The Role of Proneurotrophins in Apoptotic Signaling in Rat Brain Neurons

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ABSTRACT OF THE THESIS

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Proneurotrophins and mature neurotrophins can activate distinct signaling pathways and have opposing effects on cells: proneurotrophins induce apoptotic signaling via p75NTR while mature neurotrophins activate survival signaling by binding to Trk receptors. In the CNS, basal forebrain (BF) neurons express both p75NTR and Trk receptors. The work in this thesis demonstrates that proneurotrophins can induce loss of BF neurons through p75NTR, even in the presence of activated Trk receptors. Moreover, proNGF inhibits the phosphorylation of Akt induced by BDNF, suggesting that proNGF induces apoptotic signaling and simultaneously blocks survival signaling activated by BDNF. Phosphorylation of Akt can prevent proNGF-induced apoptosis, suggesting that regulation of Akt phosphorylation may be a critical point of interaction between survival and death signaling.

PTEN (phosphatase and tensin homologue deleted on chromosome ten) is a dual-specificity phosphatase that can act as an antagonist to the PI3 kinase/Akt pathway. ProNGF induces an increase in PTEN in BF neurons, even in the presence of BDNF, suggesting that proNGF might block survival signaling through PTEN. In the presence of BDNF, proNGF was unable to induce apoptosis when PTEN activity was inhibited both in vitro and in vivo.

Also, the PTEN inhibitor blocked proNGF-induced inhibition of Akt phosphorylation by BDNF, suggesting that PTEN is a crucial factor mediating the balance between p75-induced apoptotic signaling and Trk-mediated survival signaling.

Taken together, the interaction of proneurotrophin-p75NTR and mature neurotrophin-Trk systems is partially determined by the balance of PTEN and Akt which eventually causes the cell to die or survive.

Dedication

I would like to dedicate my thesis to my parents,

Song Jiquan and Wang Shuzhen.

It is the love from them to support me for last eight years.

Acknowledgement

I would like to sincerely thank my advisor, Dr. Wilma J. Friedman, for all the guidance she gave to me during my thesis work. As a real scientist, she opens the gate leading to the scientific world for me.

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List of Abbreviations

AD	actinomycin-D
Akt/PKB	protein kinase B
BDNF	brain-derived neurotrophic factor
BF	basal forebrain
CC3	cleaved caspase 3
CREB	cAMP regulatory element binding protein
CNS	central nervous system
CK1	casein kinase 1
CK2	casein kinase 2
CHX	cycloheximide
DD	death domain
ECD	extracellular domain
ER	endoplasmic reticulum
Erk1/2	extracellular signal-related protein kinase 1 and 2
ICD	intracellular domain
GSK3β	glycogen synthase kinase 3β
HPC	hippocampus
LY	LY294002
MMP	matrix metalloproteinase
NGF	nerve growth factor
NgR	Nogo receptor
NT-3	neurotrophin-3
NT-4/5	neurotrophin-4/5
NT-6	neurotrophin-6

NT-7	neurotrophin-7
PD	PD98059
PDK1	3-phosphoinositide-dependent protein kinase 1
PFA	paraformaldehyde
p75NTR	p75 neurotrophins receptor
PIP3	phophatidylinositol-3,4,5-trisphosphate
PIP2	phosphatidylinositol-3,4-biphosphate
PI3K	phosphatidylinositol-3-kinase
PKB	protein kinase B
PKC	protein kinase C
PNS	peripheral nervous system
PTEN	phosphatase and tensin homologue deleted on chromosome 10
TNF	tumor necrosis family
Trks	tropomyosin-related kinase family of receptors

I. Introduction:

The nervous system is a complex system that is developed and maintained in a highly regulated manner. Factors involved in this process include neurotrophins, a family of growth factors critical for multiple aspects of neuronal development. Functions of neurotrophins include supporting survival, neurite outgrowth, synapse formation, cell migration, proliferation, and inducing apoptosis (Lewin and Barde, 1996; Bibel and Barde, 2000; Huang and Reichardt, 2001).

Neurotrophin functions are mediated by two categories of receptors, the tropomyosin-related kinase (Trk) family of receptor tyrosine kinases and the p75 neurotrophin receptor (p75NTR). These receptors can activate distinct signaling pathways that have very different consequences. Trk and p75NTR can also collaborate to mediate neurotrophic effects (Hempstead et al., 1991; Mahadeo et al., 1994; Verdi et al., 1994).

All four neurotrophins are produced as precursors and can be cleaved by convertases, which include furin and convertases 1 and 2 (PC1 and PC2), to produce mature neurotrophins. Proneurotrophins have been shown to have apoptotic effects on cells (Beattie et al., 2002; Harrington et al., 2004), which are mediated by p75NTR activated signaling (Lee et al., 2001; Teng et al., 2005). Sortilin, a member of the Vps family of receptors facilitates the binding between proneurotrophins and p75NTR (Nykjaer et al., 2004).

1. Neurotrophins and their receptors:

1.1 Neurotrophins

The neurotrophin family in mammals includes four members: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-3, and neurotrophin (NT)-4/5 (Levi-Montalcini and Hamburger, 1951;

Barde et al., 1982; Ernfors et al., 1990; Hohn et al., 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990; Hallbook et al., 1991; Funakoshi et al., 1995). NT-6 and NT-7 have also been identified, but only in fish (Gotz et al., 1994; Nilsson et al., 1998). NGF was the first factor to be discovered and characterized (Levi-Montalcini and Hamburger, 1951; Levi-Montalcini et al., 1954; Cohen, 1960). It is a 26kDa homodimeric protein, with each monomer consisting of 118 residues. The three other neurotrophins share similar protein structure and gene sequence with NGF (Reichardt, 2006).

Among all the neurotrophins, NGF has been most extensively characterized. It supports survival and maturation of different populations of neurons, such as sympathetic and sensory neurons in the peripheral nervous system (PNS) and striatal cholinergic neurons in the central nervous system (CNS) (Snider, 1994; Kew et al., 1996; Conover and Yancopoulos, 1997). Implantation of NGF-secreting carcinoma or salivary glands into chick embryos increased the number of cervical ganglion neurons (Levi-Montalcini and Hamburger, 1951). A blocking antibody against to NGF caused ablation of sensory neurons and sympathetic ganglia (Levi-Montalcini and Booker, 1960). In animals with heterozygous NGF gene, there was less cholinergic innervation in hippocampus (Chen et al., 1997).

In the CNS, NGF can be produced by neurons such as pyramidal and dentate granule neurons (Ayer-LeLievre et al., 1988; French et al., 1999). Astrocytes and microglia were also found to produce NGF after injury (Arendt et al., 1995; Elkabes et al., 1996). In the PNS, NGF can be synthesized and secreted by sympathetic and sensory target organs including vascular smooth muscle cells, testis, ovary, and pituitary (Levi-Montalcini et al., 1996). The produced NGF can be retrogradely transported to neuronal cell bodies to

support survival and differentiation (Korsching, 1993). In addition to target organs, neurons can also synthesize neurotrophins. For example, BDNF can be synthesized by several populations of sensory neurons (Brady et al., 1999), and NGF can be produced by trigeminal sensory neurons (Barde et al., 1982; DiCicco-Bloom et al., 1993; Davis et al., 1998).

1.2 The Trk receptors and their signaling pathways

The Trk receptors are a family of proteins including TrkA, TrkB and TrkC. Each neurotrophin has a preference for specific Trk receptors (Fig 1): NGF activates TrkA (Kaplan et al., 1991b; Kaplan et al., 1991a; Klein et al., 1991a), BDNF and NT-4/5 activate TrkB (Klein et al., 1991b; Squinto et al., 1991), and NT-3 activates TrkC (Lamballe et al., 1991). In addition, TrkA can also bind NT-3 and NT-4/5, but with lower affinities than NGF (Kaplan et al., 1991b).

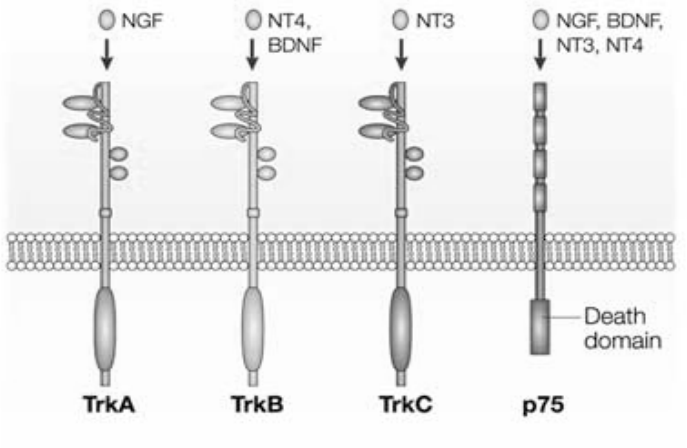

Figure 1. Neurotrophins and their receptors. Neurotrophins bind selectively to specific Trk receptors, while p75NTR can bind with all four neurotrophins.
M.V. Chao, Nature Reviews Neurosciences, 2003

Trk receptors were found in both neuronal and non-neuronal systems. In the CNS, these receptors are widely expressed in different areas including the cortex, cerebellum, basal forebrain, hippocampus and diencephalon

(Barbacid, 1994). Outside the nervous system, Trk receptors were found in arteries, tooth buds, palate, and the submaxillary gland (Barbacid, 1994).

In their extracellular domain, Trk receptors have cell-adhesion motifs, three tandem leucine-rich motifs, flanked by cysteine clusters and two immunoglobulin-like domains in the membrane-proximal region (Fig 2). Trk receptors bind neurotrophins through their second immunoglobulin-like domain (Urfer et al., 1998).

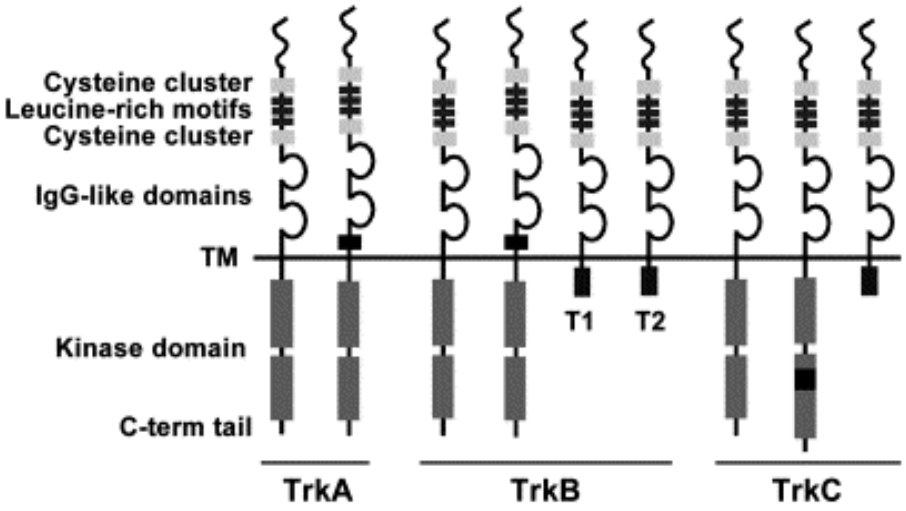

Figure 2. Schematic of structure of Trk receptors. The extracellular domains (ECDs) of Trk receptors contain two cysteine-rich region flanking a leucine-rich repeat, followed by two immunoglobulin (IgG)-like domains in the juxtamembrane region. The intracellular domain contains tyrosine kinase domain. Alternative splicing produces TrkB and TrkC variants lacking most of the intracellular domain.

