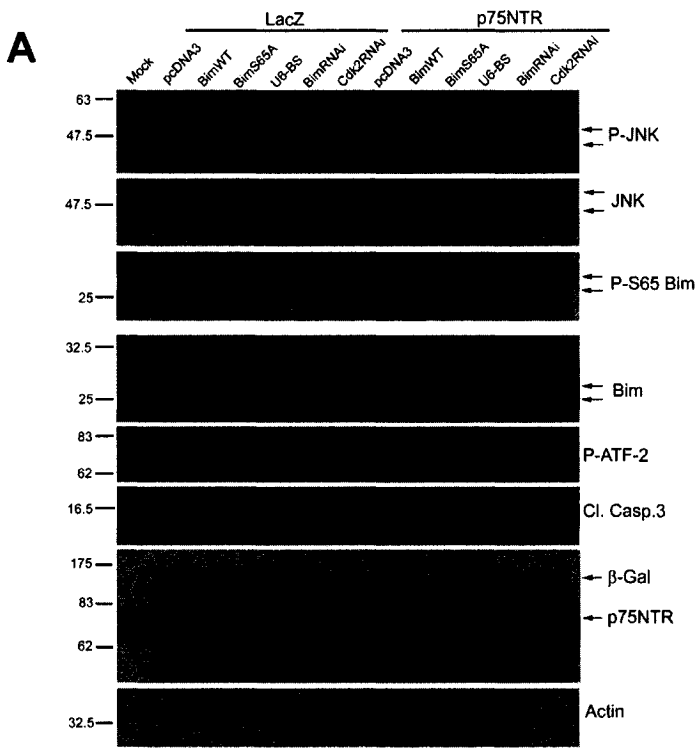
Actin

Figure 5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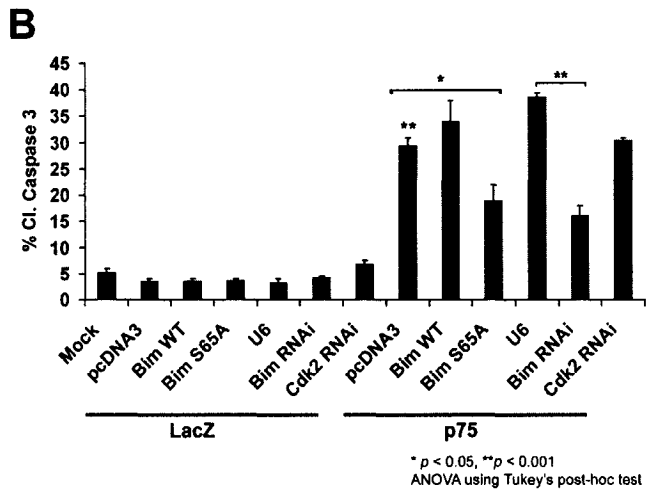
B
% Cl. Caspase 3
45
40
35
30
25
20
15
10
5
0
*
**
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Mock
pcDNA3
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Bim S65A
U6
Bim RNAi
Cdk2 RNAi
pcDNA3
Bim WT
Bim S65A
U6
Bim RNAi
Cdk2 RNAi
LacZ
p75
* p < 0.05, **p < 0.001
ANOVA using Tukey's post-hoc test

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Appendix

Cellular/Molecular

Apoptosis Induced by p75NTR Overexpression Requires Jun Kinase-Dependent Phosphorylation of Bad

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The p75 neurotrophin receptor (p75NTR), a member of the tumor necrosis factor receptor superfamily, facilitates apoptosis during development and after injury to the CNS. The signaling cascades activated by p75NTR that result in apoptosis remain poorly understood. In this study, we show that overexpression of p75NTR in primary cortical neurons, in pheochromocytoma cell line (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 p75NTR to the phosphorylation of BH3-domain-only family members.

Key words: apoptosis; jun kinase; neurotrophin; receptor; trk; cell death

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 (tyrosine kinase A), 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; Chao, 2003). 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 after 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 (Gu et al., 1999; Tournier et al., 2000; Wang et al., 2001; 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 neurotrophin receptor-interacting MAGE homolog (NRAGE) (Salehi et al., 2000), neurotrophin receptor-interacting factor (NRIF) (Casademunt et al., 1999), and p75NTR-associated cell death executor (NADE) (Mukai et al., 2000), facilitate p75NTR-dependent apoptosis. We have recently

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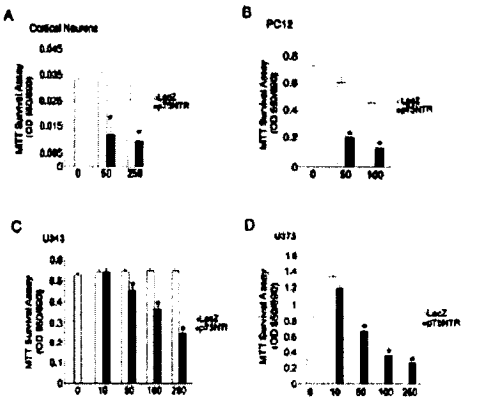

Figure 1. Overexpression of p75NTR reduces survival in a variety of cell types. *A*, Cortical neurons; *B*, PC12; *C*, U343 (wild-type p53); *D*, U373 (mutant p53) cells were infected with increasing multiplicities of infection (MOI) of LacZ or p75NTR recombinant adenovirus and then analyzed for survival by the MTT assay (see Materials and Methods). Error bars indicate SD. Results were analyzed for statistical significance by ANOVA (Tukey's HSD multiple comparison). Statistically significant differences of $p < 0.001$ are indicated by an asterisk.

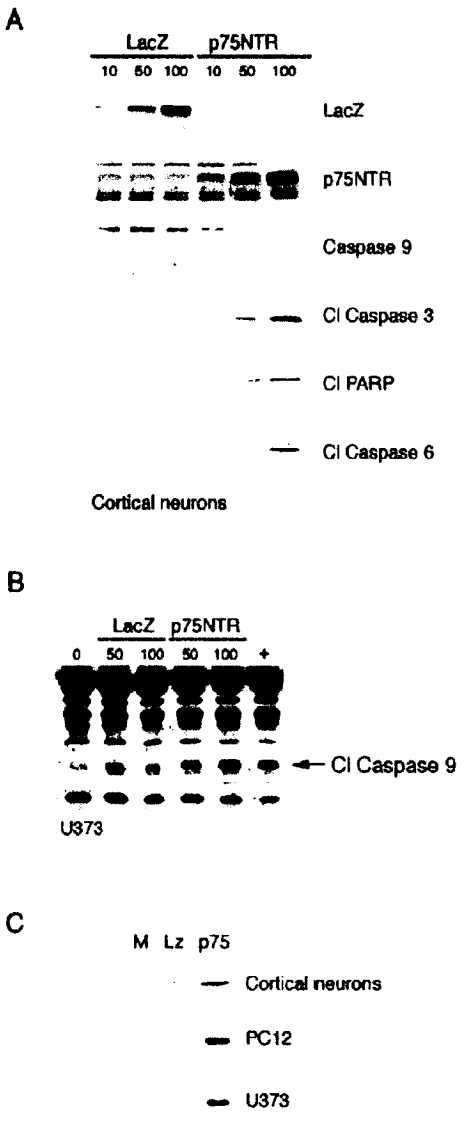

Figure 2. p75NTR activates caspases and induces accumulation of cytosolic cytochrome *c*. *A*, Cortical neurons infected with 10, 50, or 100 MOI of LacZ or p75NTR recombinant adenovirus were lysed and analyzed by immunoblot for levels of LacZ, p75NTR, and full-length caspase 9 protein or, using cleavage-specific antibodies, for levels of cleaved caspases 3 and 6 and cleaved PARP. *B*, U373 cells were infected with 50 or 100 MOI of either LacZ or p75NTR adenovirus for 48 hr or treated with etoposide 50 μM (+), 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. Thirty 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*.

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. Furthermore, the mechanisms used 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 pheochromocytoma, 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.

Materials and Methods

Materials. Cell culture reagents were purchased from BioWhittaker (Walkersville, MD), unless otherwise indicated. The p75NTR antibody αP1 is directed against the p75NTR intracellular domain (Roux et al., 1999). The antipeptide antibody to phosphorylated serine 128 BAD was directed against phosphopeptide C-EGMEEELpSPFRGRS conjugated to keyhole limpet cyanin and characterized as previously described (Konishi et al., 2002). The phospho-Thr83 p53 antibody was a kind gift of Ze'ev Ronai (Mount Sinai School of Medicine, New York, NY). JNK1 antibody (C-17, catalog #sc-474), the two Bad antibodies (C-20, catalog #sc-943 and N-19 catalog #sc-6542) and the actin antibody (C-2, catalog #sc-8432) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-flag antibody (M2, catalog #F-3165) was obtained from Sigma (St. Louis, MO), cytochrome *c* antibody was purchased from PharMingen (San Diego, CA) (catalog #556433), β-galactosidase (LacZ) antibody

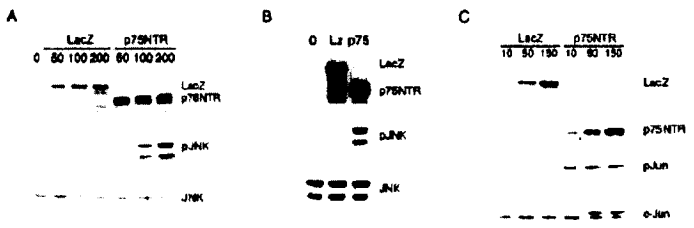

Figure 3. p75NTR activates the JNK pathway. *A*, U373 cells were infected with 0, 50, 100, or 200 MOI of control AdLacZ or with Adp75NTR. *B*, PC12 cells were injected with 0 or 50 MOI of AdLacZ or Adp75NTR. *C*, Cortical neurons were infected with 10, 50, or 150 MOI of AdLacZ or Adp75NTR. Lysates were prepared 30–48 hr after infection and examined by immunoblot for LacZ, p75NTR, phosphorylated JNK (pJNK), total JNK (SC-474 for *A*, CS-9252 for *B*), phosphorylated c-Jun (pJun), and total c-Jun as indicated.

was purchased from Promega (Madison, WI) (catalog #23781), and anti-influenza hemagglutinin (anti-HA) antibody (12CA5, catalog #1583816) was purchased from Roche (Hertforshire, UK). Phospho-Thr^{183}/Tyr^{185} JNK (G9, catalog #9255), SAPK/JNK (catalog #9252), phospho-Ser^{63} c-Jun (catalog #9261), phospho-Ser^{73} c-Jun (catalog #9164S), c-Jun (catalog #9162), caspase-9 (catalog #9502), cleaved caspase-3 (Asp175, catalog #9661), cleaved caspase-6 (Asp198, catalog #9761S), and cleaved Poly(ADP-ribose) polymerase (PARP) (Asp214, catalog #9541) -specific antibodies were obtained from Cell Signaling Technology (Beverly, MA). Cleaved caspase-9 antibody was generously provided by Merck-Frosst (Dorval, Quebec, Canada). Horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). Immunoreactive bands were detected using enhanced chemiluminescence purchased from PerkinElmer Life Sciences (Emeryville, CA). All other reagents were from Sigma, Calbiochem (La Jolla, CA), or ICN Biochemicals (Costa Mesa, CA), unless otherwise indicated.