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The mRNA of Trk receptors can be spliced in different ways to produce proteins with different intracellular domains, and these splice variants can affect ligand binding and kinase activity. The short amino-acid sequences in the juxtamembrane domain of Trk receptors are thought to be important for the activation of receptors (Wiesmann et al., 1999). It has been shown that in non-neuronal cells, the truncated isoforms of Trk receptors can present neurotrophins to neurons. In neuron populations, these receptors can bind with neurotrophins and compete with the binding between neurotrophins and

full-length Trk receptors, which attenuate the responses of cells to neurotrophins (Eide et al., 1996).

Neurotrophins directly bind to and dimerize Trk receptors, resulting in the phosphorylation of tyrosine residues on the cytoplasmic domains (Jing et al., 1992). There are ten evolutionarily conserved tyrosines on the cytoplasmic domains of Trk receptors. Three of these—Y670, Y674 and Y675—are present in the auto-regulatory loop of the kinase domain that regulates tyrosine kinase activity (Stephens et al., 1994). Phosphorylation of these residues activates the receptors. Other tyrosine residues can also be phosphorylated to create docking sites for adapter proteins containing phospho tyrosine-binding (PTB) or src-homology-2 (SH-2) motifs, which are important for signal transduction (Pawson and Nash, 2000).

Phosphorylation of Trk receptors can activate three classical signaling pathways: PI-3 kinase signaling pathway, MAPK/Erk signaling pathway and PLC-γ 1 signaling pathway (Huang and Reichardt, 2001).

PI3K/Akt signaling pathway

Phosphatidylinositol 3 kinase (PI3K) belongs to a family of intracellular lipid kinases that phosphorylate the 3'-hydroxyl position of phosphatidylinositols and phosphoinositides. The activation of phosphatidylinositol-3-kinase (PI3K) and Akt (protein kinase B) is important for the survival of several neuronal populations (Brunet et al., 2001). Inhibition of the PI3K activity blocks NGF-induced cell survival (Yao and Cooper, 1995). In both neurons and other cell types, PI3K has been found to be important for growth factor-dependent survival signaling pathways (Scheid et al., 1995; Takashima et al., 1996). Phosphorylated Trk receptors can activate PI3K through two distinct pathways. One pathway requires the binding of the

catalytic subunit of PI3 kinase to activated Ras (Downward, 1998); the other involves three adaptor proteins, Shc, Grb-2 and Gab-1. Shc can bind to phosphorylated Y490 of Trk receptors, resulting in the recruitment of Grb-2. Phosphorylated Grb-2 provides a docking site for Gab-1, which is bound by PI-3 kinase (Holgado-Madruga et al., 1997). Once PI3K is relocated to the plasma membrane, it can phosphorylate the inositol ring of membrane phosphoinositides to produce 3'-phosphorylated phosphoinositides. The phosphatidylinositol-3,4,5-trisphosphate (PIP3) is a major form of the lipid product that binds to and stimulates phosphoinositide-dependent protein kinase 1(PDK1). PDK1 can then bind and phosphorylate Akt (Alessi et al., 1997).

Akt was cloned by three independent labs in 1991 (Bellacosa et al., 1991; Coffer and Woodgett, 1991; Jones et al., 1991b). In mammals, there are three Akt isoforms that have been identified: Akt1, Akt2 and Akt3. These three isoforms are expressed differently: Akt1 is expressed in all the organs except the kidney, liver and spleen; Akt2 is expressed at high levels in muscle, intestinal organs and reproductive tissues; Akt3 is expressed mainly in the brain and testis (Coffer and Woodgett, 1991; Jones et al., 1991a; Nakatani et al., 1999).

Different models have been established for the activation process of Akt. A common important step is the relocalization of Akt from the cytoplasm to the plasma membrane where PIP3 can facilitate the activation of Akt. In one previous study, when Akt was permanently located to the plasma membrane, it exhibited constitutive kinase activity (Franke et al., 1995).

The phosphorylation of Thr-308 and Ser-473 is required for the activation of Akt (Alessi et al., 1996). PDK1 (3-phosphoinositide-dependent

protein kinase1) can phosphorylate Akt at Thr-308, however, PDK1 is constitutively active. There is no significant change of its activity in the presence or absence of survival factors (Alessi et al., 1997). In addition, PDK1 itself can not phosphorylate Akt at Ser-473 suggesting that another regulatory mechanism exists.

A later study showed that PDK1 can interact with another kinase, PRK-2. In the complex form, PDK-1/PRK-2 is able to phosphorylate Akt at both Thr-308 and Ser-473 (Balendran et al., 1999; Currie et al., 1999)). Moreover, the activity of PDK-1/PRK-2 can be increased by PI3K-generated PIP3, suggesting that the PDK-1/PRK-2 complex is responsible for growth factor-induced Akt regulation (Currie et al., 1999).

Akt targets several downstream factors, including pro-apoptotic proteins and transcription factors, as a key regulator of cell survival (Reichardt, 2006). For example, it binds and phosphorylates Bad, a pro-apoptotic member of the Bcl-2 family of proteins (Datta et al., 1997). When not phosphorylated, Bad binds and inhibits the activity of anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-X_L (Zha et al., 1996).

MAPK/Erk signaling

Both survival and differentiation of neurons can be mediated by the MAPK/Erk signaling pathway (Grewal et al., 1999). The adaptor protein, Shc, can bind to phosphorylated Y490 on a Trk receptor and then become phosphorylated. Phospho-Shc will bind to the Grb2-Sos complex and bring Sos and Ras together. The association of Sos and Ras can activate Ras by switching Ras-GDP to Ras-GTP (Basu et al., 1994; McCormick, 1994). Ras then activates the protein kinase Raf. Raf phosphorylates and activates MAP kinase kinase (MEK1) at serine 217 and serine 221. The activated MEK1 can

then phosphorylate extracellular signal-related kinase 1 and 2 (Erk1/2), which are two members of MAP kinase family (Jaiswal et al., 1994). After the activation of Erk1/2, the protein is translocated to the nucleus and phosphorylates several transcription factors including Elk-1 (Miranti et al., 1995). Activated Elk-1 can interact with the transcription factor serum response factor (SRF) and the cAMP regulatory element binding protein (CREB) to cause the gene transcription that is essential for initiation and maintenance of differentiation (Ginty et al., 1994; Gille et al., 1995).

PLC-γ1 signaling

When Trk is phosphorylated on Y785, a direct consequence is the activation of phospholipase C (Vetter et al., 1991). There are several isoforms of phospholipase C, including PLC-β, PLC-γ and PLC-δ. Among them, only PLC-γ1 has been shown to be activated by Trk (Middlemas et al., 1994). After activation, PLC-γ1 can then catalyze the hydrolysis of phosphatidylinositol (4, 5) P2 and produce diacyl-glycerol and inositol (1,4,5) P3, which induces the release of intracellular calcium and activation of protein kinase C (PKC) (Lee and Rhee, 1995; Rhee, 2001). PKC is important for regulating proliferation, differentiation, transformation, and apoptosis in cells (Newton, 1995). It has also been shown that PLC-γ1 can mediate PIP2 depletion, which leads to the hypersensitization of TRP channel (Prescott and Julius, 2003).

1.3 P75 neurotrophin receptor (NTR) and its signaling pathways

The p75 neurotrophin receptor was initially cloned by two different groups at almost the same time from human and rat (Chao et al., 1986; Radeke et al., 1987). The two forms were highly homologous. P75NTR was later identified as a member of the tumor necrosis factor (TNF) receptor

superfamily, a group that also includes tumor necrosis factor receptor 1 (TNFR1), TNFR2, Fas, RANK and CD40 (Baker and Reddy, 1998).

After cleavage of the signal peptide, human p75NTR is a 399 amino acid protein. It is a single transmembrane protein with an intracellular carboxy-terminal and several O-linked carbohydrates in the juxtamembrane stalk domain. The extracellular domain contains four repeated modules of six cysteines, which is a unique feature of the TNF receptor superfamily members. These cysteine regions create the binding site for neurotrophins (Yan and Chao, 1991). The intracellular domain of p75NTR can be palmitoylated and phosphorylated on cysteine, serine and threonine (Grob et al., 1985; Barker et al., 1994). These modifications may play a role in protein-protein interaction, the folding of the receptor and the intracellular localization of p75NTR.

P75NTR can bind to NGF with low affinity and was thus initially named the low affinity NGF receptor (Chao et al., 1986; Radeke et al., 1987). As more members of neurotrophin family were discovered, p75NTR was found to bind all neurotrophins with approximately equal affinity (Fig.1) (Rodriguez-Tebar et al., 1990; Squinto et al., 1991; Rodriguez-Tebar et al., 1992). One of the common signaling features that TNF receptors share is the ability to control cell viability by regulating apoptosis through associating with cytoplasmic adaptor proteins. The intracellular domain of p75NTR contains regions that can recruit other proteins. The juxtamembrane domain of p75NTR might also play a role during this recruitment. In addition, p75NTR signaling can vary in different systems. Research in the last ten years has shown that many signaling pathways can be activated by p75NTR. In some cells, p75NTR signaling can promote survival, while in others it can mediate

apoptosis (Barrett and Bartlett, 1994). It also has been reported that p75NTR mediated signaling can affect axonal outgrowth both in vivo and in vitro (Bentley and Lee, 2000).

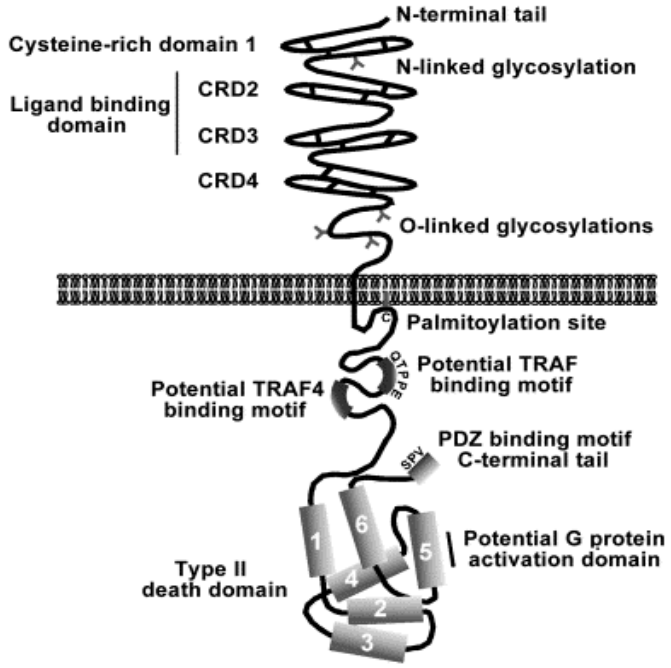

Figure3. Schematic of structure of p75NTR. P75NTR is a type I transmembrane receptor with an extracellular domain that has four cysteine-rich domains (CRDs), and multiple O- and N-linked glycosylation sites. The intracellular domain has a palmitoylation site at cysteine 279, two potential TRAF-binding sites, a type II death domain, a potential G protein activating domain, and a PDZ domain binding motif.

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The most well-documented survival pathway mediated by p75NTR involves the activation of NFκB, a transcription factor that, when activated, translocates to the nucleus and activates target genes related to various functions such as inflammatory response and apoptosis suppression (Baldwin, 1996; Wang et al., 1996). In both embryonic sensory and sympathetic neurons, neurotrophins have been found to promote p75NTR-dependent activation of NFκB and therefore support the survival of neurons (Maggirwar et al., 1998; Hamanoue et al., 1999).

However, more extensive data has been reported on p75NTR and its role in apoptotic signaling. P75NTR-mediated death has been observed in

many different systems. For example, p75NTR mediates NGF-induced death of cultured oligodendrocytes (Casaccia-Bonnefil et al., 1996). Apoptosis was also observed upon BDNF-induced activation of p75NTR in post-natal sympathetic neurons in culture (Casaccia-Bonnefil et al., 1996; Bamji et al., 1998; Trim et al., 2000). In CNS areas not expressing TrkA, high doses of NGF can induce apoptosis in embryonic chick retina neural precursor cells (Frade et al., 1996), as well as the death of hippocampal neurons via p75NTR (Friedman, 2000).

Two p75NTR knockout mice have been generated by deleting exon III or IV of the p75NTR gene (Lee et al., 1992; von Schack et al., 2001). Both mutants express a short receptor lacking the extracellular domain of the protein, and therefore do not bind NGF. However, the transmembrane and cytoplasmic domains of p75NTR were retained. Mice with the exon IV mutant are smaller compared to wild type or exon III mutant and exhibit posterior limb ataxia. Both mutants show a severe loss of peripheral sensory neurons and peripheral nerve volume (Lee et al., 1992; von Schack et al., 2001). However, p75NTR knockout mice do not manifest a dramatic behavioral phenotype compared to wild type (Lee et al., 1992; von Schack et al., 2001).

P75NTR can interact with different receptors to mediate various signaling pathways. Without p75NTR, Trk receptors have slow association and dissociation kinetics with neurotrophins (Hempstead et al., 1991). With p75NTR as a co-receptor, the affinity of TrkA with NGF can increase 25-fold (Mahadeo et al., 1994). The disruption of binding between p75NTR and NGF inhibits NGF-induced TrkA activation (Barker and Shooter, 1994; Lachance et al., 1997).

Other receptors with which p75NTR interacts are the Nogo receptor (NgR) and sortilin. The Nogo receptor complex includes Nogo receptor, Lingo and p75NTR. The activated p75NTR-NgR complex leads to the inhibition of axon outgrowth (Wang et al., 2002; Wong et al., 2002). The p75NTR-sortilin receptor complex has been shown to have a high affinity for proneurotrophins and cause apoptosis. The pro-domain of proNGF or proBDNF binds to sortilin, while the mature domain binds to p75NTR (Nykjaer et al., 2004; Teng et al., 2005).

The intracellular domain (ICD) of p75NTR is similar to that of Fas and tumor necrosis factor receptor I (TNFR I). All of these receptors have a death domain (DD) on their ICD that includes six α helices. The death domain is an intracellular homology domain that is important for apoptotic signaling. It has been divided into two types: type I and type II, based on their similarity and spacing between α helices (Cleveland and Ihle, 1995). P75NTR was found to have a type II death domain while Fas and TNFR I have the type I death domain (Liepinsh et al., 1997).

The p75NTR intracellular domain does not have intrinsic kinase activity, and signaling is believed to be transmitted through the recruitment of adaptor proteins. Several adaptor proteins have been reported to be associated with p75NTR including NRIF, NRAGE, NADE, TRAF, RIP-2 (Casademunt et al., 1999; Ye et al., 1999; Mukai et al., 2000; Salehi et al., 2000; Khursigara et al., 2001). The broad spectrum of these proteins and their activities makes the signaling pathways mediated by p75NTR very difficult to understand, and categorizing p75NTR into the known classical signaling pathways is a complicated process. It is still unclear if both the juxtamembrane region and death domain are required for p75NTR-mediated

cell death, or if each domain signals independently (Coulson et al., 2000; Roux and Barker, 2002).

Upon the binding of neurotrophins to p75NTR, several signaling pathways can be activated. One important downstream factor is Jun kinase (Casaccia-Bonnefil et al., 1996), which can be activated by phosphorylation. Activated Jun kinase causes the release of cytochrome c from the mitochondria. This causes the activation of caspases and eventually induces cell death. The caspases involved during this process include caspase 9,6, and 3 (Dechant and Barde, 1997; Martinou et al., 1999; Friedman, 2000; Troy et al., 2002).

P53 was also shown to be activated by p75NTR-induced signaling. P53 is known to be important for the apoptotic process of cells. The downstream target of p53 includes Bax, which is a pro-apoptotic factor (Yeiser et al., 2004). In PC12 cells, Cdc42 is also part of p75NTR-JNK signaling. Dominant negative Cdc42 can inhibit cell death (Bazenet et al., 1998).

The Rho GTPase family can also be activated by p75NTR. RhoA was shown to be activated by p75NTR, which inhibited neurite outgrowth (Yamashita et al., 1999). A study from the same lab showed that p75NTR displaced RhoA from RhoGDI, facilitating the release of RhoA (Yamashita and Tohyama, 2003).

P75NTR intracellular domain (ICD) cleavage has been shown to be necessary for signal transduction. The cleaved ICD is translocated to the nucleus (Jung et al., 2003; Kanning et al., 2003). There are two sequential cleavages involving different proteinases: α-secretase is responsible for cleaving the extracellular part of the protein, whereas intracellular region cleavage is mediated by γ-secretase. After cleavage, the ICD of the protein is

released and transferred to the nucleus (Jung et al., 2003; Zampieri et al., 2005). In one study, during proBDNF and BDNF-induced apoptosis of sympathetic neurons, p75NTR ICD was cleaved by γ-secretase. NRIF, one of the proteins that binds with ICD of p75NTR, was then translocated into the nucleus (Kenchappa et al., 2006). This cleavage was shown to be necessary for the NRIF translocation and apoptosis (Kenchappa et al., 2006; Volosin et al., 2008). Although the specific mechanisms of p75NTR cleavage are not fully understood, these findings suggest multiple mechanisms could be involved in p75NTR-mediated signaling.

P75NTR can also have ligands other than neurotrophins. Recent studies have shown that β-amyloid and the aggregated prion peptide can bind with p75NTR to induce the death of cells. β-amyloid can induce p75NTR to trimerize and active JNK. Aggregated prion peptide-induced death involves the activation of caspase 8 (Sotthibundhu et al., 2008). Since the accumulation of soluble amyloid β protein in the brain is one of the reasons for the symptoms of Alzheimer's disease, this observation strongly supports a possible role for p75NTR in the neuronal loss in Alzheimer's disease.

2. Proneurotrophins

2.1 Proneurotrophins and protein processing and sorting

All neurotrophins are synthesized as precursor proteins, pre-pro-neurotrophins, which have an N-terminal signaling peptide and a pro-peptide. The pre-pro-NGF is a 31-35 kDa protein (Fig. 4). After the signal peptide is removed, proNGF is produced as a 32 kDa protein. In either trans-Golgi, or secretory granules, proneurotrophins can be glycosylated on the pro-region and cleaved by protein convertases to form mature neurotrophins (Edwards et al., 1988). There are two N-glycosylated sites and three separate sequences

of two or more contiguous basic amino acids in the pro-region. The cleavage site is located after these sequences. The subsequent processes for NGF and BDNF are different. NGF is mainly secreted by a constitutive pathway and partially by a regulated pathway. However, the release of BDNF is highly regulated. The main portion of BDNF is normally stored intracellularly. Only upon activation can it be secreted by cells (Mowla et al., 1999; Balkowiec and Katz, 2000). There have been multiple studies about the functions of the pro-region of the neurotrophins during this sorting process. Two conserved amino acid segments within the NGF pro-region were shown to be critical for the expression and secretion of biologically active NGF (Suter et al., 1991). It was also shown that the pro-region of NGF facilitated folding and disulfide bond formation of the protein (Rattenholl et al., 2001b). The C-terminal part of pro-region has been shown to play an important role for neurotrophin processing (Rattenholl et al., 2001a). The pattern of processing and secretion of NGF and BDNF were exchanged when their propeptides were exchanged, suggesting that the pro-region of neurotrophins was important for the processing efficiency (Nomoto et al., 2007).

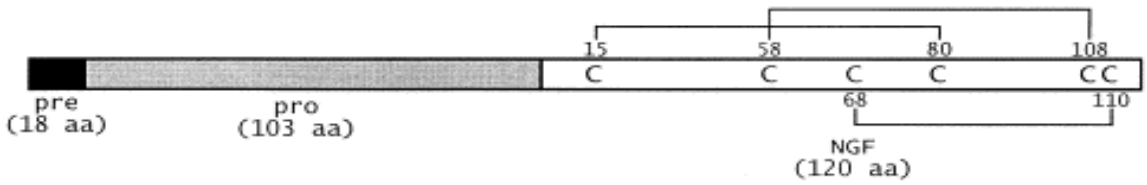


Figure 4. Schematic of pre-pro-NGF structure with the disulfide pattern. Pre indicates the signal peptide, pro the pro-peptides.

Rattenholl A el al. (2001) J Mol Biol 305:523-533

2.2 Proneurotrophins and cell death

Significant level of proNGF and proBDNF were found in various tissue extracts, suggesting that proneurotrophins might have functions other than

protein processing and sorting (Fahnestock et al., 2001). Using a cleavage-resistant proNGF, it was found that proNGF was a high affinity ligand for p75NTR, and this binding caused cell death (Lee et al., 2001). Sortilin, a member of the Vps10p family of receptors, was required to act as a co-receptor with p75NTR for proNGF-binding (Nykjaer et al., 2004; Teng et al., 2005; Volosin et al., 2006).

In different systems, it has been shown that proneurotrophins have apoptotic effects on cells. In PC12 cells, sympathetic and basal forebrain neuronal cultures, proNGF causes cell death via p75NTR (Lee et al., 2001; Volosin et al., 2006). Additionally, in cultured sympathetic neurons, proBDNF has been shown to induce the death of cells through the p75NTR-sortilin receptor complex (Teng et al., 2005). ProNGF was also shown to induce the death of oligodendrocytes after spinal cord injury in vivo (Beattie et al., 2002). When the binding between proNGF and p75NTR is blocked, there is a rescue of injured adult corticospinal neurons (Harrington et al., 2004). Moreover, the infusion of anti-proNGF can rescue hippocampal neuronal death after seizures (Volosin et al., 2008).

One potential source of proNGF is astrocytes. In respond to peroxynitrite or after seizures, proNGF was found be increased in astrocytes (Volosin et al., 2006; Domeniconi et al., 2007).

2.3 Proneurotrophins and sortilin receptor

Sortilin belongs to the Vps10p family of receptor. Five members of the family have been found in vertebrates so far (Willnow et al., 2008). Different members of the family have different distributions in the nervous system. Sortilin has been found in the cerebral cortex, hippocampus, and cerebellum (Sarret et al., 2003; Nykjaer et al., 2004). These receptors were considered to

function as sorting proteins. However, later studies suggested that these proteins could be involved with neuronal diseases including Alzheimer's disease (Grupe et al., 2006; Rogaeva et al., 2007).

The study of proneurotrophin-induced cell death through p75NTR-sortilin receptor complex revealed the necessary role of sortilin for the function of proneurotrophins. The binding between the pro-domain of proneurotrophins and sortilin strengthened the ligand receptor interaction (Nykjaer et al., 2004). Also, sortilin could facilitate p75NTR internalization, which is important for the proneurotrophin-induced cell death (Gargano et al., 1997). In sortilin knockout mice, decreased neuronal apoptosis in the retina was observed (Jansen et al., 2007).