Plasmids and recombinant adenovirus. Preparation of recombinant adenovirus expressing enhanced green fluorescent protein (AdGFP), β-galactosidase (AdLacZ), full-length p75NTR (Adp75NTR), the Flag-tagged JNK-binding domain of JNK interacting protein (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 sucrose gradients, 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 by targeting the sequence GGGAGCATCGTTCAGCAGCAGC in rat BAD, 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, Montreal, Quebec, Canada) and maintained in 5% CO_2 at 37°C in either DMEM or RPMI medium supplemented with 10% fetal calf serum (FCS) (Clontech, Cambridge, UK), 2 mM L-glutamine, and 100 μg/ml penicillin–streptomycin. Neuroblastoma cell lines (SY5Y, SKNAS, 15N, and NGP) were provided by Dr. David Kaplan (University of Toronto, Toronto, Canada) 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 μg/ml penicillin–streptomycin, and 100 μg/ml G418. Cell lines were plated 18–24 hr before transfection and typically harvested 24–48 hr after infection. Primary cortical cultures were prepared from embryonic day 14 (E14)–E16 CD1 mouse telencephalon as described previously (Bhakar et al., 2002). Neuronal cultures were infected before plating and maintained in Neurobasal media (Invitrogen, Gaithersburg, MD) supplemented with 1× B27 supplement (Invitrogen), 2 mM L-glutamine, and 100 μg/ml penicillin–streptomycin. PC12 cells were plated onto poly-L-lysine-coated plates and transfected using Lipofectamine2000 as directed by the manufacturer (Invitrogen). Cell lines were infected with adenovirus 24 hr 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, and 20 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.4), and then resuspended in 500 μl Buffer B (Buffer A plus 5% Percoll, 0.01% digitonin, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride). A sample of this suspension was retained as total cell lysate. The remainder was incubated on ice for 15 min and then centrifuged at 2500 × *g* for 10 min 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 radioimmunoprecipitation assay buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride) and analyzed for protein content using the BCA assay (Pierce, Rockford, IL). 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, and 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 phosphospecific antibodies that 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 4 hr of a 48 hr 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 nonspecific 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 ANOVAs 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 hr later mRNA was isolated using the RNEasy Mini kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). 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′ CCAGACGGAA-

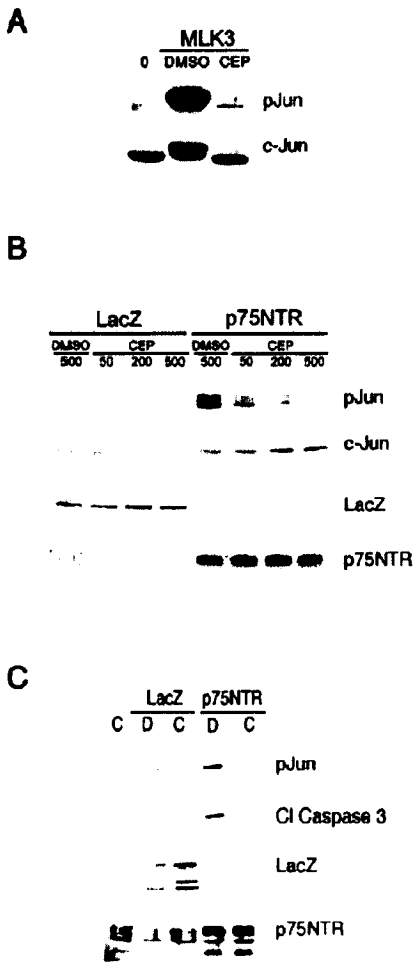

Figure 4. Inhibition of MAP3K signaling attenuates apoptosis induced by p75NTR. *A*, U373 cells infected with 100 MOI of MLK3 adenovirus or left uninfected (0) were treated 47 hr later with DMSO or CEP1347 at 200 nM for 1 hr. Cells were harvested, and lysates were subjected to immunoblot analysis for phospho-Ser[63] c-Jun (pJun) and total 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 hr 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 CEP1347 (*C*) or DMSO (*D*) at the time of infection, and lysates were prepared 48 hr later and analyzed by immunoblot for levels of p75NTR, LacZ, phospho-Ser[63] c-Jun (pJun), and cleaved caspase 3 (cl. caspase 3).

GATAAAGCGTAACAG; 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. Five percent of the cDNA prepared was used in a 25 μl 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 hr after transfection and fixed 24 hr later using 4% paraformaldehyde in PBS. Cells were blocked in TBS supplemented with 5% donkey serum and 0.3% Triton X-100 for 30 min and then incubated for 18 hr 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 hr 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 were analyzed for statistical significance by ANOVA (Tukey's honestly significant difference (HSD) 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 1 shows that primary mouse cortical neurons, PC12 pheochromocytoma 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 death 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 (Fig. 2*A*,*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 caused by adenoviral toxicity because 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

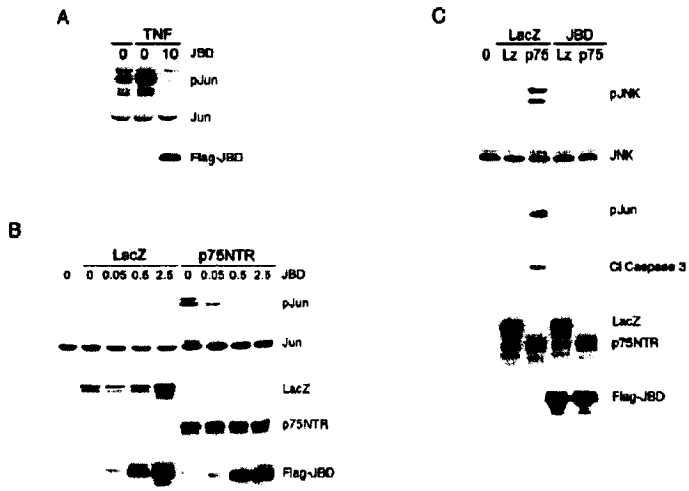

Figure 5. Activation of the JNK pathway is required for p75NTR-mediated caspase activation. Immunoblots for phospho-Ser[63] c-Jun (pJun), c-Jun, Flag-JIP, LacZ, p75NTR, phospho-Thr[183]/Tyr[185]-JNK (pJNK), JNK, and cleaved caspase 3 were performed as indicated on lysates from U373 cells treated with TNF 20 ng/ml that were either left uninfected (0) or infected with JBD-JIP adenovirus (JBD) at 10 MOI (*A*), 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 (*B*), and PC12 cells infected with 50 MOI of LacZ or p75NTR adenovirus supplemented with LacZ or JBD-JIP (JBD) adenovirus (both at 5 MOI) (*C*).

immunoblot. Figure 2*C* 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 (Fig. 3*A*,*B*) and induced a dose-responsive increase in the phosphorylation of c-Jun, a JNK target (Fig. 3*C*). These results indicate that 75NTR-induced JNK activation is a consistent feature 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, an MAP3K identified as a target of CEP1347 (Maroney et al., 2001; Roux et al., 2002). Figure 4*A* shows that the compound almost completely blocked the robust c-Jun phosphorylation induced by this kinase. 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) (Fig. 4*B*,*C*) (data not shown). These findings indicate that reductions in MAP3K and JNK signaling attenuates apoptosis induced by p75NTR yet suggest that blockade of a nonpreferred 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 JIP1 scaffolding molecule (AdJBD) was used to inhibit JNK activity *in vivo*. This JIP1 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 tumor necrosis factor (TNF)α, a well characterized JNK pathway inducer (Fig. 5*A*). Subsequent studies established that AdJBD was equally effective in blocking c-Jun phosphorylation induced by p75NTR expression (Fig. 5*B*). 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 the p75NTR-induced apoptotic cascade (Fig. 5*C*) (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 6*A* 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 hr after adenovirus infection. Figure 6*B* shows that phosphorylated JNK was first detected 18 hr after p75NTR infection and increased further by 24 and 30 hr. Cleaved caspase 3 was detectable 24 hr after infection, but c-Jun phosphorylation showed a significant lag, and an elevation in phospho-Jun levels were detected only after 30 hr 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 that is activated by p75NTR.

BH3-domain-only proteins directly and indirectly induce the association of Bax and Bak, which in turn facilitate 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 apoptotic signaling 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

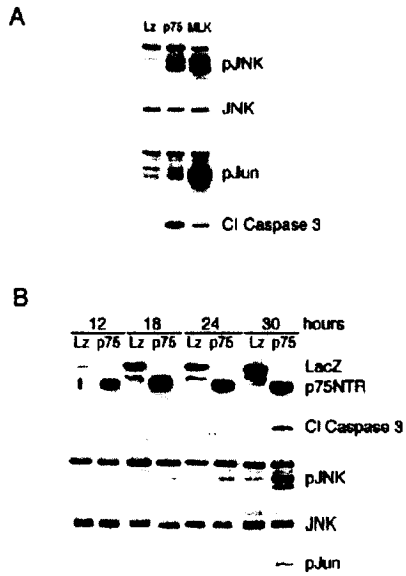

Figure 6. p75NTR-induced caspase 3 cleavage does not correlate with phosphorylation of c-Jun. PC12 cells were infected with 50 MOI of AdLacZ (Lz), Adp75NTR (p75), or AdMLK3 (MLK) recombinant adenovirus, and lysates were prepared at 30 hr after infection (*A*, *C*), or at 12, 18, 24, and 30 hr after infection (*B*). Lysates normalized for protein content were analyzed for LacZ, p75NTR, cleaved caspase 3, phospho-Thr^{183}/Tyr^{185}-JNK (pJNK), total JNK, phospho-Ser^{63} c-Jun (pJun), and total c-Jun protein levels by immunoblot as indicated.

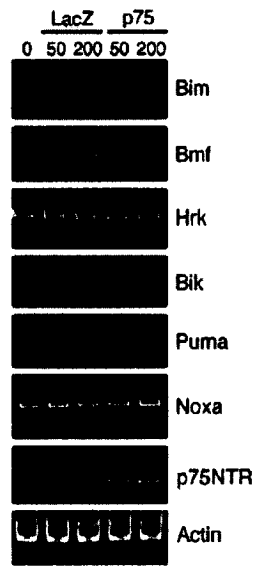

Figure 7. p75NTR does not transcriptionally regulate BH3-domain-only proteins. Cortical neurons were infected with 0, 50, or 200 MOI of LacZ or p75NTR (p75) adenovirus, and 24 hr later mRNA was isolated as described in Materials and Methods. RT-PCR was performed using primers directed against Bim, Bmf, Hrk, Bik, Puma, Noxa, p75NTR, and Actin as indicated.

each of these family members were readily detected in both cell types examined, but p75NTR-dependent increases in their levels were not detected (Fig. 7) (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 were examined by immunoblot. Figures 8, *A* and *B*, shows that p75NTR expression had little effect on the levels or phosphostatus of monomeric Bad (~25 kDa) but rather induced the accumulation of a higher molecular weight species (~75 kDa). This product was detected by two antibodies directed against distinct epitopes in Bad (N19, C20) as well as by a phosphospecific 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 Ad-JBD, lysed and examined by Bad immunoblot. Figure 8*C* 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 caspase activation, 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 24 hr of virus infection by scoring transfected cells for the presence of cleaved caspase 3. Figure 9 shows that expression of the dominant-negative Bad serine 128 mutant allele confers significant protection from p75NTR-induced caspase 3 cleavage, suggesting 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 and PC12 cells (supplementary Fig. 2, available at www.jneurosci.org) and then used to reduce Bad levels in PC12 cells before adenovirus infection. PC12 cells were transfected with GFP alone or with GFP together with the Bad-RNAi plasmid and, 48 hr later, were infected with either p75NTR or LacZ adenovirus for 24 hr. PC12 cells transfected with Bad-RNAi do not generate pyknotic nuclei (data not shown) and are highly resistant to p75NTR-induced caspase-3 cleavage (Fig. 9), indicating a crucial role for Bad in the p75NTR apoptotic pathway.

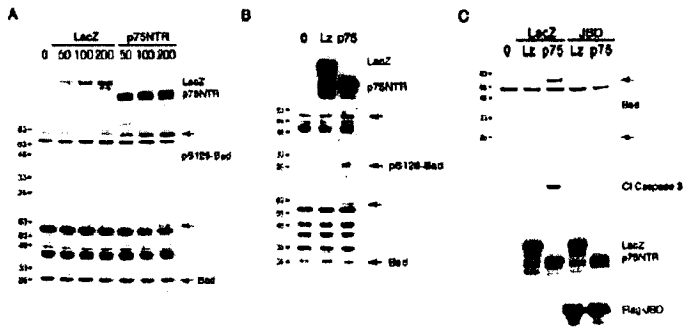

Figure 8. p75NTR activates JNK-dependent phosphorylation and oligomerization of Bad. *A*, U373 cells were infected with 0, 50, 100, or 200 MOI of LacZ or p75NTR adenovirus, and lysates were analyzed by immunoblot for LacZ, p75NTR, phospho-Ser ^{128} Bad, and Bad (C-20, shown; N19, data not shown). *B*, PC12 cells were left uninfected (0) or were infected with LacZ (Lz) or p75NTR (p75) adenovirus at 100 MOI, and lysates were analyzed by immunoblot for LacZ, p75NTR, phospho-Ser ^{128} 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.

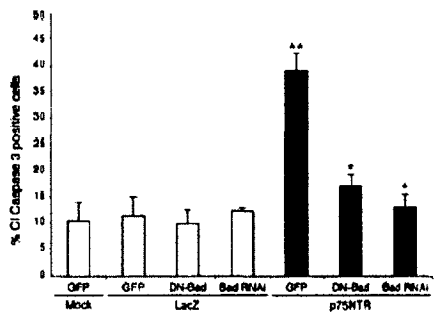

Figure 9. Bad is required for p75NTR-induced apoptosis. PC12 cells were transfected with GFP plasmid alone or with GFP plasmid together with plasmids encoding DN-Bad (S128A) or expressing Bad-RNAi. Cells were infected 48 hr later with LacZ or p75NTR adenovirus and, at 24 hr after infection, were fixed and immunostained for cleaved caspase 3 as described in Materials and Methods. Control experiments established that cleaved caspase 3 immunoreactivity correlates with TUNEL staining and is thus a valid surrogate for direct measurement of apoptosis (see supplementary Fig. 1, available at www.jneurosci.org). 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.

Discussion

The mechanisms used by p75NTR to induce apoptosis are unique and bear little similarity to cell death signaling pathways used 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 death-inducing signaling complex that facilitates FAS-associated death domain-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, whereas 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 nonpreferred 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 raise the possibility that enzymes in the JNK pathway may provide feasible targets for inhibiting p75NTR-induced apoptosis after 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 distinct pro-apoptotic BH3-domain-only family members, such as Noxa and Puma (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 required for p75NTR-dependent caspase activation.

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 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 are sequestered in the cytosol by binding to dynein light chain or myosin V (Puthalakath et al., 1999, 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 cytoskeletal association 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, and the observation that p75NTR induces phosphorylation of BAD at Serine 128 suggests that p75NTR promotes apoptosis by opposing survival factor signals that suppress the cell death machinery. p75NTR activation also 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 p75NTR activation to the phosphorylation 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, p75NTR readily induces apoptosis in U373 cells that lack functional p53 (Fig. 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.