2.4 The proteolytic processing of proneurotrophins

The conversion of proneurotrophins to mature neurotrophins is under the strict regulation of a protease cascade. After being synthesized as pre-pro-proteins, the signal peptide is removed in the endoplasmic reticulum (ER) to produce pro-form proteins. The cleavage of the pro-domain of the protein to produce mature neurotrophins can be performed in different ways, either intracellularly or extracellularly. Furin is responsible for intracellular cleavage. However, there are still substantial amounts of proneurotrophins released into extracellular environment (Yang et al., 2009). Several matrix metalloproteases (MMPs) and plasmin have been shown to be involved in the extracellular cleavage of proneurotrophins (Fig. 5) (Bruno and Cuello, 2006).

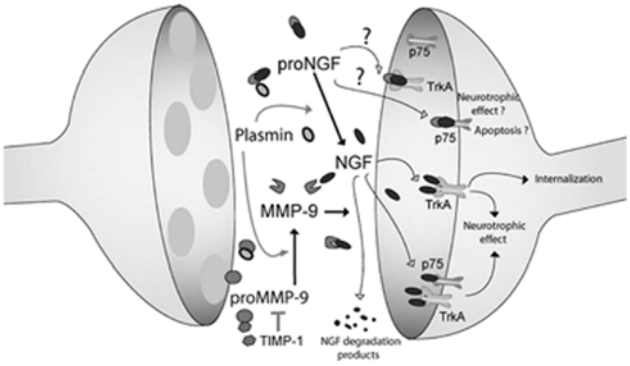


Figure 5. Schematic of extracellular cleavage of proNGF. Intracellular stored proNGF, plasminogen, tPA, neuroserpin, proMMP-9, and TIMP-1 are released into the extracellular environment after neuronal stimulation. ProNGF is converted into mature NGF under the control of the enzyme cascade.

Bruno MA, Cuello AC (2006) Proc Natl Acad Sci U S A 103:6735-740

3. PTEN and proneurotrophin-induced death signaling

3. 1 PTEN and PI3 kinase

PTEN (phosphatase and tensin homologue deleted on chromosome 10) was discovered in 1997 by two independent labs (Li et al., 1997; Steck et al., 1997). Due to a high frequency of mutations of the PTEN gene in various tumors, PTEN has been widely studied as a tumor suppressor. In multiple primary human cancers, PTEN was found to be inactive (Ali et al., 1999). This loss of function study also showed that this decreased PTEN activity lead to syndromes including developmental disorders, neurological deficits, and higher chances of breast, thyroid, and endometrial cancers (Saal et al., 2008).

PTEN specifically dephosphorylates the 3-position of the inositol ring (Maehama and Dixon, 1998). PTEN substrates include several phosphatidylinositol phosphates, such as PI(3)P, PI(3,4)P2, and PI(3,4,5)P3. PTEN dephosphorylates PIP3, converting it to PIP2, thereby antagonizing the activity of PI3 kinase (Stambolic et al., 1998). The accumulation of PIP3 leads to the phosphorylation and activation of the downstream factor Akt. Activation of this pathway is important for the survival, growth, and proliferation of cells.

The balance between PTEN and PI3K can determine the amount of PIP3 and assure the proper function of PI3K-Akt pathway (Fig. 6).

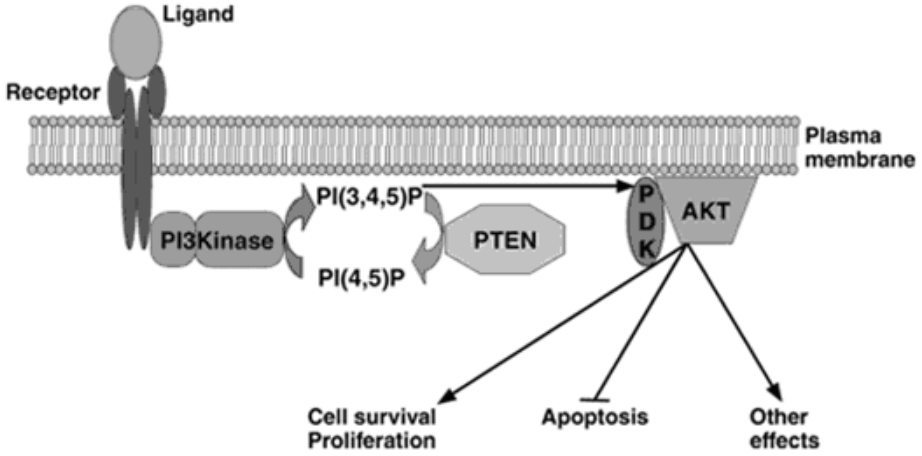


Figure 6. PTEN and PI3 kinase pathway. PI3K converts PIP2 into PIP3 which facilitates the activation of Akt. Akt then targets multiple downstream factors to support cell survival. PTEN dephosphorylates PIP3 into PIP2, thereby blocking PI3K-Akt pathway.
Planchon, S. M. et al. J Cell Sci 2008;121:249-253

The function of PTEN is non-redundant, and no other protein has been identified that can act as a substitute (Stambolic et al., 1998). Thus, PTEN is a master regulator of the PI3K-Akt pathway. Mutations of the PTEN gene have been shown to cause constitutive activation of Akt and tumorgenesis in human tissues (Vazquez and Sellers, 2000), also suggesting that it plays a necessary role in suppressing tumors.

3.2 Regulation of PTEN

PTEN can be regulated at different levels. PTEN transcription can be up-regulated by factors such as peroxisome proliferation-activated receptor γ (PPAR γ) and the early growth-regulated transcription factor-1 (EGR-1), while c-Jun can down-regulate PTEN transcription (Patel et al., 2001; Virolle et al., 2001; Hettinger et al., 2007).

Post-translationally, PTEN protein can be subjected to several modifications including phosphorylation, acetylation, oxidation, and ubiquitination (Salmena et al., 2008). Phosphorylation at certain residues decreases protein stability and the activity of the protein. Six phosphorylation

sites, includingThr366, Ser370, Ser380, Thr382, Thr383, and Ser 385 have been shown to be involved in suppressing PTEN activity (Maccario et al., 2007). Phosphorylation of PTEN on these sites closes protein conformation, decreasing their capacity to interact with other proteins. Furthermore, PTEN phosphorylation shields the amino acids responsible for the electrostatic interaction between PTEN and the cell membrane (Leslie and Downes, 2004).

Multiple kinases have been found to be responsible for the phosphorylation of PTEN. Casein kinase 2(CK2) phosphorylates PTEN on Ser 370 and Ser 385, decreasing PTEN activity by 30%. CK2 inhibitor was shown to decrease PTEN phosphorylation (Vazquez and Sellers, 2000). Another study revealed that glycogen synthase kinase 3β (GSK3β) might also phosphorylate PTEN (Maccario et al., 2007). Since GSK3β is downstream of Akt and Akt can inhibit GSK3β, this kinase could be part of a negative feedback loop for the PI3K pathway. Neither CK2 nor GSK3β can phosphorylate PTEN on Ser380, Thr382 or Thr383 (collectively referred to as the STT cluster), which are critical for PTEN activity, suggesting that there may be more kinases involved in PTEN phosphorylation.

3.3 PTEN and apoptosis

Because the PI3K-Akt pathway is known to be important for cell survival, by inhibiting this pathway, PTEN has been shown to be able to increase apotosis. Overexpression of PTEN was shown to reduce the activation of Akt in different tumor cells (Lu et al., 1999; Wick et al., 1999). The level of phosphorylation of Bad and GSK-3β is also decreased when PTEN is overexpressed (Myers et al., 1998). In vivo studies showed that knocking out PTEN in mouse embryonic fibroblast cells made these cells resistant to apoptotic stimulation. However, when PTEN activity was restored,

these cells became sensitive to the apoptotic treatments again (Stambolic et al., 1998).

3.4 PTEN and neuronal death

PTEN is widely expressed in the brain, especially in Purkinje neurons, olfactory mitral neurons and large pyramidal neurons (Lachyankar et al., 2000; Perandones et al., 2004). PTEN is involved in multiple functions in the central nervous system including neuronal survival, migration, differentiation and synaptogenesis. PTEN also plays an important role in neuronal injury process. In cultured cerebellar granule neurons, potassium deprivation plus serum deprivation decreases PTEN mRNA level (Kyrylenko et al., 1999). PTEN +/- neural precursor cells showed increased resistance to oxidative stress-induced apoptosis (Conner et al., 2003). In vivo studies showed that phosphorylated PTEN levels were increased in damaged brains (Omori et al., 2002). In an experimental rat model where chronic gestational animals were exposed to ethanol, it was found that there was a correlation between increased PTEN expression and increased neuronal death (Xu et al., 2003). A similar study showed that hypothermia protected neurons from death and this effect might have been due to the increased level of phosphorylated PTEN (Zhao et al., 2005). Conditionally deleting PTEN showed that the brain size of knockout mice increased over time compared with wild-type (Backman et al., 2001; Kwon et al., 2001).

3.5 The nuclear PTEN and cytoplasmic PTEN

Earlier PTEN studies focused on the cytoplasmic PTEN since it is mainly observed in the cytosol close to the cell membrane. However, recent studies found PTEN is also located in the nucleus in different cell types including primary neurons, endothelial cells and normal follicular thyroid cells

(Sano et al., 1999; Gimm et al., 2000). Nuclear PTEN was mainly observed when cells were quiescent while cytoplasmic PTEN was found in dividing cells, suggesting that nuclear PTEN is necessary for tumor suppression (Whiteman et al., 2002). Expression of PTEN in the nucleus correlates with a decrease in MAPK phosphorylation and cyclin D1 levels, whereas PTEN nuclear-localization-defective mutants do not show this relationship (Chung et al., 2006). This suggests that nuclear PTEN might specifically regulate these two events. The nuclear PTEN might also be involved in PTEN-related apoptosis, since expression of wild-type nuclear PTEN increases the activity of caspase 3 (Chung and Eng, 2005).

PTEN has been shown to be shuttled between the nucleus and cytosol. GFP-PTEN constructs can enter the nucleus by passive diffusion (Liu et al., 2005). The uneven distribution of PTEN between the nucleus and cytosol is possibly caused by cytosolic PTEN interacting proteins that sequester PTEN in the cytosol while free PTEN diffuses into nucleus (Planchon et al., 2008).

4. Basal forebrain cholinergic neurons, Alzheimer's disease and proneurotrophins

The basal forebrain (BF) includes several structures located on the ventral side of the forebrain. These structures include the nucleus basalis, diagonal band, medial septum and substantia innominata (Fig. 7). The diagonal band can be further divided into the horizontal limb and the vertical limb. The medial septum and diagonal band are continuous structures and can not be separated anatomically.