In this and previous studies, we have used p75NTR overexpression as an experimental paradigm to study p75NTR-induced apoptosis. p75NTR levels often rise dramatically after neuronal injury, and the injury-induced accumulation of p75NTR is tightly associated with neuronal death *in vivo* (Roux et al., 1999; Troy et al., 2002). However, although p75NTR overexpression is a convenient paradigm for analyzing downstream, it is important to emphasize that p75NTR binds multiple ligands, and these are certain to modulate its activity. In particular, recent studies indicate that the pro-form of NGF is a potent activator of p75NTR-induced apoptosis *in vitro* and *in vivo* (Lee et al., 2001; Beattie et al., 2002) and a crucial next step will be to directly assess the effects of proneurotrophins on p75NTR signaling events.

p75NTR plays a prominent role in nervous system apoptosis, particularly after trauma, and identification of the pro-apoptotic signal transduction mechanisms activated by the receptor are beginning to emerge. In this study, we show that p75NTR-dependent JNK activation is required for caspase activation. We demonstrate that p75NTR-dependent JNK activation induces phosphorylation and activation of Bad, a BH3-domain-only protein, and we show that Bad is required for apoptosis induced by p75NTR.

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Supplementary Figure 1
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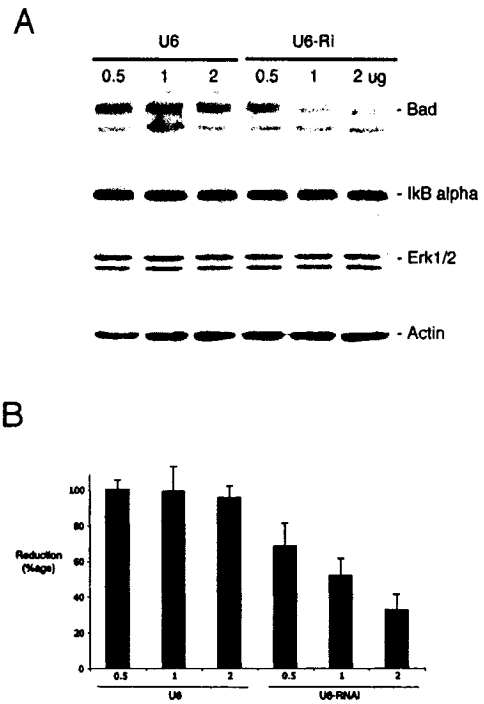

Validation of Bad RNA Interference Vector. (A) PC12 cells were transfected with increasing amounts of the U6-driven RNAi plasmid or a corresponding parental control vector and analyzed for reductions in endogenous Bad, IkB alpha, Erk1/2 or Actin, as indicated. Bad levels were reduced but levels of actin, IkB alpha and Erk1/2 were unchanged. (B) Bad protein knockdown was quantified on immunoblots by densitometry. Data shown represents average and standard deviation of three independent experiments. U6 = pcDNA3 + U6 promoter, U6-RL1 = pcDNA3 + U6 promoter driving Bad RNAi.

Supplementary Figure 2
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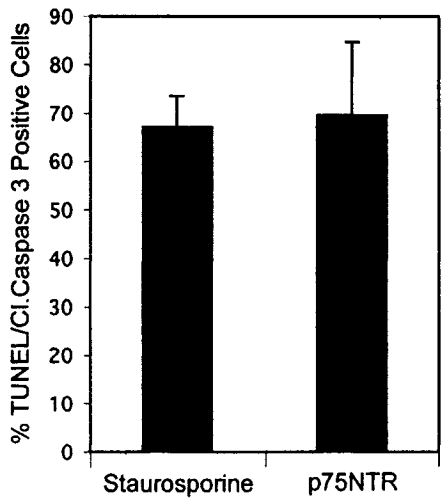

Cleaved Caspase 3 Immunoreactivity Correlates with TUNEL. PC12 cells were exposed to staurosporine (10 ug.ml) or were infected with p75NTR adenovirus (100 MOI) and 24 hours later were fixed and immunostained for TUNEL and cleaved Caspase 3. Cells were scored for coincidence of TUNEL and Caspase 3 cleavage by a blinded observer (n = 300 cells/condition).

Cellular/Molecular Neuroscience

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Characterization of the JNK-Bim$_{EL}$ Signaling Pathway in p75^{NTR}-Induced Apoptosis

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Abstract

The c-Jun N-terminal kinase (JNK) signaling pathway plays a critical role in mediating apoptosis in the nervous system. However, the mechanisms by which JNK triggers neuronal apoptosis remain incompletely understood. Recent studies suggest that in addition to inducing transcription of pro-apoptotic genes, JNK also directly activates the cell death machinery. Here, we report that JNK catalyzed the phosphorylation of the BH3-only protein Bim_{EL} at serine 65, both *in vitro* and *in vivo*. The JNK-induced phosphorylation of Bim_{EL} at serine 65 promoted the apoptotic effect of Bim_{EL} in primary cerebellar granule neurons. We also characterized the role of the JNK-Bim_{EL} signaling pathway in apoptosis that was triggered by overexpression of the p75 neurotrophin receptor ($p75^{NTR}$). We found that activation of $p75^{NTR}$ induced the JNK-dependent phosphorylation of endogenous Bim_{EL} at serine 65 in cells. The genetic knockdown of Bim_{EL} by RNA interference or the expression of a dominant interfering form of Bim_{EL} significantly impaired the ability of activated $p75^{NTR}$ to induce apoptosis. Taken together, these results suggest that JNK-induced phosphorylation of Bim_{EL} at serine 65 mediates $p75^{NTR}$-induced apoptosis. Our findings define a novel mechanism by which a death-receptor pathway directly activates the mitochondrial apoptotic machinery.

Key words

Apoptosis; survival; neuron; BH3-only; signal transduction; neurotrophin receptor; protein kinase

Programmed cell death of neurons is an indispensable part of the development of the nervous system and contributes to neuronal loss in neurologic injury and disease. Two canonical cell death pathways have been characterized: an apoptotic pathway that is triggered by the activation of cell-surface death-receptors and leads to direct activation of the caspase cascade, and a cell-intrinsic apoptotic pathway that requires the release of mitochondrial proteins for caspase activation (Strasser et al., 2000; Kaufmann and Hengartner, 2001).

The pro-apoptotic BH3-only proteins play an important role in the activation of the cell-intrinsic cell death program by acting as sentinels of cellular damage (Huang and Strasser, 2000). Once activated, some BH3-only proteins including Bid are thought to directly activate the pro-apoptotic multidomain Bcl-2 family members, while others including Bad interact with and inhibit the pro-survival Bcl-2 family members including Bcl-2 and Bcl-X_L (Gross et al., 1999; Huang and Strasser, 2000).

Among the BH3-only proteins, Bim (Bcl-2 interacting mediator of cell death) is of particular interest to studies of apoptosis in the nervous system. Developmentally-regulated neuronal apoptosis is delayed in mice in which the *bim* gene is disrupted (Putcha et al., 2001; Whitfield et al., 2001), suggesting a critical role for Bim in neuronal cell death. An important question raised by these studies is how Bim-induced death is regulated in neurons. Previously, it has been shown that the apoptotic stimuli of growth factor and neuronal activity withdrawal induce *bim* transcription in sympathetic and cerebellar granule neurons, in part through the c-Jun N-terminal kinase (JNK)-mediated phosphorylation and activation of the transcription factor c-Jun (Harris and Johnson, 2001; Putcha et al., 2001; Whitfield et al., 2001).

Bim exists in three major isoforms that are generated by alternative splicing: Bim_{EL}, Bim_L, and Bim_S. Among the distinct Bim isoforms, Bim_L and Bim_{EL} are the predominant isoforms in neurons (O'Reilly et al., 2000). Interestingly, Bim_{EL} is constitutively expressed in many neuronal cell types suggesting that Bim_{EL} function might be regulated by post-translational modifications in neuronal cells upon exposure to an apoptotic stimulus (O'Reilly et al., 2000). Recent studies suggest that Bim is phosphorylated in response to several apoptotic stimuli, including growth-factor withdrawal in primary neurons (Lei and Davis, 2003; Putcha et al., 2003).

The stress-activated protein kinases (SAPKs) including JNK and p38MAPK are commonly activated in neurons in response to apoptotic stimuli (Mielke and Herdegen, 2000; Harper and LoGrasso, 2001). In addition to mediating trophic factor withdrawal-induced apoptosis, the JNK signaling pathway is a critical mediator of neuronal cell death upon activation of the p75 neurotrophin receptor ($p75^{NTR}$) (Friedman, 2000; Harrington et al., 2002; Roux and Barker, 2002). A widely held view is that activation of $p75^{NTR}$ in the absence of stimulation of the Trk family of neurotrophin receptors, constitutes an important trigger of apoptosis (Frade et al., 1996; Kaplan and Miller, 2000). $p75^{NTR}$ is thought to play a critical role during development and following pathogenic stimuli in the adult brain (Yaar et al., 1997; Bamji et al., 1998; Roux et al., 1999; Dechant and Barde, 2002)

The downstream effector pathways of $p75^{NTR}$-activated and JNK-mediated neuronal apoptosis remain to be characterized. In contrast to trophic factor withdrawal-induced apoptosis, in which JNK-mediated c-Jun-dependent transcription plays a critical role, in $p75^{NTR}$-induced cell death JNK activation is required yet activation of c-Jun appears to be dispensable (Harrington et al., 2002; Palmada et al., 2002; Troy et al., 2002). These findings raise the

interesting possibility that $p75^{NTR}$-activated JNK might directly phosphorylate components of the mitochondrial cell death machinery and thereby induce neuronal apoptosis.

In this study, we have delineated a novel mechanism by which JNK mediates $p75^{NTR}$-induced neuronal apoptosis. We found that JNK catalyzes the phosphorylation of the BH3-only protein Bim_{EL} *in vitro* and *in vivo* at the distinct site of serine 65. The phosphorylation of Bim_{EL} at serine 65 promotes the pro-apoptotic activity of Bim_{EL} in primary neurons. Overexpression of $p75^{NTR}$ was found to induce JNK-mediated phosphorylation of endogenous Bim_{EL} at serine 65. In addition, we demonstrated that Bim_{EL} and JNK-induced phosphorylation of BimEL at serine 65 are required for $p75^{NTR}$-induced apoptosis. Together, these findings define the JNK-Bim_{EL} signaling pathway as a novel link between death receptor activation and the cell death machinery.

Material and Methods

Plasmids and Reagents

Full-length Bim_{EL} cDNA was cloned by RT-PCR from RNA isolated from rat cerebellar granule neurons. Bim_{EL} cDNA was then subcloned into the polylinker of the bacterial GST gene fusion vector pGEX-4T-3 (Amersham Pharmacia Biotech, Piscataway, NJ) and into the polylinker of the mammalian expression vector pCDNA3 (Invitrogen, Carlsbad, CA) with an NH_2-terminal HA-tag. Point mutations of Ser-55, Ser-65, Ser-73, Ser-100, and Thr-112 were made using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Similarly, the splicing donor site for Bim_L (T_{126} to G, without amino acid change), and the BH3-domain of Bim_{EL} (Leu-150 to Ala and Asp-155 to Ala) were mutated. Mutations were verified by sequencing. Activated MKK3 was purchased from Stratagene. The activated MEKK1 plasmid has been previously described (Lee et al., 1997). The dominant negative JNK plasmid was kindly provided by Dr. Yang Shi (Harvard Medical School, Boston, MA).

The U6/BS-Bim RNA interference vector was designed as described (Gaudilliere et al., 2002; Sui et al., 2002). The sense strand of the encoded hairpin RNA was designed to specifically target the 20-nucleotide region GGTATTTCTCTTTTGACACAG in Bim RNA. The targeted region in Bim RNA showed no significant homology with any other gene by BLAST.

Preparation of recombinant adenovirus expressing green fluorescent protein (AdGFP), beta-galactosidase (AdLAcZ), full-length $p75^{NTR}$ ($Adp75^{NTR}$), the Flag-tagged JNK-binding domain of JIP1 (AdJBD) have been previously described (Roux et al., 2002). All adenoviruses were amplified in 293A cells and purified on a sucrose gradient as described (Roux et al., 2002). Titers are expressed as plaque forming units.

To generate the phospho65-Bim antibody, a phosphopeptide of the sequence CLAPPApSPGPFATR (Tufts Synthesis Facility, Tufts Medical School, Boston, MA), was synthesized and coupled to keyhole limpet hemocyanin using the Imject Maleimide Activated mcKLH Kit (Pierce, Rockford, IL). The antigen was injected into New Zealand White rabbits (Covance Research Products, Denver, PA), from which serum was collected approximately every three weeks. Serum was affinity-purified by subsequently passing it on an Immunopure immobilized protein A column (Pierce) and an agarose-iodoacetyl column (Pierce) to which a synthetic peptide of the sequence CLAPPASPGPFATR (Tufts Synthesis Facility) was coupled. The final eluate was desalted and concentrated using Amicon Ultra centrifugal filter devices (Millipore, Bedford, MA).