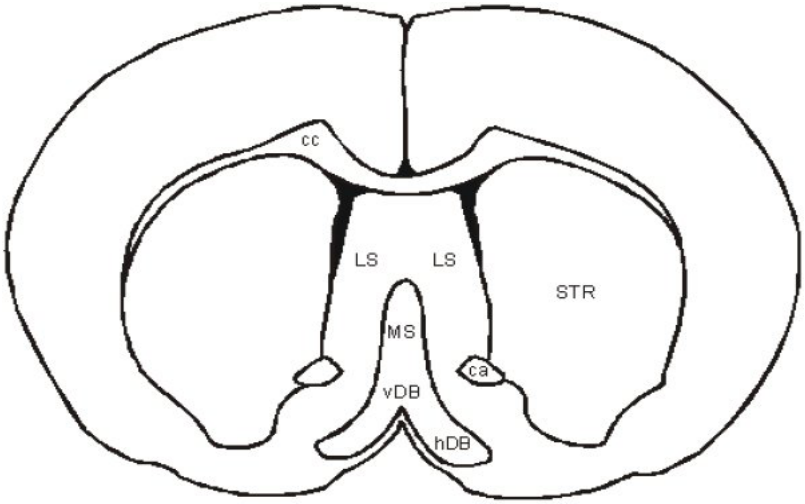


Figure 7. Coronal section of the basal forebrain. Abbreviations: MS = medial septum; vDB = vertical limb of the diagonal band; hDB = horizontal limb of the diagonal band; STR = striatum; ca = commissura anterior; cc = corpus callosum.
Paxinos G and Watson. Academic Press, 1986

The cholinergic neurons in the BF are important for learning and memory (Conner et al., 2003). These cells can send axons to various areas of the brain and provide the main source of acetylcholine innervation (Baratta et al., 1996). One target of BF axons is the hippocampus (Melander et al., 1985), where they regulate neuronal development and activity (Semba, 2000).

BF cholinergic neurons express all three Trk receptors, and neurotrophins are important for the development and maintenance of BF neurons (Seiler and Schwab, 1984). Neurotrophins are retrogradely transported to BF cell bodies through projections between the BF and hippocampus (Seiler and Schwab, 1984; DiStefano et al., 1992). BF astrocytes and oligodendrocytes can also release neurotrophins to support the development of BF neurons (Dai et al., 2003; Wu et al., 2004).

BF cholinergic neurons comprise one of a few populations of CNS neurons that express p75NTR throughout life (Hefti et al., 1985). P75NTR knock-out mice have an increased number of BF cholinergic neurons (Van der Zee et al., 1996; Yeo et al., 1997), suggesting that p75NTR may be important

for developmental basal forebrain cell death. In addition, proneurotrophins induce the loss of BF neurons via p75NTR signaling pathway in culture (Volosin et al., 2006).

In Alzheimer's disease, one salient characteristic is the early and progressive loss of BF neurons. The number of cholinergic markers including choline acetyltransferase, muscarinic and nicotinic acetylcholine receptor and acetylcholine was found to be decreased (Nordberg, 1992; Bierer et al., 1995). Although the mechanism of neuronal death remains unclear, significant amounts of proNGF have been found in the Alzheimer's brain (Fahnestock et al., 2001; Peng et al., 2004), suggesting that proneurotrophins may contribute to the loss of basal forebrain neurons via p75NTR.

5. Hippocampus and neurotrophins

The hippocampus has been shown to be important in the formation of new memories (Scoville and Milner, 1957). It is a C-shaped structure located in the medial temporal lobe, comprised of the hippocampus proper and the dentate gyrus (fig. 8). For rats, the hippocampus proper includes CA1, CA2 and CA3. In human, there are four parts: CA1, CA2, CA3 and CA4. The CA areas are composed of high density of pyramidal cells while the dentate gyrus is composed of granule cells.

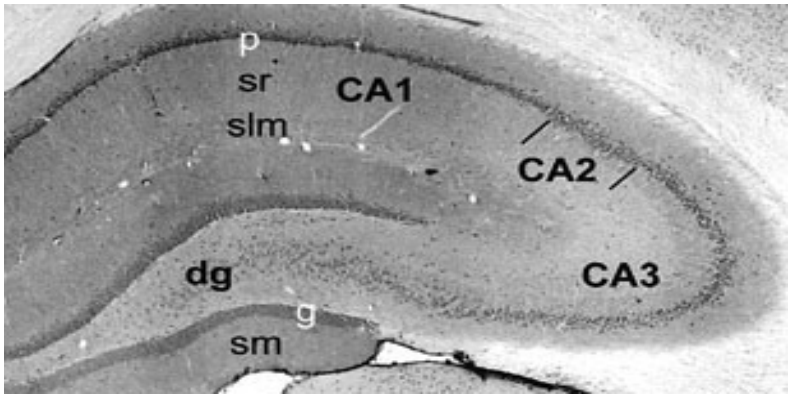


Figure 8. Coronal section of rat hippocampus. Abbreviation: dg = dentate gyrus; g = granular cell layer; p = pyramidal cell layer; slm = stratum lacunosum moleculare; sm = stratum moluculare; sr = stratum radiatum.

(Kauselmann et al., 1999)

All four neurotrophins have been shown to be produced by hippocampal neurons to support the survival of local neurons and afferent neurons from areas such as the basal forebrain and locus coeruleus (Hefti et al., 1985; Friedman et al., 1993; Arenas and Persson, 1994). TrkB and TrkC, but not TrkA have been found to be expressed in hippocampal neurons (Ernfors et al., 1992; Ip et al., 1993). P75NTR is highly expressed in hippocampal neurons during both embryonic and postnatal stages (Buck et al., 1988; Lu et al., 1989).

6. Retrograde transport of neurotrophin signaling

During development, axonal processes can grow very long distances to receive neurotrophins from remote target cells. The signal induced by neurotrophins can be retrogradely transported to cell bodies to support the survival of innervating neurons. Trk receptors located at axon terminals can be activated by neurotrophins, and this signal can be propagated through axons to cell bodies (Kaplan and Miller, 2000). One important protein that can be activated by Trk is Erk5, which can then be translocated to the nucleus to support the survival of cells (Watson et al., 2001). In contrast, if Trk receptors located at cell bodies are activated by neurotrophins, Erk1 and 2 will be

activated and translocated from the cytoplasm to the nucleus to support cell survival (English et al., 1999; Finkbeiner, 2000).

7. Crosstalk between survival and death signaling

Trk-mediated signaling pathways have been shown to be mostly involved in survival and differentiation of cells, while in many cases signaling pathways activated by p75NTR promote apoptosis (Reichardt, 2006). These two receptor systems have their own preferred ligands: mature neurotrophins bind to Trk receptors while proneurotrophins have a high affinity for p75NTR. Different downstream factors can be activated by these two types of receptors. Trk receptors activate both the PI3K-Akt and MAPK pathways, which support cell survival, whereas p75NTR signaling activates JNK and caspases, causing cell death.

Trk receptors and p75NTR can work in both synergistic and antagonistic ways, depending on the type of cells or environmental circumstances (Schweigreiter, 2006). Acting as a co-receptor, p75NTR increases the affinity and selectivity of binding between mature neurotrophins and Trk receptors (Bibel et al., 1999). However, if both Trk and p75NTR signaling are induced by mature NGF, Trk signaling dominates over p75NTR (Friedman, 2000). Collectively, these data suggest a crosstalk between Trk and p75NTR-mediated signaling.

II. Research Aims:

1. **Determine if proNGF can cause the loss of BF neurons via the p75NTR- mediated signaling pathway**. ProNGF has been shown to induce apoptosis via p75NTR in different systems (Beattie et al., 2002). Can proNGF

cause the death of BF neurons since they express both Trk receptors and p75NTR?

2. **Determine if sortilin plays a necessary role in proNGF-induced apoptosis in BF neurons**. Sortilin was found to be a co-receptor with p75NTR to mediate cell death induced by proNGF in sympathetic neurons (Nykjaer et al., 2004). It is important to show if sortilin is necessary for proNGF-induced death in BF neurons.

3. **Identify crosstalk that may occur between signaling pathways activated by cleaved versus proneurotrophins in BF neurons**. Mature neurotrophins are important for the survival of neurons by activating survival pathways such as the PI3 kinase pathway, while proNGF and proBDNF have been found to induce death of cells via p75NTR. When BF neurons are exposed to both cleaved and proneurotrophins, is there crosstalk between survival and death signaling pathways, and if so, which pathway predominates and how?

4. **Determine if PTEN is necessary for suppression of survival signaling induced by mature neurotrophins**. PTEN is a phosphatase that can dephosphorylate PIP3 into PIP2, inhibiting the activation of Akt. Since Akt is one of the important downstream factors of survival signaling activated by mature neurotrophins, we hypothesized that PTEN could antagonize survival signaling induced by mature neurotrophins.

III. Material and Methods:

1. Reagents: BDNF was a gift from C.F.Ibanez (Karolinska Institute, Stokholm, Sweden). Recombinant murine proNGF and proBDNF were provided by B.L. Hempstead (Weill Medical College of Cornell University, New

York, New York). Neurotensin was purchased from Bachem (Torrance, CA). Anti-p75 (Ig 192) was purchased from Chemicon (Temecula, CA); other anti-p75 antisera were generously provided by M.V.Chao (Skirball Institute, New York University, New York, New York), L.F.Reichardt (University of California, San Francisco, CA), and B. Carter (Vanderbilt University, Nashville, TN). Rabbit Anti-$TrkB_{in}$ antiserum and recombinant adenovirus vector encoding constitutively activated ras (ras V12) were from David Kaplan (Toronto, Ontario, Canada); Chicken anti-$TrkB_{in}$ was purchased from Promega (Madison, WI). Antibodies to p-JNK, JNK, p-Trk, p-Akt, Akt, p-Erk, Erk, cleaved caspase-3, cleaved caspase-6, PTEN, p-PTEN, as well as the inhibitors to PI3 kinase, LY294002, and Mek1, PD98059, were purchased from Cell Signaling Technologies (Beverly, MA). PTEN inhibitor (bpV(pic)) was from Calbiochem (San Diego, CA). Anti-sortilin (anti-neurotensin3) polyclonal antibody was from Alpha Diagostics (San Antonio, TX) and from BD Biosciences (Mountain View, CA). The secondary antibodies were Alexa 488 and Alexa 594 anti-rabbit and anti-mouse antibodies purchased from Invitrogen. Poly-D-lysine, glucose, putrescine, progesterone, transferring, selenium, insulin were purchased from Sigma (St. Louis, MO). Rat collagen was from Roche Diagnostics (Indianapolis, IN).

2. Neuronal cultures: Pregnant rats were sacrificed by exposure to CO2 and soaked in 80% ethanol for 10 min. Embryonic day 16 (E16) rat fetuses were taken under sterile conditions and kept in PBS on ice. Basal forebrains were dissected and dissociated in serum free medium (SFM). The cells were then plated on tissue culture dishes that were pre-coated overnight with poly-D-lysine (0.1mg/ml). The cells were maintained in serum free medium for 5 days at 37°C (Farinelli et al., 1998; Friedman, 2000)

3. Real time PCR (QPCR): BF neurons were cultured for five days before RNA was isolated using TRIZOL reagent (Invitrogen). CDNA was synthesized by using superscript reverse transcriptase (Invitrogen). QPCR was performed on LightCycler 480 (Roche) by using Sybrgreen master mix.

4. Small interfering RNA experiment: PTEN small interfering RNA (siRNA) with a 5' thiol on the sense strand was purchased from Dharmocon RNAi Technologies. Sequences for the sense strand were: GATGGCTGTCATGTCTGGGAG. The siRNA was mixed with Penetratin1 and heated for 15 min at 65°C and incubated for 1 hour at 37 °C. Before treated cells with siRNA, the sequences were heated at 65°C for 15 min.

5. Western blot analysis: After 5 days in culture, the neurons were treated for 30min or 4h with vehicle, mature neurotrophins or proneurotrophins. Lysis buffer containing 120mM Tris, 2% SDS, 10% glycerol and protease inhibitor was added into dishes to harvest cells. Proteins from equal amount of neurons were run on a 10% or 12% polyacrylamide gel and then transferred to nitrocellulose membrane. The blots were blocked by 5% nonfat milk and then incubated with primary antibody overnight. After washing 3 times with TBST for 15 minutes each, the blots were processed with secondary antibody for 1 hour at room temperature. The membrane was washed 3 times with TBST before being visualized using either the ECL kit (Pierce) or scanned by Typhoon 9410 (Amersham Biosciences) or Odyssey infrared imaging system (LI-COR Bioscience).

6. Neuron survival assay: After 5 days in culture, the neurons were treated overnight with different neurotrophins with or without their inhibitors. The medium was removed and lysis buffer containing 10% triton, 1M $MgCl_2$, and 0.2% bromphenol blue was added to cell culture. With a hemacytometer, the

intact nuclei were counted. Only the nuclei that were phase bright and had a clearly defined limiting membrane were counted. For each treatment, triplicate samples were counted (Friedman, 2000).

7. Immunocytochemistry: Cells cultured in 8-compartment slide wells were treated with ligand and then fixed with 4% paraformaldehyde for 30 minutes. Before primary antibody incubation, the cells were permeabilized and blocked with PBS/0.3%Triton/5%goat serum for 30 minutes at room temperature. Different primary antibodies were applied to the slides overnight at 4°C. The slides were washed three times with PBS for 15 minutes. The secondary antibodies Alexa 488 (green) and 594 (red) (1:500) were applied to the slides in the dark for 1 hour and then washed three times with PBS for 15 minutes and mounted. Hoechst 33342 dye (1 μg/ml; Sigma) was added into PBS during the last wash to stain nuclei. The apoptotic cells were identified by chromatin condensation and clumping in the nucleus. The slides were examined by fluorescence microscopy (Troy et al., 2002).

8. Pilocarpine-induced seizures: Male Sprague Dawley rats (250-275g) were cannulated 1 week before the induction of seizures. Animals were sedated with ketamine/xylazine and placed in a stereotaxic. Two cannulas were implanted bilaterally into the hippocampus. After one week of recovery, animals were pretreated with methyl-scopolamine (1mg/kg, s.c; Sigma-Aldrich) for half a hour before being treated with pilocarpine hydrochloride (350 mg/kg, i.p.; Sigma-Aldrich). After 1 hour of status epilepticus, diazepam (10 mg/kg; Abbott Laboratories) and phenytoin were injected to stop seizure.

After seizures, PTEN inhibitor bpv(pic) was injected into one side of hippocampus and saline was injected into another side of hippocampus twice a day until the rats were perfused 3 days after the seizures.

All animal studies were conduced using the National Institute of Health guidelines for the ethical treatment of animals with approval of the Rutgers Animal Care and Facilities Committee.

9. Immunocytochemistry of the hippocampal sections: Rats were anesthetized with ketamine/xylazine and perfused transcardially by saline and then by 4% paraformaldehyde. The brain was removed and fixed in 4% paraformaldehyde for 2 hours before being cryoprotected in 30% sucrose for 2 days. After the brain had totally sunk down in sucrose solution, it was sectioned on a cryostat (Leica) and mounted onto charged slides. The slides were then immunostained with antibodies to p75NTR and cleaved caspase 3 as described previously.

IV. Results:

1. ProNGF induces the death of BF neurons through p75NTR-sortilin receptor complex:

Expression of Trk receptors and p75NTR are highly overlapped in the basal forebrain in vivo. It was known that basal forebrain neurons express all three Trk receptors and p75NTR (Seiler and Schwab, 1984). But the precise distribution of Trk receptors and p75NTR in basal forebrain is unknown. Since we are interested in the potential interaction between p75NTR-mediated signaling and Trk-mediated signaling, it is important and necessary to determine their localization. Basal forebrain sections from adult rat were double stained with each of the Trk receptors and p75NTR. Trk receptors and p75NTR were found to be strongly colocalized in these sections (Fig. 9, Table 1).

ProNGF binds with p75NTR and induces the death of BF neurons in the absence of mature neurotrophins. In the presence of a Trk receptor, p75NTR acts as a co-receptor to facilitate the binding between Trks and mature neurotrophins. P75NTR-mediated apoptotic signaling does not manifest because of either its low affinity with mature neurotrophins or due to being sequestered in the Trk-p75NTR receptor complex. Since TrkA receptors are not expressed in hippocampal neurons, high doses of mature NGF induce the death of cells via p75NTR (Friedman, 2000).

In basal forebrain neurons, mature neurotrophins cannot cause the death of cells due to protection from Trk signaling. However, proneurotrophins are able to induce apoptosis in sympathetic neurons due to their high affinity to p75NTR (Lee et al., 2001). We hypothesized that proNGF would induce the death of BF neurons via p75NTR as well. To test our hypothesis, E16 rat BF neurons were cultured for 5 days then treated overnight with vehicle, mature NGF, or proNGF. ProNGF, but not mature NGF, was found to cause a 40% loss of BF neurons, suggesting that proneurotrophins can activate apoptotic signaling in BF neurons. When BF neurons were treated with a combination of proNGF and a blocking antibody to p75NTR, the cells were protected from death, indicating that p75NTR mediates this effect (Fig. 10).

Sortilin is colocalized with p75NTR in BF neurons. One of the important features of binding between proneurotrophins and p75NTR is the involvement of another receptor: sortilin. Binding with the pro-domain of the proneurotrophins, sortilin acts as a co-receptor with p75NTR to mediate apoptosis caused by proneurotrophins (Nykjaer et al., 2004). To examine the potential function of sortilin in proNGF-induced death signaling in the basal forebrain, BF neurons were double stained with p75NTR and sortilin

antibodies. Strong colocalization of p75NTR and sortilin was found in both healthy and apoptotic BF neurons (Fig.11 A).

Sortilin is necessary for proNGF-induced death in BF neurons. Since sortilin is colocalized with p75NTR in basal forebrain, it was important to examine if sortilin was necessary for proneurotrophin-induced cell death. BF neurons were treated overnight with proNGF and proBDNF in the presence or absence of anti-sortilin (anti-Neurotensin Receptor III (NTR)). Anti-sortilin is an antibody that binds to its extracellular domain and competes with proNGF binding, efficiently blocking proneurotrophin-induced neuronal loss. Cells treated with both proneurotrophins and anti-sortilin showed significantly less cell death than those treated with proneurotrophins alone. Neurotensin, another ligand for sortilin, was also used to compete with proneurotrophins for sortilin binding. After one hour of neurotensin treatment, we found that neurotensin could protect BF neurons in a dose-dependent manner: 4μM neurotensin had no effect while 40μM neurotensin significantly reduced cell death. Together, these data indicate that p75NTR and sortilin are both necessary for proneurotrophin-induced apoptosis in BF neurons (Fig.11 B, C).

2. ProNGF induces the death of BF neurons even in the presence of activated Trk receptors:

ProNGF causes the loss of BF neurons in the presence of BDNF. From our previous data, we found that most p75NTR positive cells also express Trk receptors. Can proNGF induce the death of BF neurons when Trk signaling is activated? To address this question, BF neurons were grown for 5 days in the presence of BDNF (10ng/ml) from the time of plating before overnight treatment with vehicle, mature NGF or proNGF. ProNGF, but not mature NGF, caused a loss of 40% of BF neurons, suggesting that the activation of

TrkB signaling does not protect BF neurons from proNGF-induced death. Treating cells with a blocking antibody to p75NTR protected neurons from death, suggesting that proNGF induced the death of BF neurons via p75NTR (Fig. 12).

ProNGF induces apoptosis of BF neurons expressing TrkB in the presence of BDNF. Since the basal forebrain is not a homogeneous population of neurons, it is possible that the neurons that die are the ones that express TrkA or TrkC, not TrkB. In order to study individual cells, BF neurons were grown in slide wells for 5 days in the presence of BDNF from the time of plating. BDNF was re-added to ensure that TrkB was activated before vehicle, mature NGF, or proNGF were used to treat cells for 4 hours. The cells were then double immunostained with antibodies to p75NTR (192 IgG) and TrkB receptor (anti-$TrkB_{in}$). The apoptotic cells were identified by nuclear morphology as indicated by Hoescht staining. In proNGF-treated populations, we found that cells expressing both p75NTR and TrkB receptor were also apoptotic, demonstrating that proNGF induced the death of neurons expressing both p75NTR and TrkB receptor (Table 2; Fig.13 a).

ProNGF induces the death of BF neurons even in the presence of phosphorylated Trk receptor. While proNGF was found to induce cell death in cells expressing Trk receptors, it remained unclear if death occurred when Trk was activated. BF neurons were treated with BDNF for five minutes to activate Trk receptors, followed by a five hour treatment with proNGF, which we had previously established as when the morphology of apoptotic nuclei could be observed. Cells were then double stained with p75NTR and p-Trk antibodies, and dying cells were identified using Hoechst staining. Among dying cells, we

found colocalized p75NTR and p-Trk staining, indicating that proNGF can induce apoptosis even in the presence of activated Trk (Fig.13 b).

3. Mature and proneurotrophins induce distinct signaling pathways in BF neurons:

BDNF induces the phosphorylation of Akt and Erk in BF neurons. Trk receptors can activate the PI3 kinase pathway and MAP/Erk pathway to support survival and differentiation of cells (Patapoutian and Reichardt, 2001). In order to confirm this effect in BF neurons, cultured BF neurons were treated with BDNF or proNGF for 30 min. The phosphorylation of Akt and Erk were examined by western blot analysis. BDNF, not proNGF, induced Akt and Erk phosphorylation in BF neurons, indicating the activation of PI3 kinase pathway and MAP/Erk pathway by BDNF (Fig.14 A).

ProNGF induces the phosphorylation of JNK and cleavage of caspases 3 and 6 in BF neurons. The phosphorylation of JNK and the cleavage of caspases 3 and 6 are required for apoptotic signaling activated by mature neurotrophins via p75NTR (Yoon et al., 1998; Friedman, 2000; Wang et al., 2001). To determine if proNGF uses the same signaling pathway to induce death of BF neurons, cultured BF neurons were treated with proNGF to activate the p75NTR signaling pathway. ProNGF induced JNK phosphorylation and cleavage of caspases 3 and 6 in BF neurons, indicating activation of the same downstream factors as observed in apoptosis induced by mature neurotrophins (Fig.14 B, C).

ProNGF induces the activation of caspase 3 in the presence of BDNF. To test if proNGF can induce the activation of caspases in the presence of BDNF, BF neurons were grown in the presence of BDNF for 5 days then treated with either proNGF, additional BDNF, or a combination of the two.

Western blot analysis shows that caspase 3 was activated by proNGF even in the presence of BDNF, further confirming that proNGF can induce apoptotic signaling even in the presence of activated Trk signaling (Fig. 15).

4. Survival and death signaling pathways interact downstream of Trk receptors, but upstream of Akt and Erk in BF neurons:

ProNGF causes apoptosis in the presence of p-Trk, but not in neurons with p-Akt or p-Erk. BF neurons were grown in the presence of BDNF from the time of plating for 5 days. BDNF was then re-added to activate Trk signaling, followed by four-hour treatment with proNGF to induce apoptosis. The cells were then double immunostained with either p-Trk and p-Akt, p-Erk and p-Akt, or cleaved caspase 3 and p-Akt. Hoechst dye was used in all cases to identify apoptotic cells. Apoptotic cells were found to express cleaved caspase 3, but not p-Akt or p-Erk. ProNGF induced about 30% of p-Trk positive neurons to die while none of them expressed p-Akt. Furthermore, neurons that showed both p-Trk and p-Akt staining were not apoptotic. Together, these data suggest that the activation of downstream survival signaling proteins such as Akt can protect neurons from proNGF-induced death (Fig 16, Table 3).