Other antibodies used in this study include polyclonal anti-Bim antibody (Stressgen, Victoria, BC, Canada, Cat# AAP-330), polyclonal anti-Bim antibody (Calbiochem, San Diego, CA, Cat# 202000), monoclonal anti-GST antibody (Santa Cruz, Santa Cruz, CA, Cat# sc-138), polyclonal anti-HA antibody (Santa Cruz, Cat# sc-805), polyclonal phospho-JNK antibody (Cell Signaling, Beverly, MA, Cat# 9251), polyclonal JNK antibody (Upstate Biotechnology, , Lake Placid, NY, Cat# 06-748), polyclonal phospho-p38MAPK antibody (Cell Signaling, Cat# 9211), polyclonal phospho-c-Jun antibody (Cell Signaling, Cat# 9261), monoclonal 14-3-3 beta antibody (Santa Cruz, Cat# sc-1657), monoclonal p42 MAP kinase antibody (Cell Signaling, Cat# 9107), monoclonal anti-beta-galactosidase antibody (Promega, Madison, WI, Cat# Z3781), monoclonal anti-actin antibody (Santa Cruz, Cat# sc-8432), and a polyclonal antibody against cleaved caspase-3 (Cell Signaling, Cat# 9661). The p75NTR antibody has been previously described (Roux et al., 1999). Secondary antibodies conjugated to horseradish peroxidase or cyanine dye were purchased from Jackson Immunoresearch Laboratories (West Grove, PA).

The JNK inhibitor II (SP600125) and the p38MAPK inhibitor (SB203580) were purchase from Calbiochem (EMD Biosciences Inc., San Diego, CA)

In Vitro Kinase Assays

In vitro kinase assays were done as described in the Upstate Biotechnology procedure for the JNK1α1 protein kinase assay. Briefly, JNK1α1 (Upstate Biotechnology), 5 μg of recombinant GST-Bim, 100 μM ATP, and 5 μCi of [γ-^{32}P]ATP in 2 mM MOPS, pH 7.2, 2.5 mM β-glycerol phosphate, 0.5 mM EGTA, 0.1 mM sodium orthovanadate, 0.1 mM DTT, and 15 mM magnesium chloride were incubated at 30°C for 1 h. The reactions were stopped by adding SDS sample buffer and boiling. Samples were analyzed by 15% SDS-PAGE, Coomassie staining, and autoradiography. In the experiments depicted in Fig. 2*B*, no radiolabeled ATP but 200 μM ATP was used, and the samples were analyzed by SDS-PAGE and immunoblotting.

Mass Spectrometry

Following SDS-PAGE and Coomassie staining, the JNK-phosphorylated Bim_{EL} band was cut from the gel and digested with trypsin in-gel. Peptides were separated by nanoscale microcapillary high performance liquid chromatography as described (Stemmann et al., 2001). Eluting peptides were ionized by electrospray ionization and analyzed by an LCQ-DECA ion trap mass spectrometer as described (Stemmann et al., 2001).

Cell Culture, Transfection, and Infection of 293T and PC12 cells

Human embryonic kidney (HEK) 293T cells (American Type Culture Collection, Manassas, VA) were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Carlsbad, CA) supplemented with 10% fetal calf serum (Gibco),

2 mM L-glutamine, and 100 μg/ml penicillin/streptomycin. Rat pheochromocytoma PC12rtta cells were maintained at 37°C in 10% CO_2 in air in DMEM (Hyclone, Logan, UT) supplemented with 6% horse serum (BioWhittaker, East Rutherford, NJ), 6% bovine calf serum (BioWhittaker), 1% glutamine, and 1% penicillin/streptomycin.

Cell lines were plated 18 to 24 h prior to transfection and harvested 24 h after transfection. HEK293T transfections were performed by a standard calcium phosphate transfection method. PC12rtta cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. 48 h post-transfection, PC12rtta were infected with 100 MOI of adenovirus per well unless otherwise indicated.

Western Analysis

Proteins from cell extracts were separated by polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes (Schleicher&Schuell, Keene, NH). Membranes were blocked for 1 h in a Tris-buffered saline solution (TBS) containing 0.02% to 0.05% Tween (TBST) and 5% (w/v) dried skim milk powder. All primary antibody incubations were performed in TBST supplemented with 5% bovine serum albumin (USB) for 1 h at room temperature, except for incubations with the phospho-BimS65 antibody, which were done in blocking solution overnight at 4°C. Antibody binding was detected by enhanced chemiluminescence (ECL, Perkin Elmer Life Sciences, Boston, MA) using a secondary antibody conjugated to horseradish peroxidase (dilution: 1:20,000).

Survival Assays in Cerebellar Granule Neurons

Cerebellar granule cells were prepared as described (D'Mello et al., 1993; Shalizi et al., 2003) and transfected using a calcium phosphate transfection method. For the experiments depicted in Fig. 4, cultures from postnatal day 6 that were in culture for 8 days were transfected with Bim wildtype or mutant DNA (0.1 μg/well) or the control vector pCDNA3 (0.1 μg/well), together with a plasmid encoding β-galactosidase (0.25 μg/well) and a carrier plasmid (2 μg/well pECE) in a 24-well plate. The calcium phosphate precipitate was placed on granule neurons that had been starved for 1 hr in DMEM for 20 min, and cultures were subsequently returned to conditioned medium (basal medium Eagle (BME) (Gibco) supplemented with 10% calf serum (Hyclone) and 30 mM KCl). After overnight incubation with conditioned medium, cultures were deprived of conditioned medium and left in BME in the presence or absence of insulin (10 μg/ml) for 8 h. Cultures were fixed with 4% paraformaldehyde (PFA) in PBS and subjected to indirect immunofluorescence using an antibody to beta-galactosidase (dilution 1:500). Cell survival and death were assessed in beta-galactosidase-expressing neurons based on the integrity of neurites and the morphology of the nucleus as determined using the DNA dye bisBenzimide Hoechst 33258 trihydrochloride (Sigma, Saint Louis, MO). Cell counts were done in a blinded manner (n=100 cells/condition) and analyzed for statistical significance by ANOVA (Fisher's Protected Least Significance Difference).

Caspase-3 Activation Assay in PC12 cells

PC12rtta cells were transfected with the indicated plasmids encoding either GFP, pU6/BS control vector, pU6/BS-Bim RNA interference plasmid, pU6/BS-Cdk2 RNA interference plasmid, pCDNA3 control vector, Bim wildtype BH3-domain mutant plasmid, or Bim S65A BH3-domain mutant plasmid. 48 h after transfection, cells were infected with either AdLacZ or with Adp75^{NTR} and

fixed 24 h later with 4% PFA in PBS. GFP-positive cells were scored for the presence of cleaved caspase-3 by indirect immunocytochemistry using an antibody against cleaved caspase-3 (dilution 1:1000). Transfected cells were scored by a blind observer (n=150 cells/condition) and the composite data were analyzed for statistical significance by ANOVA (Tukey's Honestly Significant Difference).

Results

JNK Phosphorylates Bim_{EL} at Serine 65 in Vitro

We first investigated whether JNK might directly regulate the BH3-only protein Bim_{EL}. Bim_{EL} contains six putative proline-directed sites for JNK-mediated phosphorylation (Fig. 1*A*). Using an *in vitro* kinase assay, we found that recombinant JNK robustly catalyzed the phosphorylation of a recombinant GST-Bim_{EL} fusion protein (Fig. 1*B*). To determine which putative phosphorylation sites are targeted by JNK, we subjected recombinant GST-Bim_{EL} that was phosphorylated by JNK to tandem mass spectrometry (LC-MS/MS). Tryptic peptides were identified that matched with the entire Bim_{EL} sequence. Phosphate-containing peptides were identified by a differential mass of +80 Da relative to the theoretical mass of unphosphorylated peptides. Analysis of the peptides revealed that JNK phosphorylated Bim_{EL} at all six putative phosphorylation sites. As an example, the MS/MS spectrum of a phosphopeptide containing phospho-serine 55 and phospho-serine 65 is shown (Fig. 1*C*).

To identify the major sites of JNK-induced phosphorylation among the six sites that were identified by mass spectrometry, we generated Bim_{EL} mutants in which the phosphorylation sites at serine 55, serine 65, and threonine 112 were replaced with alanine, and subjected the recombinant Bim_{EL} mutant proteins to an *in vitro* kinase reaction. We found that although all three Bim_{EL} mutants were phosphorylated by JNK, the phosphorylation was dramatically diminished in the Bim_{EL}S65A mutant and to a lesser degree in the other mutants (Fig. 2*A*), suggesting that serine 65 is the predominant site that is phosphorylated by JNK *in vitro*.

To establish that JNK phosphorylates Bim_{EL} at serine 65, we raised a phosphospecific antibody to recognize Bim_{EL} only when it is phosphorylated at

serine 65 (phospho65-Bim antibody). Recombinant Bim_{EL} was phosphorylated *in vitro* by JNK and then immunoblotted with the phospho65-Bim antibody or an antibody that recognizes Bim regardless of its phosphorylation state. We found that JNK robustly phosphorylated Bim_{EL} at serine 65 (Fig. 2*B*). The phospho65-Bim reactivity was specific, as it failed to recognize the Bim_{EL}S65A mutant that was incubated with JNK (Fig. 2*B*). Taken together, our results indicate that JNK phosphorylates Bim specifically at serine 65 *in vitro*.

Stress-activated Protein Kinases Phosphorylate Bim at Serine 65 in Vivo

We next determined if activation of the JNK signaling pathway induces the phosphorylation of Bim_{EL} at serine 65 within cells. We expressed in HEK293T cells either wildtype Bim_{EL} or Bim_{EL} mutants in which the *in vitro* sites of JNK-induced phosphorylation were replaced with alanine in the presence or absence of an expression plasmid encoding a truncated and constitutive active form of the kinase MEKK1 (MEKK1Δ). MEKK1 is the prototypical mitogen-activated protein kinase (MAPK) that activates the JNK signaling pathway. Extracts from transfected HEK293T cells were prepared and subjected to immunoblotting using an anti-Bim antibody.

The expression of activated MEKK1 together with Bim_{EL} in HEK293T cells resulted in the retardation of the electrophoretic mobility of Bim_{EL} (Fig. 3*A*). The electrophoretic mobility shift of Bim_{EL} reflected Bim_{EL} phosphorylation, since the shift completely collapsed upon treatment of the cell lysates with alkaline phosphatase (data not shown). Compared to wildtype Bim_{EL} the phosphorylation mutants Bim_{EL}S55A and Bim_{EL}S65A displayed reduced electrophoretic mobility shifts, and the shift was completely absent in the Bim_{EL}S55/65A mutant. In contrast to the S55/65A mutation, mutation of each of the other three *in vitro* sites of JNK-induced phosphorylation S73, S100, or T112 modestly reduced the

electrophoretic mobility shift of Bim_{EL} (Fig. 3*A*). Immunoblotting of lysates of HEK293T cells in which Bim_{EL} was expressed together with MEKK1Δ using the phospho65-Bim antibody revealed that activated MEKK1 robustly induced the phosphorylation of Bim_{EL} at serine 65 (Fig. 3*B*). Together, these results indicate that activation of the MEKK1-induced SAPK signaling pathways *in vivo* induces the phosphorylation of Bim_{EL} at serine 65.

MEKK1 has been previously shown to induce protein kinase cascades that culminate in the activation of the stress-activated MAPKs JNK and p38MAPK (Harper and LoGrasso, 2001). In HEK293T cells, activated MEKK1 did induce the phosphorylation of both JNK and p38MAPK at sites that reflect their activation as determined by immunoblotting of HEK293T extracts using antibodies that recognize the phosphorylated forms of JNK and p38MAPK specifically (Fig. 3*B*). The expression of a constitutive active form of JNK also stimulated the phosphorylation of Bim_{EL} at serine 65, although to a lesser degree than MEKK1 (data not shown), suggesting that both JNK and p38MAPK might phosphorylate Bim_{EL} downstream of MEKK1. In other experiments, the expression of a constitutive active form of the p38MAPK activator MKK3 together with p38MAPK and Bim_{EL} in HEK293T cells strongly induced the phosphorylation of Bim_{EL} at serine 65 (Fig. 3*B*). Interestingly, the expression of MKK3 activated both p38MAPK and JNK in HEK293T cells (Fig. 3*B*), consistent with the conclusion that both of these kinases might contribute to the phosphorylation of Bim_{EL} at serine 65. In agreement with this interpretation, we found that the pharmacological inhibitors SB203580 (5 μM) and SP600125 (10 μM), which inhibit respectively p38MAPK and JNK, each partially reduced the MEKK1-mediated phosphorylation of Bim_{EL} at serine 65 (data not shown).

Recently, Bim_{EL} has been suggested to be the target of the ERK1/2 signaling pathway (Ley et al., 2003). However, the expression of a constitutive

active form of the ERK1/2 activator MEK1 together with Bim_{EL} had little effect on the phosphorylation of Bim_{EL} at serine 65 (Fig. 3*B*). Together, these data suggest that among the MAPKs, the SAPKs JNK and p38MAPK may selectively induce the phosphorylation of Bim_{EL} at serine 65.