Activation of PI3 kinase and MAP kinase/Erk pathways protect BF neurons from death caused by proNGF. Because cells expressing p-Akt and/or p-Erk could not be killed by proNGF, we studied the effects of constitutively activating PI3 kinase and MAP kinase in the absence of ligand. Adenovirus expressing either GFP or myc-tagged, constitutively activated ras (ras V12) was used to infect BF neurons before proNGF treatment. In neurons infected by ras V12, both PI3 kinase and MAP kinase pathways were activated in the absence of neurotrophin. GFP positive neurons showed a similar percentage of death as uninfected neurons, while ras V12-infected

neurons were resistant to proNGF-induced cell death. Even background death was significantly reduced in ras V12-infected neurons (Fig. 17 A). Furthermore, all ras V12 infected neurons were p-Akt positive (Fig. 17 B).

Akt is more crucial than Erk for the protection of BF neurons from pro-NGF induced death. It is important to identify if these pathways are of equal importance to protection from cell death. Inhibitors of either PI3 kinase or MEK/Erk pathway were used to treat cells before the proNGF treatment. Treatment with PI3K or MEK/Erk pathway inhibitors alone did not affect the survival of BF neurons significantly. However, treatment with the PI3 kinase inhibitor (LY294002), but not the MEK inhibitor (PD98059), significantly increased the amount of cell death induced by proNGF, suggesting that Akt may be more critical for survival signaling in BF neurons compared to Erk (Fig. 18).

ProNGF inhibits the phosphorylation of Akt and Erk, but not Trk. BF neurons were grown on 6-well dishes for 5 days in the presence of BDNF from the time of plating. The cells were then treated with either BDNF or proNGF or a combination of the two. Western blot analysis was used to detect p-Trk, p-Akt and p-Erk. BDNF and proNGF induced distinct signaling pathways. When cells were treated with both ligands, proNGF inhibited the phosphorylation of Akt or Erk induced by BDNF. However, BDNF still induced the phosphorylation of Trk, suggesting there is an interaction between the survival and death signaling pathways downstream of Trk and upstream of Akt and Erk (Fig. 19).

5. ProNGF suppresses BDNF-induced survival signaling through PTEN:

ProNGF increases the level of PTEN in BF neurons in the presence and absence of BDNF. ProNGF can inhibit Akt phosphorylation in BF

neurons, but the mechanism of this inhibition is unknown. Because PTEN can act as an antagonist to the PI3K/Akt pathway, we hypothesized that proNGF may inhibit Akt phosphorylation through PTEN. In order to test this hypothesis, cultured BF neurons were treated with proNGF at different time points. ProNGF increased the level of PTEN starting from 30 minutes up to 120 minutes after treatment, suggesting proNGF can regulate PTEN protein (Fig. 20A).

Since proNGF causes the death of BF neurons in both the absence and presence of BDNF, it is necessary to know if proNGF can regulate PTEN even in the presence of BDNF. When BF neurons were treated with BDNF, proNGF or a combination of both ligands, we observed that proNGF treatment, but not BDNF treatment, significantly increased the level of PTEN protein in cells. The presence of BDNF did not affect the amount of PTEN induced by proNGF (Fig. 20B).

ProNGF increases the level of PTEN through the p75NTR-sortilin receptor complex. Our previous study indicated that proneurotrophins induced the death of BF neurons via a p75-sortilin receptor complex. Does the same receptor complex mediate the up-regulation of PTEN? In order to test this, a blocking antibody to either p75NTR or sortilin was used to inhibit proneurotrophin-receptor binding. Blocking either receptor significantly reduced the induction of PTEN, indicating that PTEN induction occurs through p75NTR-sortilin receptor binding (Fig. 21).

PTEN inhibitor reverses the inhibition of Akt phosphorylation by proNGF. In order to understand the potential role of PTEN in proNGF-induced apoptosis, we treated cultured BF neurons with a PTEN inhibitor, bpv(pic), to inhibit PTEN activity. After bpv(pic) treatment, proNGF no longer

inhibited BDNF-induced Akt phosphorylation, suggesting that PTEN is involved in blocking PI3 kinase pathway (Fig. 22A). However, proNGF still inhibited Erk activation in the presence of bpv(pic), suggesting that Erk was not the target of PTEN protein (Fig. 22B)

PTEN inhibitor blocks the effect of proNGF in BF neurons only in the presence of BDNF. Because proNGF treatment could increase the level of PTEN in BF neurons, we tested if the inhibition of PTEN could block the pro-apoptotic effects of proNGF. BF neurons were cultured in the presence of BDNF. BDNF was added again, then cells were treated with proNGF, PTEN inhibitor, or PTEN inhibitor plus proNGF. Only in the presence of BDNF could PTEN inhibitor significantly decrease the amount of cell death induced by proNGF (Fig. 22C).

Knocking down PTEN rescues BF neurons from proNGF-induced apoptosis in the presence of BDNF. In order to confirm the data from the PTEN inhibitor experiment, we developed a small penetratin-linked interfering RNA to PTEN to know down PTEN protein in cells (Fig. 23A). This siRNA was used to prevent proNGF-induced PTEN up-regulation in BF neurons (Fig. 23B). When PTEN induction was blocked by siRNA, there was a rescue of BF neurons, but only in the presence of BDNF (Fig. 23 C,D), which is consistent with our previous data.

BDNF increases non-active form of PTEN protein in BF neurons that can be blocked by proNGF. Phosphorylated PTEN is the non-active form of the protein. ProNGF treatment increased the level of PTEN in BF neurons, but not the level of p-PTEN. Do mature neurotrophins also have this effect on PTEN regulation? To test this, BF neurons were treated with BDNF at different time points. Interestingly, p-PTEN, but not PTEN levels, were

increased after treatment, suggesting that proneurotrophins and mature neurotrophins could have opposite effects on PTEN protein (Fig. 24 A).

Our previous data showed that BDNF-induced signaling can be prevented by proNGF-induced signaling. To test if BDNF-induced p-PTEN can also be blocked by proNGF, BF neurons were treated with BDNF in the absence and presence of proNGF. ProNGF blocked BDNF-induced p-PTEN up-regulation, suggesting BDNF regulation of p-PTEN can be suppressed by proNGF signaling (Fig. 24 B).

ProNGF does not regulate PTEN mRNA. Given the observation that proNGF can rapidly increase PTEN protein in BF neurons, it was necessary to address the mechanism of this regulation. It may require the synthesis of new mRNA or new protein or both. To detect the possible change of PTEN mRNA level after proNGF treatment, primers against PTEN coding sequence were generated to detect PTEN mRNA. When BF neurons were treated with proNGF at different time points, there was no significant change of PTEN mRNA, suggesting that proNGF-induced PTEN increase does not require synthesis of new mRNA (Fig. 25 A).

Protein synthesis inhibitor, but not transcription inhibitor can block the proNGF-induced PTEN increase. ProNGF regulates PTEN level by synthesizing new protein. BF neurons were treated with proNGF in the presence or absence of cycloheximide, a protein synthesis inhibitor. Cycloheximide significantly blocked the increase of PTEN, suggesting new protein synthesis was necessary for the PTEN regulation. However, actinomycin-D, an inhibitor of mRNA synthesis, could not block this up-regulation, confirming that mRNA synthesis was not required for PTEN induction (Fig. 25 B).

6. PTEN function in cell death after seizure in the hippocampus

Pilocarpine-induced seizure is a model to study the function of proneurotrophins in vivo since p75NTR is up-regulated and proNGF is increased in astrocytes in hippocampus after seizure (Volosin et al., 2008). More important, infusing an antibody to proNGF into one side of the hippocampus after seizure resulted in a significant decrease of neuronal loss in the dentate hilus when compared with an infusion of control IgG on the contralateral side. These data imply that proNGF is produced and causes neuronal death in the hippocampus after seizure.

ProNGF blocks survival signaling through PTEN in cultured hippocampal neurons. Since proNGF can use PTEN to block survival signaling in cultured neurons, it is important to address if PTEN can also play necessary role in proNGF-induced death in vivo. However, before the animal model can be used, it was necessary to confirm that proNGF can also regulate PTEN in cultured hippocampal neurons as in BF neurons. When E18 hippocampal neurons were cultured and treated with proNGF, there was an increase of PTEN protein level starting from 30 min up to 120 min, consistent with our data from BF neurons. Moreover, proNGF could also block BDNF-induced Akt activation in hippocampal neurons, further supporting that proNGF regulation of PTEN is similar in both hippocampal and basal forebrain neurons (Fig. 26).

PTEN inhibitor can rescue HPC neurons after seizures. Since PTEN can also be regulated by proNGF in hippocampal neurons, the pilocarpine-induced seizure animal model was used to study the function of PTEN in proNGF-induced death in the hippocampus. Cannulation and the pilocarpine-induced seizures were carried out as described previously (Volosin et al.,

2008). After the seizure, the PTEN inhibitor bpv(pic) was injected into one side of the hippocampus while saline was injected into the opposite side as a control for three days. On the side receiving bpv(pic), there were significantly fewer cells expressing p75NTR and cleaved caspase3, suggesting that PTEN inhibitor could rescue neuronal loss after the seizure in the hippocampus (Fig. 27).

V. Discussion

Neurotrophins have been shown to regulate multiple cellular functions such as survival, differentiation, migration, synaptic activity and apoptosis in both the CNS and PNS (Arevalo and Wu, 2006). Neurotrophins binding to Trk receptors or p75NTR leads to several signaling pathways and causes different physiological consequences. Trk signaling has been associated with survival and differentiation of cells via both the PI3 kinase and MAP kinase pathways (Patapoutian and Reichardt, 2001), while studies on p75NTR signaling have been very controversial. P75NTR-mediated signaling pathways promote survival by activating NFκB in embryonic sensory and sympathetic neurons (Maggirwar et al., 1998; Hamanoue et al., 1999). However, when p75NTR is expressed independently, it can mediate death signaling. Numerous data have demonstrated that p75NTR signaling is important in regulating apoptosis, both in developmental and pathological conditions, even though the signaling mechanism is poorly understood (Roux and Barker, 2002). In both CNS and PNS, mature neurotrophins can induce the death of cells via p75NTR in the absence of cognate Trk receptors (Frade et al., 1996; Yoon et al., 1998; Friedman, 2000).

Studies on proneurotrophins have expanded the knowledge of p75NTR death signaling. ProNGF and proBDNF bind with high affinity to the p75NTR-sortilin receptor complex and with low affinity to Trk receptors, suggesting that proneurotrophins are important ligands for the activation of p75NTR-mediated signaling(Lee et al., 2001). In different models, proNGF and proBDNF were shown to induce the death of cells via p75NTR (Lee et al., 2001; Beattie et al., 2002; Teng et al., 2005). The concentration needed for proneurotrophins (0.1-0.2nM) to induce death is much lower than the concentration needed for mature neurotrophins (2-4nM) to induce death. Considering the low concentration of neurotrophins in vivo, it is possible that proneurotrophins, not mature neurotrophins, play a very important role in p75NTR-mediated death. Moreover, proneurotrophins can be increased in astrocytes under stress conditions, which further implicates their involvement in physiological states (Volosin et al., 2006). Several studies have demonstrated that proneurotrophins induce apoptosis under pathological conditions, further supporting this hypothesis (Harrington et al., 2004; Peng et al., 2004; Pedraza et al., 2005; Volosin et al., 2006; Domeniconi et al., 2007).