To determine if activation of the SAPK signaling pathways induces the phosphorylation of endogenous Bim_{EL} *in vivo*, we tested whether exposure of cells to hyperosmotic stress, a stimulus known to activate SAPK signaling pathways, triggers the phosphorylation of Bim_{EL} at serine 65. We found that exposure of HEK293T cells to high concentrations of the hyperosmotic stimulus sorbitol induced the phosphorylation of endogenous Bim_{EL} at serine 65 (Fig. 3*C*). Inhibition of JNK and p38MAPK activity by the inhibitors SP600125 and SB203580 partially reduced the sorbitol-mediated phosphorylation of Bim_{EL} at serine 65 (Fig. 3*D*). The activity of the JNK inhibitor was confirmed by the observation that it effectively reduced sorbitol-induced phosphorylation of the transcription factor c-Jun (Fig. 3*D*). Together, these results suggest that endogenous SAPKs mediate the phosphorylation of endogenous Bim_{EL} at serine 65 *in vivo* following osmotic stress.

JNK-Induced Phosphorylation of Bim_{EL} at Serine 65 Activates the Apoptotic Function of Bim_{EL}

To determine the biological effect of the JNK-induced phosphorylation of Bim_{EL} on the function of Bim, we expressed in primary cerebellar granule neurons Bim_{EL} or Bim_{EL} mutants in which the JNK sites of phosphorylation were mutated. After transfection, granule neurons were deprived of extracellular survival factors to provide a stimulus that activates the JNK signaling pathway in neurons (Coffey et al., 2002). The expression of Bim_{EL} robustly induced apoptosis of survival factor-deprived cerebellar granule neurons (Fig. 4*A*). We

found that a dominant interfering form of JNK (JNK DN), when co-expressed with Bim_{EL}, significantly reduced Bim_{EL}-induced apoptosis in survival factor-deprived granule neurons, suggesting that the JNK signaling pathway stimulates the apoptotic function of Bim_{EL} in these neurons. Since the *in vitro* (Fig. 2*A*) and *in vivo* evidence (Fig. 3*A*) suggested that serines 55 and 65 might represent key sites of JNK-mediated phosphorylation, we first tested the effect of the double S55/65A mutation on Bim_{EL}-induced apoptosis. We found that expression of the Bim_{EL}S55/65A mutant induced significantly less neuronal apoptosis than expression of Bim_{EL} (Fig 4*A*), suggesting that JNK-induced phosphorylation of Bim_{EL} at these sites might promote Bim_{EL}-dependent apoptosis.

We next set out to determine the relative contribution of each of the two phosphorylation sites, serine 55 and 65, in Bim_{EL}-mediated neuronal apoptosis. In our initial experiments, the effect of these phosphorylation site mutants on neuronal apoptosis was somewhat variable. Remarkably, the Bim_{EL} cDNA contains internal splicing donor and acceptor sites such that the Bim_{EL} cDNA may lead to the generation of both Bim_{EL} and Bim_{L} mRNA (Shinjyo et al., 2001). According to Shinjyo et al., cells transfected with Bim_{EL} cDNA may produce both Bim_{EL} and Bim_{L} proteins (Shinjyo et al., 2001). Bim_{L}, a potent inducer of apoptosis, does not contain the JNK phosphorylation sites serines 55 and 65 and is not subject to the post-translational regulation at these sites. We therefore reasoned that in the survival assays in granule neurons, the potential expression of Bim_{L} encoded by Bim_{EL} cDNA might mask the regulatory effect of serines 55 and 65 on Bim_{EL}-induced neuronal cell death. Therefore, prior to further testing of the functional consequences of Bim_{EL} phosphorylation, we first addressed the concern that the unusual splicing of Bim_{EL} cDNA might interfere with the interpretation of the survival assays. To circumvent the potential expression of

Bim_L encoded by Bim_{EL} cDNA, we mutated the splice donor site in the Bim_{EL} expression plasmids and used these cDNAs for all subsequent survival assays.

The expression of Bim_{EL} encoded by the non-spliceable Bim_{EL} cDNA strongly induced apoptosis in survival factor-deprived granule neurons (Fig. 4*B,C*). Using non-spliceable Bim_{EL} cDNAs, we found surprisingly that the Bim_{EL} mutant in which serine 55 was replaced with an alanine (Bim_{EL}S55A) induced apoptosis in neurons as effectively as Bim_{EL} (Fig. 4*B*). By contrast, the non-spliceable Bim_{EL} S65A mutant (Bim_{EL}S65A) strikingly reduced neuronal apoptosis to levels that were similar to those in control vector-transfected neurons (Fig. 4*B*). In other experiments we found that replacement of all five potential JNK phosphorylation sites by alanine reduced the apoptotic effect of Bim_{EL} but not more effectively than the single S65A mutation (Fig. 4*B* and data not shown). Taken together, our findings suggest that JNK-induced phosphorylation of Bim_{EL} at serine 65 promotes the pro-apoptotic activity of Bim_{EL} in primary cerebellar granule neurons.

To begin to characterize how the phosphorylation at serine 65 promotes Bim_{EL}-dependent apoptosis, we expressed a Bim_{EL} mutant in which serine 65 was replaced with aspartate to mimic phosphorylation at this site (Bim_{EL}S65D). We found that Bim_{EL}S65D robustly induced apoptosis in survival factor-deprived neurons (Fig. 4*B*). Under these conditions, the apoptotic effect of Bim_{EL}S65D was slightly greater than that of Bim_{EL}, but the difference did not reach statistical significance. While Bim_{EL}-mediated apoptosis was inhibited by the expression of JNK DN (Fig. 4*A*), we found that co-expression of JNK DN had little effect on Bim_{EL}S65D-induced cell death (data not shown). These results are consistent with the interpretation that the phosphorylation of Bim_{EL} at serine 65 acts downstream of JNK in promoting neuronal apoptosis.

We next asked if phosphorylation of Bim_{EL} at serine 65 might have an effect on the apoptotic effect of Bim_{EL} under survival conditions. We found that activation of the IGF-I receptor and its downstream survival pathways by high doses of insulin (Dudek et al., 1997) significantly reduced Bim_{EL}-induced apoptosis (Fig. 4*C*). However, insulin failed to inhibit the apoptotic effect of Bim_{EL}S65D (Fig. 4*C*). These results raise the possibility that the phosphorylation at serine 65 might promote Bim_{EL}'s apoptotic function by opposing survival factor inhibition of Bim_{EL}-mediated apoptosis.

The JNK-Bim_{EL} Signaling Pathway in $p75^{NTR}$-induced Apoptosis

Having identified the JNK-induced phosphorylation of Bim_{EL} at serine 65 as a mechanism for JNK to directly activate the cell death machinery, we next investigated if the JNK-Bim_{EL} signaling pathway is a critical mediator of specific stimuli known to induce apoptosis in the nervous system. Recent studies suggest that JNK triggers neuronal apoptosis upon activation of the neurotrophin receptor p75 ($p75^{NTR}$) via a c-Jun-independent mechanism (Palmada et al., 2002; Bhakar et al., 2003). These observations suggest that $p75^{NTR}$ might directly activate the cell death machinery via the JNK-Bim_{EL} signaling pathway.

To characterize the potential role of the JNK-Bim_{EL} signaling pathway in $p75^{NTR}$-mediated apoptosis, we employed a well-established paradigm of overexpression of full-length $p75^{NTR}$ to trigger $p75^{NTR}$-dependent cell death (Roux et al., 2001; Wang et al., 2001; Bhakar et al., 2003). We infected PC12 cells with adenovirus encoding full-length $p75^{NTR}$ or control LacZ protein and immunoblotted the PC12 cell extracts with the phospho65-Bim antibody or an anti-Bim antibody that recognizes Bim_{EL} regardless of its phosphorylation state. Expression of $p75^{NTR}$ induced the phosphorylation of Bim_{EL} at serine 65 (Fig. 5*A*). To determine if $p75^{NTR}$-induced phosphorylation of Bim_{EL} at serine 65 is

dependent on JNK activation, we coexpressed with p75 the JNK binding domain (JBD) of the scaffold protein JIP that functions as a dominant negative inhibitor of JNK signaling (Harding et al., 2001). The expression of JBD blocked $p75^{NTR}$-induction of JNK activity and cell death (Bhakar et al., 2003) (data not shown). We found that JBD expression also blocked the $p75^{NTR}$-induced phosphorylation of Bim_{EL} at serine 65 (Fig. 5*A*). Together, these results suggest that JNK mediates $p75^{NTR}$-induced phosphorylation of Bim_{EL} at serine 65.

To investigate if Bim_{EL} mediates $p75^{NTR}$-induced apoptosis, we employed a DNA template-based method of RNA interference (RNAi) to reduce the levels of endogenous Bim_{EL} in PC12 cells. Expression of Bim hairpin RNA but not the control RNAi plasmid or the expression of hairpin RNA to the unrelated protein Cdk2 significantly reduced endogenous Bim_{EL} protein levels as determined in transfection experiments in PC12 cells (Fig. 5*B*). To determine the effect of Bim_{EL} knockdown on $p75^{NTR}$-induced apoptosis, we transfected PC12 cells with GFP alone or GFP together with the distinct RNAi plasmids and then infected these cells with the $p75^{NTR}$ or control adenovirus. We found that the genetic knockdown of Bim_{EL} significantly reduced the $p75^{NTR}$-induced apoptosis of PC12 cells as assayed by measuring the cleavage of caspase-3 (Fig. 5*C*). Together, these data suggest that Bim_{EL} plays a crucial role in $p75^{NTR}$-induced cell death.

To test directly if the phosphorylation of Bim_{EL} at serine 65 mediates $p75^{NTR}$-induced cell death, we tested if a mutant allele of Bim_{EL} in which serine 65 was replaced by an alanine acts in a dominant negative manner to inhibit the $p75^{NTR}$-mediated apoptotic response. Since Bim_{EL} is a potent inducer of cell death, we replaced serine 65 with alanine in the context of a full length Bim_{EL} in which the BH3-domain was also mutated. Overexpression of Bim_{EL} harboring two BH3-domain mutations but that was otherwise wildtype including an intact serine 65 failed to alter the ability of $p75^{NTR}$ to induce cell death (Figure 5*C*). By

contrast, the expression of Bim_{EL} containing both the BH3-domain mutations and the S65A mutation remarkably inhibited $p75^{NTR}$-induced cell death (Figure 5*C*). These results suggest that the non-phosphorylatable BimEL protein acts in a dominant negative manner to inhibit $p75^{NTR}$-induced cell death. Taken together with the Bim_{EL} knockdown results, our findings support the conclusion that JNK-induced phosphorylation of Bim_{EL} at serine 65 mediates $p75^{NTR}$-induced cell death.

Discussion

In this study, we have defined a novel mechanism by which activation of JNK by several distinct stimuli, including activated $p75^{NTR}$, mediates the phosphorylation and activation of the BH3-only protein Bim_{EL}. The JNK-induced phosphorylation of Bim_{EL} at the distinct site of serine 65 promoted the apoptotic function of Bim_{EL} in primary neurons. The genetic knockdown of Bim_{EL} by RNA interference or the expression of a dominant interfering form of Bim_{EL} in which serine 65 was replaced by an alanine significantly inhibited apoptosis induced by $p75^{NTR}$ overexpression. Together, our findings suggest that JNK-induced phosphorylation and activation of Bim_{EL} mediates $p75^{NTR}$-induced cell death.

Activation of JNK has long been implicated in neuronal apoptosis (Mielke and Herdegen, 2000; Harper and LoGrasso, 2001). JNK is thought to induce neuronal apoptosis via transcription-dependent and -independent mechanisms. JNK-induced phosphorylation and activation of the transcription factor c-Jun that in turn mediates the expression of pro-apoptotic proteins has been extensively characterized (Ham et al., 2000). Accumulating evidence suggests that in addition to transcription-dependent mechanisms, JNK also directly activates the cell death machinery (Tournier et al., 2000; Lei et al., 2002). However, the connections between JNK and the cell death machinery remain to be elucidated. Our results in this study suggest that JNK phosphorylation of Bim_{EL} at serine 65 promotes Bim_{EL}-mediated apoptosis. We have shown previously that JNK phosphorylates and thereby activates the BH3-only protein Bad (Donovan et al., 2002). Together, our findings suggest that BH3-only proteins are critical targets of JNK, whose phosphorylation directly activates the apoptotic machinery.

The mechanism by which JNK-mediated phosphorylation leads to the activation of Bim_{EL} remains to be characterized. In non-neuronal cells, Bim has been shown to be bound to dynein light chain (DLC1) and thereby sequestered

to the cytoskeleton (Puthalakath et al., 1999). Apoptotic stimuli are thus believed to cause the disruption of the Bim-DLC1 complex thereby allowing Bim to function at the mitochondria. UV-activated JNK catalyzes the phosphorylation of exogenous Bim_L at threonine 56, which lies within the DLC1-binding motif, thereby releasing Bim from DLC1 (Lei and Davis, 2003). However, a role for DLC1 in regulation of BimEL function in neuronal cells remains unclear. The primary site of Bim_{EL} localization in neurons appears to be the mitochondria, and the JNK-induced phosphorylation of Bim_{EL} has not been shown to alter Bim_{EL} localization (Putcha et al., 2003)(EBE Becker and A Bonni, data not shown). Together, these data suggest that JNK activates Bim_{EL} in neuronal cells by a mechanism other than the release of Bim_{EL} from the cytoskeleton. Current investigations are underway to characterize the mechanism by which the phosphorylation of Bim_{EL} at serine 65 promotes the apoptotic function of Bim_{EL}.

In our studies of regulation of Bad, we found that JNK-induced phosphorylation of Bad at serine 128 opposes Akt-inhibition of Bad-mediated apoptosis (Donovan et al., 2002; Konishi et al., 2002). In an analogous manner, our results obtained in survival assays performed in primary neurons support the hypothesis that JNK-induced phosphorylation of Bim_{EL} at serine 65 promotes the apoptotic function of Bim_{EL} in neurons in part by opposing Akt-promoted cell survival. Insulin, a survival signal that robustly induces Akt (Dudek et al., 1997), blocked wildtype Bim_{EL}- but not Bim_{EL}S65D-mediated cell death in granule neurons (Fig. 4*B*), suggesting that JNK-induced phosphorylation of Bim_{EL} at serine 65 counteracts Akt-mediated neuronal survival. The mechanisms underlying the antagonism of Akt and the JNK-Bim_{EL} signaling pathway remain to be characterized. Akt has been reported to inhibit the activation of JNK in neurons by interacting with JIP1 (Kim et al., 2002), raising the possibility that Akt might inhibit JNK-induced phosphorylation of Bim_{EL} at serine 65. An alternative

interpretation of our results is that the serine 65 phosphorylation might counteract the ability of an insulin-induced survival signal to directly inhibit Bim_{EL}-induced apoptosis.

Although we focus in this study on the phosphorylation of Bim_{EL} by JNK, the p38MAPK signaling pathway might also play a role in phosphorylating Bim_{EL} at serine 65. Similar to JNK, p38MAPK has been reported to be involved in a number of neuronal cell death paradigms including growth factor withdrawal and exposure to glutamate (Mielke and Herdegen, 2000; Harper and LoGrasso, 2001). It will be interesting to determine whether the JNK and p38MAPK signaling pathways have overlapping functions in phosphorylating Bim_{EL} at serine 65 under the same apoptotic conditions, or whether distinct stimuli activate these signaling pathways to differentially to induce the phosphorylation of Bim_{EL}.

One cell death trigger that is specifically associated with the activation of JNK is activation of the neurotrophin receptor $p75^{NTR}$ (Harrington et al., 2002). In contrast to other extrinsic cell death receptor pathways, $p75^{NTR}$ activation does not induce caspase-8 and subsequent cleavage of Bid, but $p75^{NTR}$ has been suggested to directly activate the cell-intrinsic apoptotic machinery at the mitochondria (Roux and Barker, 2002). In this study, we show that overexpression of $p75^{NTR}$ induces the JNK-mediated phosphorylation of Bim_{EL} at serine 65 and thereby triggers apoptosis. We have recently found that the $p75^{NTR}$-JNK pathway also stimulates the phosphorylation of Bad at serine 128 and thus activates Bad-mediated apoptosis (Bhakar et al., 2003). Therefore, we propose that one mechanism by which activation of $p75^{NTR}$ leads to apoptosis is the JNK-mediated direct phosphorylation of BH3-only proteins. Consistent with our results, Putcha *et al.* found in the course of our studies that Bim_{EL} is phosphorylated downstream of the JNK signaling pathway in neurons (Putcha et al., 2003). However, while Putcha *et al.* focus on the phosphorylation of Bim_{EL}

following trophic factor withdrawal, we here provide evidence that Bim_{EL} is phosphorylated upon activation of $p75^{NTR}$, thereby linking this cell-death receptor pathway directly to the cell-intrinsic apoptotic machinery.

$p75^{NTR}$ has been implicated in several neuropathological conditions including focal ischemia, stroke, axotomy, and neurodegenerative disorders (Dechant and Barde, 2002). It will be interesting to determine whether the JNK-induced phosphorylation of Bim_{EL} at serine 65 contributes to neuronal cell loss under these neuropathological conditions.

Acknowledgments

We thank R. Tomaino and S. Gygi for analysis of JNK-phosphorylated Bim_{EL} by mass spectrometry, S. Vasquez for technical assistance, and A. Shalizi for critical reading of the manuscript. This work was supported by an American Federation for Aging Research (AFAR)/Pfizer grant (A.B.) and by a National Institutes of Health grant R01-NS41021-01 (A.B.). A.B. is the recipient of a career development award from the Burroughs Wellcome Fund, a fellowship from the Alfred P. Sloan Foundation, a Robert H. Ebert Clinical Scholar Award from the Esther A. and Joseph Klingenstein Fund, and a Sidney Kimmel Foundation Award. E.B.E.B. is supported by the Boehringer Ingelheim Fonds and an Albert J. Ryan Foundation award.

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Figure legends

Fig 1. JNK phosphorylates Bim_{EL} *in vitro*. (*A*) The structure of Bim_{EL} is shown schematically. The location of the six putative proline-directed phosphorylation sites, the dynein binding motif (DBM), the BH3-only domain, and the hydrophobic domain (ϕ) are indicated. (*B*) Recombinant GST-Bim_{EL} was incubated with recombinant JNK1α1 and subjected to an *in vitro* kinase assay using [γ-^{32}P]ATP. Phosphorylated GST-Bim_{EL} was separated by SDS-PAGE, stained with Coomassie Blue (lower panel), and analyzed by autoradiography (upper panel). Degradation products of bacterially expressed GST-Bim_{EL} are indicated with an asterisk (*). (*C*) Representative tandem mass spectrum of one of the phosphopeptides (amino acids 51-71) derived from JNK-phosphorylated GST-Bim_{EL}. Fragment ions in the spectrum were sequenced from both the N- and C-termini (b- and y-type ions). Both serine 55 and serine 65 show additional mass from a phosphate residue.

Fig. 2. JNK phosphorylates Bim_{EL} at the distinct site of serine 65 *in vitro*. (*A*) Recombinant GST-Bim_{EL} (Bim_{EL}) and recombinant Bim_{EL} phosphorylation mutants GST-Bim_{EL}S55A (55A), GST-Bim_{EL}S65A (65A), and GST-Bim_{EL}T112A (112A) were incubated with no kinase or recombinant JNK1α1 and subjected to an *in vitro* kinase assay using [γ-^{32}P]ATP. Phosphorylated substrates were separated by SDS-PAGE, stained with Coomassie Blue (lower panel), and analyzed by autoradiography (upper panel). Degradation products of bacterially expressed GST-Bim_{EL} are indicated with an asterisk (*). (*B*) Recombinant GST, GST-Bim_{EL} (Bim_{EL}) and GST-Bim_{EL}S65A (65A) were subjected to an *in vitro* kinase assay with recombinant JNK1α1. Phosphorylated substrates were separated by SDS-PAGE and immunoblotted with the phospho65-Bim, Bim, or

GST antibody. Degradation products of bacterially expressed proteins are marked with an asterisk (*).

Fig. 3. JNK phosphorylates Bim_{EL} *in vivo*. (*A*) HEK293T cells were transfected with 1 μg of wildtype Bim_{EL} (Bim_{EL}) or the Bim_{EL} phosphorylation mutants Bim_{EL}S55A (55A), Bim_{EL}S65A (65A), Bim_{EL}S73A (73A), Bim_{EL}S100A (100A), Bim_{EL}T112A (112A) alone or together with 0.5 μg activated MEKK1 (MEKK1Δ). The electrophoretic mobility of the Bim_{EL} proteins was assessed by SDS-PAGE and immunoblotting with the HA antibody. (*B*) HEK293T cells were transfected with 1 μg of wildtype Bim_{EL} or vector control alone or together with 0.5 μg activated MEKK1 (MEKK1Δ), p38MAPK and activated MKK3, or activated MEK1. Lysates of transfected cells were immunoblotted with the phospho65-Bim, Bim, phospho-JNK (P-JNK), or phospho-p38MAPK (P-p38MAPK) antibody. Untagged Bim_{EL} is indicated with an asterisk. *(C)* HEK293T cells were treated with sorbitol (500 mM) for 3 h. Lysates of cells were immunoblotted with the phospho65-Bim, Bim, phospho-JNK (P-JNK), or phospho-p38MAPK (P-p38MAPK) antibody. (*D*) HEK293T cells were treated with sorbitol (500 mM) for 3 h in the presence of vehicle or the p38MAPK and JNK inhibitors SB203580 (5 μM) and SP600125 (10 μM), respectively. Lysates of cells were immunoblotted with the phospho65-Bim, Bim, phospho-c-Jun (P-c-Jun), phospho-JNK (P-JNK), JNK, phospho-p38MAPK (P-p38MAPK), or 14-3-3 antibody. Aspecific bands recognized by the phospho65-Bim antibody are indicated with an asterisk.

Fig. 4. Phosphorylation of Bim_{EL} at serine 65 promotes Bim_{EL}-mediated apoptosis. (*A*) Cerebellar granule neurons were transfected with control vector (V), Bim_{EL}, or Bim_{EL}S55/65A (55/65A) and beta-galactosidase (left panel) or with control vector (V) or a dominant interfering form of JNK (JNK DN) together with

Bim_{EL} and beta-galactosidase (right panel). One day after transfection, cultures were switched from full medium to basal medium in the absence of survival factors. Cells were fixed after 8 h and subjected to indirect immunofluorescence. Percent apoptosis is represented as mean ± S.E. The expression of $Bim_{EL}S55/65A$ induced significantly less apoptosis than Bim_{EL} (n=3; ANOVA; $p<0.0005$). Expression of JNK DN significantly reduced Bim_{EL}-induced apoptosis (n=3; ANOVA; $p<0.005$). (*B*) Cerebellar granule neurons were transfected with plasmids encoding non-spliceable cDNAs of Bim_{EL}, $Bim_{EL}S55A$ (55A), $Bim_{EL}S65A$ (65A), $Bim_{EL}S65D$ (65D), or control vector (V) and beta-galactosidase. Cultures were treated as described above and analyzed by indirect immunofluorescence. The expression of $Bim_{EL}S65A$ induced significantly less apoptosis than Bim_{EL} and $Bim_{EL}S65D$ (mean ± S.E.; n=4; ANOVA; $p<0.01$ and $p<0.0005$). The expression of $Bim_{EL}S55A$ did not reduce the apoptotic effect compared to wildtype Bim_{EL} (mean ± S.E.; n=6; ANOVA; $p=0.4$). (*C*) Cerebellar granule neurons were transfected with plasmids encoding non-spliceable cDNAs of wildtype Bim_{EL}, $Bim_{EL}S65D$ (65D), or control vector (V) and beta-galactosidase. One day after transfection, cultures were switched from full medium to basal medium in the presence (Ins) or absence (-) of insulin (10 μg/ml) to activate the IGF-I receptor. Cells were fixed after 8 h and subjected to indirect immunofluorescence. Activation of the IGF-I receptor reduced Bim-mediated apoptosis (mean ± S.E.; n=4; ANOVA; $p<0.0001$), but had little effect on $Bim_{EL}S65D$-induced apoptosis ($p>0.04$).

Fig. 5. $p75^{NTR}$-induced apoptosis activates the JNK-dependent phosphorylation of Bim_{EL} and is dependent on Bim_{EL}. (*A*) PC12 cells were infected with $p75^{NTR}$ or control adenovirus (LacZ) together with either 5 or 15 MOI of JBD-JIP (J) or control (L) adenovirus. Lysates were immunoblotted with phospho65-Bim or Bim

antibody. Aspecific bands recognized by the phospho65-Bim antibody are indicated with an asterisk. (*B*) Lysates of PC12 cells transfected with increasing amounts of control vector (U6), Bim_{EL} RNA interference plasmid (Bim RNAi), or control RNAi plasmid (Cdk2 RNAi) were immunoblotted with the Bim (upper panel), p42 MAP kinase (Erk1/2, middle panel), or actin antibody (lower panel). (*C*) PC12rtta cells were transfected with the indicated plasmids and subsequently infected with $p75^{NTR}$ (p75) or control adenovirus (LacZ). 24 h post-infection, cells were fixed and immunostained for cleaved caspase-3. '**' indicates a difference of $p<0.001$ between $p75^{NTR}$-infected, pCDNA3-transfected cells (bar 8) and mock or LacZ-infected cells (bars 1-7), and $p75^{NTR}$-infected, U6-transfected cells (bar 11) and Bim RNAi-transfected cells (bar 12). '*' indicates a difference of $p<0.05$ between $p75^{NTR}$-infected, pCDNA3-transfected cells (bar 8) and Bim_{EL}S65A-transfected cells (bar 10), as determined by ANOVA.

Figure 1

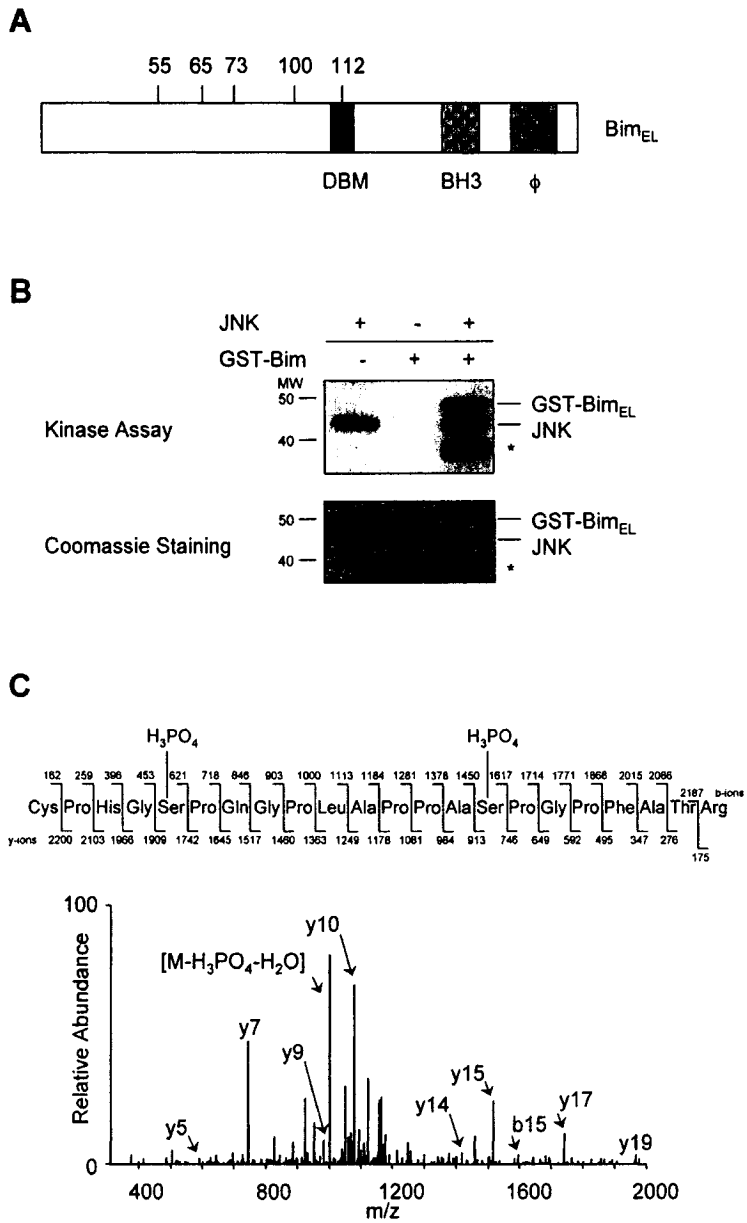
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Figure 2

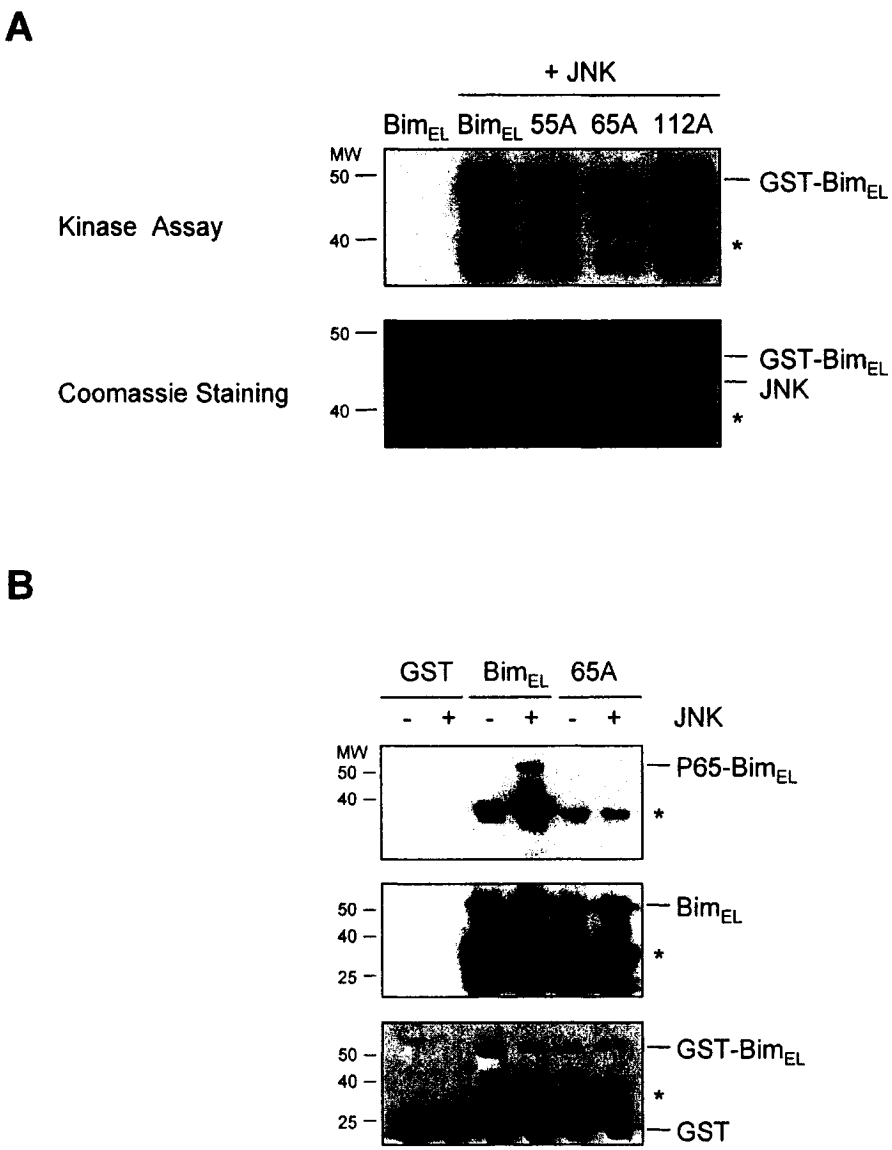
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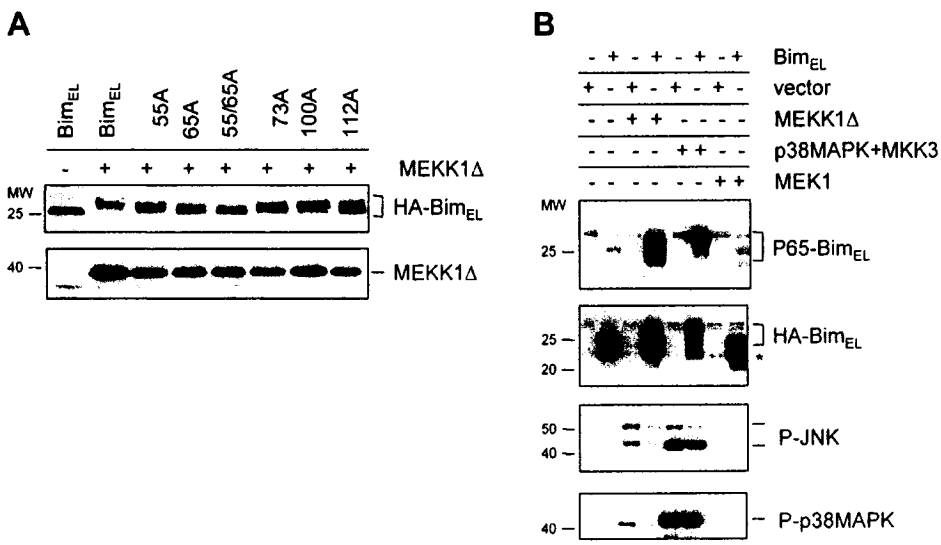
Figure 3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P-p38MAPK

Figure 4

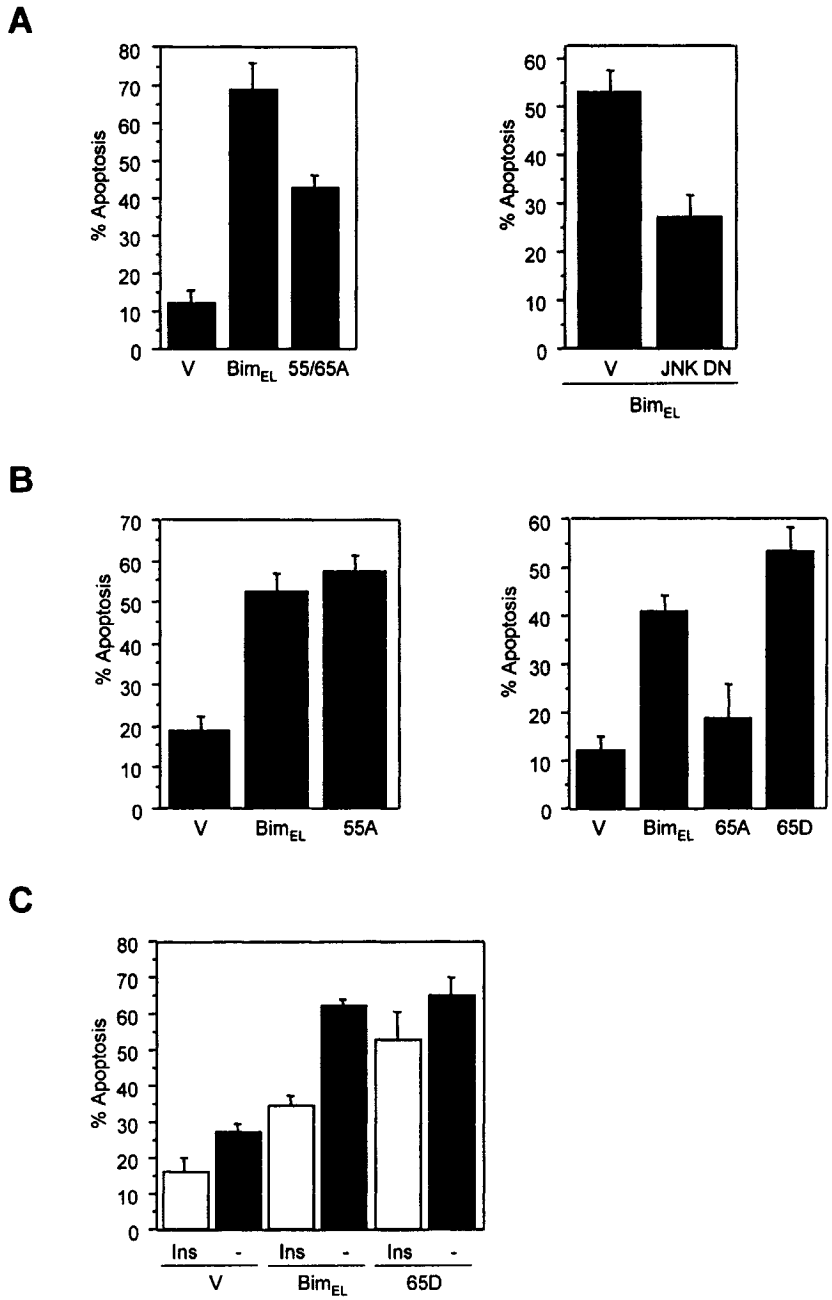
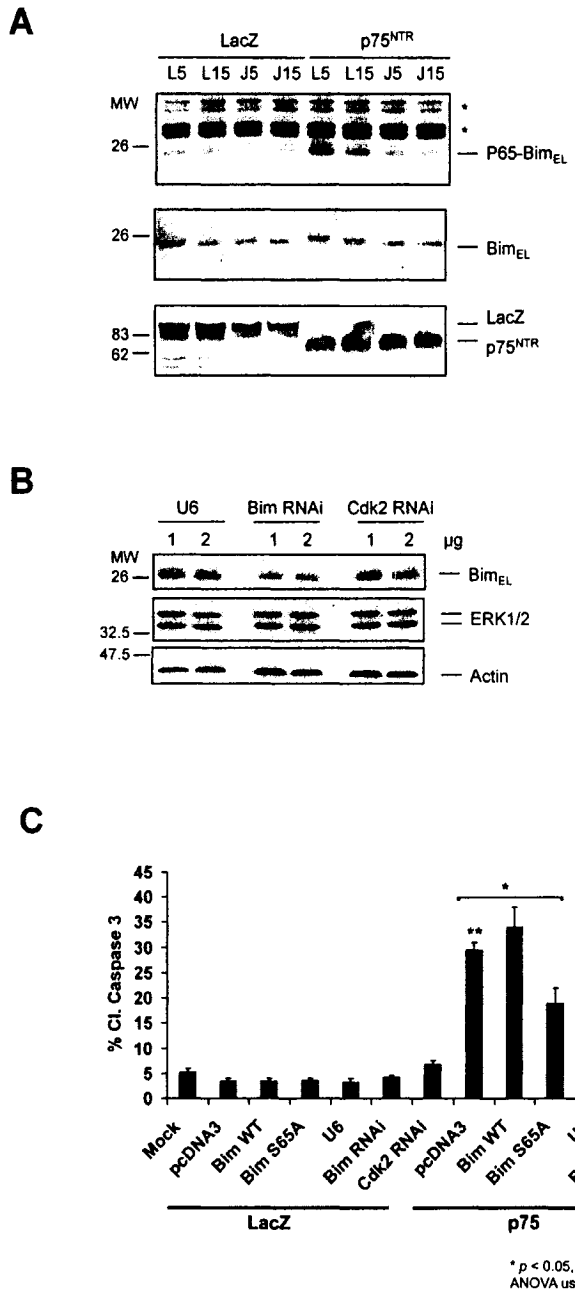


Figure 5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p75NTR
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U6
Bim RNAi
Cdk2 RNAi
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BimEL
32.5
47.5
ERK1/2
Actin
C
% Cl. Caspase 3
Mock
pcDNA3
Bim WT
Bim S65A
U6
Bim RNAi
Cdk2 RNAi
LacZ
p75
* p < 0.05, ** p < 0.001
ANOVA using Tukey's post-hoc test

New Application.

5 d) List the section / subsection numbers where significant changes have been made

N/A

5 e) KEYWORDS: Using keywords only, list the procedures used on animals (e.g. anaesthesia, breeding colony, injection IP, gavage, drug administration, major survival surgery, euthanasia by exsanguination, behavioural studies). For a more complete list of suggested keywords refer to Appendix 1 of the Guidelines (www.mcgill.ca/rgo/animal).

anesthesia, analegics, tissue dissecton, neuronal primary cultures, transgenic mice, mouse breeding, genotyping, euthanization, polyclonal antibodies, exsanguinations, euthanization

6. Animals Use data for CCAC

6 a) Purpose of Animal Use (Check one):

1. ☒ **Studies of a fundamental nature/basic research**
2. ☐ **Studies for medical purposes relating to human/animal diseases/disorders**
3. ☐ **Regulatory testing**
4. ☐ **Development of products/appliances for human/veterinary medicine**
5. **If for Teaching, use the Animal Use Protocol form for Teaching (www.mcgill.ca/rgo/animal)**

6 b) Will field studies be conducted? NO ☒ YES ☐ If yes, complete "Field Study Form"
Will the project involve the generation of genetically altered animals? NO ☐ YES ☒ If yes, complete SOP #5
Will the project involve breeding animals? NO ☐ YES ☒ If breeding transgenics/knockouts, complete SOP #4

7. Animal Data

7 a) 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 as well as during neuronal injury states such as chemically-induced seizures or peripheral nerve transsections. 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 either rat or mice embryos) or in situ (within transgenic animals).

7 b) 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)

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 have developed injury models in that past in order to study the in vivo signaling mechansims of the p75 NTR. Although rats will be used to derive primary neurons because of the ability of these primary neuronal cultures to survive more readily in culture than those derived from other species, in certain cases during our experimentation, we can only use primary neurons derived from our transgenic animals. Rabbits are the preferred species for small scale antibody production, given their blood volume.

7 c) Description of animals

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.

If more than 6 columns are needed, please attach another page

	Sp/strain 1	Sp/strain 2	Sp/strain 3	Sp/strain 4	Sp/strain 5	Sp/strain 6
Species	mouse	mouse	mouse	mouse	mouse	
Supplier/Source	In-house colony	In-house colony	In-house colony	In-house colony	In-house colony	
Strain	p75 exon-3 NTR (-/-) (C57/Bl6)	p75 exon-4 NTR (-/-) (C57/Bl6)	p75-ICD transgenic (C57/Bl6)	p75-Hore element LacZ reporter (C57/Bl6)	PIN1 transgenic(-/-) (C57/Bl6)	
Sex	both	both	both	both	both	
Age/Wt	all	all	all	all	all	
# To be purchased	none	none	none	none	none	
# Produced by in-house breeding	all	all	all	all	all	
# Other (e.g.field studies)	N/A	N/A	N/A	N/A	N/A	
#needed at one time	25	25	25	25	25	
# per cage	4	4	4	4	4	
TOTAL# /YEAR	50	50	50	50	50	

	Sp/strain 1	Sp/strain 2	Sp/strain 3	Sp/strain 4	Sp/strain 5	Sp/strain 6
Species	rabbit	rat	rat			
Supplier/Source	Charles River	Charles River	Charles River			
Strain	New Zealand White	Sprague Dawley	Sprague Dawley			
Sex	NA	female	female			
Age/Wt	> 4 months	175-200 g and pregnant	175-200 g			
# To be purchased	10	50	100			
# Produced by in-house breeding	0	0	0			
# Other (e.g.field studies)	0	0	0			
#needed at one time	2	1	25			
# per cage	1	2	2			
TOTAL# /YEAR	10	50	100			

7 d) Justification of Animal Usage: BASED ON THE EXPERIMENTAL OBJECTIVES OF THE PROJECT, describe the number of animals required for one year. Include information on experimental and control groups, # per group, and failure rates. For breeding, specify how many adults are used, number of offspring produced, and how many offspring are used in experimental procedures. Use the table below when applicable. The arithmetic explaining how the total of animals for each column in the table above is calculated should be made clear. *Space will expand as needed.*

1) 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 25 animals per colony. With aging, maintaining colonies of this size will require 50 animals per year for each mouse colony described above.
2) 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/year.
3) We produce one culture per week form embryonic rat tissue. We will therefore require 1 timed pregnant female each week to produce these cultures, a total of 50 rats per year. Pilocarpine-induced seizure experiments will involve 25 rats per experiment. We expect to do 4 experiments per year for a total of 100 rats per year (Grand total = 50 + 100 = 150/year).

7d table) ***The following table may help you explain the animal numbers listed in the 7c table:***

(Table will expand as needed)	Sp/strain 1	Sp/strain 2	Sp/strain 3	Sp/strain 4	Sp/strain 5	Sp/strain 6
Test agents or procedures						
# of animals per group						
Dosage / route of administration						
# of endpoints						
Other variables (sex,genotypes...)						
Total number of animals per year						

8. Animal Husbandry and Care

8 a) If projects involves non-standard cages, diet and/or handling, please specify

NA

8 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 ☐ **if yes, specify:**

8 c) Indicate area(s) where animal use procedures will be conducted:

Building: MNI **Room:** operating room

Indicate area(s) all facilities where animals will be housed:

Building: MNI **Room:** 865 (mice) and 867 (rats)

If animal housing and animal use are in different locations, briefly describe procedures for transporting animals:

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/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	☐	**Collection of Amphibian Oocytes UACC#9**	☐
Anaesthesia in rodents UACC#2	☒	**Rodent Survival Surgery UACC#10**	☒

Analgesia in rodents UACC#3	☒	**Anaesthesia & Analgesia Neonatal Rodents UACC#11**	☐
Breeding transgenics/knockouts UACC#4	☒	**Stereotaxic Survival Surgery in Rodents UACC#12**	☐
Transgenic Generation UACC#5	☒	**Euthanasia of Adult & Neonatal Rodents UACC#13**	☒
Knockout/in Generation UACC#6	☐	**Field Studies Form**	☐
Production of Monoclonal Antibodies UACC#7	☐	**Phenotype Disclosure Form**	☐
Production of Polyclonal Antibodies UACC#8	☒	**Other, specify:**	☐

10. Description of Procedures

10 a) . IF A PROCEDURE IS COVERED BY AN SOP, WRITE "AS PER SOP", NO FURTHER DETAIL IS REQUIRED.

FOR EACH EXPERIMENTAL GROUP, DESCRIBE ALL PROCEDURES AND TECHNIQUES, WHICH ARE NOT PART OF THE SOPs, 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, etcAppendix 2 of the Guidelines (www.mcgill.ca/rgo/animal) provides a sample list of points that should be addressed in this section.

1) We will produce primary cultures of peripheral and central neurons from normal embryonic rats and normal and transgenic embryonic mice. Pregnant mothers are first sacrificed in CO2 chambers. Neonatal mice are then sacrificed by decapitation and superior cervical ganglia, hippocampi, or brain cortices dissected, dissociated and grown in tissue culture incubators.
2) For genotyping of transgenic animals, 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 (see Internal MNI SOP #T97-018 attached).
3) Status epilepticus (SE) seizures will be induced using the cholinergic agonist pilocarpine. Prior to seizure induction, adult mice (30 g) or rats (175g) will be injected with methyl scopolamine (1 mg/kg; I.P.) to prevent the peripheral autonomic effects of pilocarpine. Fifteen minutes later, animals will be injected with pilocarpine hydrochloride (280 mg/kg; I.P.). This dose has been used successfully in the past (see project. #3605) to trigger seizures. After one hour, each animal will be injected with diazepam (10 mg/kg; I.P.) in order to terminate the seizure episode. Administration of pilocarpine produces initial major tonic clonic convulsions. It is at this time pilocarpine is potentially dangerous to the animal. Immediately after this pilocarpine injection, animals are monitored closely for as least one hour for any signs of distress. Distress is eliminated by the immediate injection of diazepam (10 mg/kg; I.P.) Seizure activity which develops later consists of facial automatisms and head nodding. These symptoms are not life threatening Using this protocol, the mortality rate is < 5%. Animals will be allowed to survive 1 to 7days post-surgery after which time they will be sacrificed by CO2 chambers.
4) For sciatic nerve transections, adult mice (30 g) or rats (175 g) will be anathesized using a ketomine/xylazine mixture (DOSE). A small incision will be made (mice = > 5 mm; rats > 10 mm) and the sciatic nerve exposed. Using Iris scissors, the sciatic nerve will then be cut followed by suturing of the skin and topical analgesics. Animals will be monitored every hour for 6 hours for any signs of mutilation. Animals will be allowed to survive 1 to 7days post-surgery after which time they will be sacrificed by CO2 chambers. Sciatic nerves will be removed and analyzed.
5) Polyclonal antibodies will be produced as per the McGill University Faculty of Medicine Standard Protocol for Generation of Polyclonal Antibodies in Rabbits (see SOP# 8 attached). Animals will be allowed to survive 1 to 7days post-surgery after which time they will be sacrificed by CO2 chambers.

10 b) Experimental endpoint – for each experimental group indicate survival time

For pilocarpine-induced seizures and sciatic nerve transections, animals will be allowed to survive for 1 to 7 days post-surgery.

10 c) Clinical endpoint – describe the conditions, complications, and criteria (e.g. >20% weight loss, maximum 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)

For seizures, if animal is in distress even after diazapam administration, the animal will be euthansized. For sciatic nerve transection, if the animal shows signs of mutilation, the animal will be euthansized.

10 d) Specify person(s) who will be responsible for animal monitoring and post-operative care *(must also be listed in section 4)*

Name: Dr. Alberto Ramos	**Phone #:** 398-3212

10 e) Pre-Anesthetic/Anaesthetic/Analgesic Agents: List all drugs that will be used to minimize pain, distress or discomfort. ***Table will expand as needed.***

Species	Agent	Dosage (mg/kg)	Total volume(ml)	Route	Frequency

			per administration		

10 f) Administration of ALL other 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.***

Species	Agent	Dosage (mg/kg)	Total volume(ml) per administration	Route	Frequency
mice	methyl scopolamine	1 mg/kg	100 ul	IP	once
mice	pilocarpine	280 mg/kg	100 ul	IP	once
mice	diazepam (post seizure)	10 mg/kg	100 ul	IP	once

10 g) Method of Euthanasia

Specify Species	
	☐ **Anaesthetic overdose, list agent/dose/route:**
	☐ **Exsanguination with anaesthesia, list agent/dose/route:**
	☐ **Decapitation without anaesthesia *** ☐ **Decapitation with anesthesia, list agent/dose/route:**
	☐ **Cervical dislocation without anaesthesia *** ☐ **Cervical dislocation with anaesthesia, list agent/dose/route:**
Rats, mice	☒ **CO_2 chamber**
	☐ **Other, specify:**
	☐ **Not applicable, explain:**

*** For physical method of euthanasia without anaesthesia, please justify:**

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: ☒

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

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

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

Agent name			
Dosage			
Route of administration			
Frequency of administration			
Duration of administration			
Number of animals involved			
Survival time after administration			

12 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.

12 d) Describe potential health risk (s) to humans or animals:

None

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

NA

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

Name	Department	Check appropriate classification				Fellow
		Investigator	Technician & Research Assistant	Student		
				Undergraduate	Graduate	
Dr. Phil Barker	**Neurology and Neurosurgery**	X				
Genevieve Dorval	**Neurology and Neurosurgery**		X			
Kathleen Dickson	**Neurology and Neurosurgery**		X			
Dr. Wai Chi Ho	**Neurology and Neurosurgery**					X
Dr. Joe Makkerh	**Neurology and Neurosurgery**					X
Dr. Emily Vereker	**Neurology and Neurosurgery**					X
Dr. Alberto Ramos	**Neurology and Neurosurgery**					X
Dr. Daniel Auld	**Neurology and Neurosurgery**					X
Jacqueline Boutilier	**Neurology and Neurosurgery**				X	
Jenny Howell	**Neurology and Neurosurgery**				X	
Amir Saleki	**Neurology and Neurosurgery**				X	

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

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

Name: **Kathleen Dickson** Phone No: work: **398-3212** Cell: **793-2125**

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 biolc 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 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-d or sodium hypochlorite solution (5.25% bleach diluted 1:10).
- Containers/equipment leaving the lab will be decontaminated with 1% bleach or 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 plastic "Broken Glass" containers.
- Organic/caustic chemicals are stored in a reinforced cabinet and used in a fume hood.

Sharps are disposed in impermeable sealed plastic containers; glass in sealed plastic containers.

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 towels and then applying 1% bleach from the periphery inwards.
- After a 30 minute incubation period in the applied bleach, the paper towel will be disposed of in a biohazard bin and subsequently autoclaved.
- Spills onto clothing will be decontaminated by autoclaving.

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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.

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

   Yes.

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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**	**67000-ADO**	**Dec. 12/02**
			NU440-400	**67062-ADO**	**Dec. 13/02**
			NU440-400	**67064-ADO**	**Dec. 13/02**