The processing of proneurotrophins has been studied as well. After removal of the signal peptide from the endoplasmic reticulum, the pro-form of neurotrophins can be cleaved by several different proteases to yield the mature forms. Intracellularly, proneurotrophins can be cleaved by furin, a major processing enzyme of the secretory pathway (Seidah et al., 1996). Extracellularly, several matrix metalloproteinases (MMP) and plasmin were found to cleave proneurotrophins (Bruno and Cuello, 2006). Collectively, these data indicate that proneurotrophins are under a strictly controlled regulation system.

Basal forebrain cholinergic neurons are crucial for memory and learning processes (Conner et al., 2003). One of the early hallmarks of Alzheimer's disease is the loss of BF neurons (Whitehouse et al., 1982), but the mechanism of this neuronal death remains unclear. Since BF neurons are one of a few CNS populations that express p75NTR throughout life, it is possible that p75NTR signaling plays an important role for the death of these neurons. Our data demonstrating that eliminating p75NTR rescues BF neurons supports this hypothesis. The next question is which ligand activates this process, mature neurotrophins or proneurotrophins? Given that BF neurons express all Trk receptors, mature neurotrophins do not cause cell death via p75NTR because Trk receptors can be activated to protect cells. However, proneurotrophins have a high affinity to p75NTR and low affinity to Trk receptors, suggesting they may be the ligand for p75NTR-mediated death in the basal forebrain. In vivo studies also showed that proNGF is increased in the cortex of the Alzheimer's brain, which implies that proneurotrophins may activate p75NTR-mediated cell death under this pathological condition (Fahnestock et al., 2001; Peng et al., 2004).

Sortilin was shown to facilitate the binding between proneurotrophins and p75NTR. In this thesis, we have demonstrated that proNGF and proBDNF induce death of BF neurons via the p75NTR-sortilin receptor complex in the basal forebrain. In order to further confirm the necessity of sortilin, another sortilin ligand, neurotensin, was used to compete with sortilin binding. Neurotensin blocked the effect of proneurotrophins in a dose-dependent manner, also implying that sortilin is involved in signaling. When double staining of p75NTR and sortilin was performed in BF neurons, we were expecting that colocalization might be only seen in apoptotic neurons.

However, coexpression of p75NTR and sortilin was seen in both healthy and apoptotic cells, suggesting coexpression of p75NTR and sortilin alone is not sufficient to determine if the neuron is going to die or survive.

In previous studies of sympathetic neurons, it was found that proNGF could not induce cell death in the presence of activated Trk receptors (Teng et al., 2005). Our data showed different results: in the presence of BDNF, proNGF and proBDNF caused 40% BF neuronal death, suggesting that Trk signaling could not protect BF neurons from the apoptotic effects of proneurotrophin treatment.

Survival and death signaling in BF neurons

Trk receptors and p75NTR mediate opposite effects on cells. The interaction between these two receptor systems has not been fully addressed. Before the identification of apoptotic effects of proneurotrophins, the study of the interaction between Trk and p75NTR mediated signaling focused on survival and death effects of mature neurotrophins. As a co-receptor, p75NTR can facilitate the binding between mature neurotrophins and Trks to support the survival of cells. In cells expressing p75NTR alone, however, mature neurotrophins binding to p75NTR cause cell death. When cells express both Trks and p75NTR, survival signaling dominates death signaling. This may be caused by the competition between the receptors and their downstream factors. The high affinity between mature neurotrophins and their cognate Trk receptors leads to the activation of the Trks prior to the activation of the p75NTR. Once Trk signaling is activated, one of its downstream factors, Akt, can inhibit the apoptotic signaling pathway. Together, survival signaling, not apoptotic signaling, can proceed. Given that p75NTR plays a critical role in apoptosis, in cells expressing both Trks and p75NTR, how does p75NTR

mediate death? The question of a ligand for p75NTR-induced death in physiological conditions was controversial.

The discovery of new functions for proneurotrophins made significant progress on this issue. Unlike mature neurotrophins, proneurotrophins have a high affinity for p75NTR and a low affinity to Trks. The death effect of proNGF and proBDNF in different systems further suggests the potential potent function of proneurotrophins.

The availability of both Trk and p75NTR signaling in BF neurons allows us to address the possible crosstalk between survival and death signaling pathways activated by mature and proneurotrophins, respectively. Previous studies have demonstrated that activated Trk receptors protect neurons from apoptotic signaling mediated by p75NTR in sympathetic neurons (Teng et al., 2005). However, our data indicates that proNGF can cause the death of BF neurons even in the presence of an activated Trk receptor. To further investigate the interaction between survival and death signaling pathways, BF neurons were exposed to both BDNF and proNGF to determine if there is competition between the signaling pathways activated by these ligands, and if so, which pathway would gain preference. Interestingly, proNGF inhibited the phosphorylation of Akt and Erk, inducing apoptosis, while BDNF could not block the cleavage of caspase 3 and 6 induced by proNGF. These results suggest that proneurotrophins could cause the death of BF neurons by both activating death signaling and blocking survival signaling. Since the phosphorylation of Trk receptors was not blocked by proNGF, the interaction point between survival and death signaling pathways may exist between Trk and Akt and Erk. Moreover, when BF neurons were treated with both BDNF and proNGF and then immunostained with antibodies to cleaved-caspase 3

and p-AKT or p-Erk, it showed two distinct populations: healthy neurons expressed p-Akt and/or p-Erk staining, and apoptotic cells expressed cleaved-caspase 3 staining. There was no overlap between these two populations, suggesting that proNGF may block survival signaling to cause BF neuronal death. However, once Akt and/or Erk were activated, the cells were protected from death.

Akt and Erk belong to the PI3 kinase and MAP kinase pathway, respectively. We sought to determine the effects of constitutively activating these pathways without neurotrophin binding. To address this question, we infected BF neurons with adenovirus expressing either GFP or constitutively activated ras, which could activate both PI3 kinase and MAP kinase. In infected cultures, proNGF could not induce cell death. Conversely, when inhibitors to either PI3 kinase (LY294002) or MAP kinase (PD98059) were used to treat BF neurons before proNGF treatment, we found that inhibition of PI3 kinase significantly increased the effect of proNGF, suggesting Akt plays a more prominent role in protecting BF neurons from the death.

PTEN and proneurotrophin-induced death signaling

One of the important inhibitory mechanisms of the Akt pathway is mediated by PTEN, a dual-specificity phosphatase. PTEN decreases the amount of PIP3 necessary for the activation of Akt, blocking the PI3K-Akt pathway and facilitating cell death (Stambolic et al., 1998). Because PTEN antagonizes Akt activity, and because Akt phosphorylation is significantly decreased by proNGF, we hypothesized that proNGF activity could decrease Akt phosphorylation via PTEN activation.

Because no data has shown that PTEN can be regulated by proneurotrophins, we determined if this regulation existed. In our system,

PTEN was increased by proNGF as early as 30 min, suggesting proNGF could regulate PTEN. Moreover, same receptor complex was required for PTEN up-regulation and proneurotrophin-induced neuronal loss. The presence of BDNF could not block this induction, which was consistent with the observation that proneurotrophin signaling dominates Trk signaling in BF neurons. As expected, both a pharmacological inhibitor and siRNA to PTEN significantly decreased death caused by proNGF in the presence of BDNF. Taken together, this demonstrates that proNGF might signal via PTEN to inhibit Trk-mediated survival signaling.

The interaction between survival and death signaling pathways determines if a neuron dies or survives. In proneurotrophin-induced death, inhibiting Akt activity lets the apoptotic signaling reach downstream levels and cause cell death. Our data indicates that PTEN performs this inhibitory effect upon survival signaling as part of the proneurotrophin-induced cell death.

The information about the regulation of PTEN activity is limited. One important mechanism is through phosphorylation. The phosphorylation on certain residues make the protein unstable and less active (Maccario et al., 2007). BDNF can increase the phospho-form of PTEN implies that mature neurotrophins might also be part of the PTEN regulation. By switching PTEN from its active to non-active form, mature neurotrophins can decrease PTEN activity, which is consistent with the survival effects of mature neurotrophins. Additionally, proNGF treatment can block this PTEN phosphorylation. Mature and proneurotrophins have opposite effects on PTEN protein, suggesting that PTEN plays a role in the interaction between the survival and death signaling pathways.

PTEN activity and proNGF-induced cell loss after seizure

The function of PTEN under physiological conditions has been studied in different animal models. PTEN is critical for apoptosis in vivo (Stambolic et al., 1998). Our lab has used pilocarpine-induced seizures as a model to study the proneurotrophin-induced neuronal loss in vivo. ProNGF was show to be increased in astrocytes after seizure in hippocampus (Volosin et al., 2006). Infusing an antibody to proNGF into the hippocampus prevented the neuronal loss in p75NTR positive cell population (Volosin et al., 2008), suggesting that the proNGF-p75NTR system is at least partially responsible for neuronal loss after seizure. These data support the theory that proneurotrophins are important for cell death in the nervous system after seizures.

Because our in vitro data suggested that PTEN is part of proNGF-induced death signaling, it was important to test if this is also happening in vivo. However, in our animal model, we tested the hippocampus, not basal forebrain. In order to confirm the role of PTEN in HPC neurons, cultured hippocampal neurons were used to test proNGF-PTEN regulation. Up-regulation of PTEN and blockage of Akt activation were observed in hippocampal neurons, suggesting that PTEN can be regulated by proNGF in both hippocampus and basal forebrain. By infusing PTEN inhibitor (bpv(pic)) into the hippocampus after seizure, neuronal loss was significantly reduced, indicating that PTEN also plays an important role in proNGF-induced death after seizure.

Our in vivo data indicates that PTEN plays an important role in the interaction of survival signaling and death signaling. It has been shown that PTEN is expressed widely in nervous system. Further data support that proneurotrophins are important for apoptosis during development and under the pathological conditions. However, the cellular mechanism of

proneurotrophin-induced death is not fully addressed. The fact that PTEN inhibitor can rescue hippocampal neurons after seizure strongly supports that PTEN is one of the downstream factors of proNGF-induced apoptotic signaling in the body.

VI. Conclusion

After nervous system injury, p75NTR expression levels can increase, and this increase is correlated with the induction of apoptosis (Bagum et al., 2001). Data from different labs support that p75NTR can mediate cell death after injury (Ernfors et al., 1989; Armstrong et al., 1991; Kokaia et al., 1998; Bagum et al., 2001). However, the identity of the ligand that induces this signaling pathway has been a key question. Studies on proneurotrophins have shed a light on this issue. In both in vitro and in vivo systems, it has been shown that proneurotrophins could induce the cell death through the p75-sortilin receptor complex (Lee et al., 2001; Nykjaer et al., 2004; Teng et al., 2005; Volosin et al., 2006; Volosin et al., 2008).

Since the identification of the apoptotic function of proneurotrophins, the study of interaction of survival and death signaling pathways has been expanded. Previously, the interaction was focused on the dual-role of p75NTR in both survival and death signaling pathways. Introducing proneurotrophins into this system makes it more complete. Two systems: proneurotrophins-p75NTR and mature neurotrophins-Trks, can be expressed and function in the same cell and at the same time. The interaction between these two systems can occur at different levels. As more information of proneurotrophin-induced signaling is obtained, the crosstalk between two systems becomes an important issue. It is likely the neurotrophin-induced effects are determined by

the final balance between two systems. There could be multiple levels of crosstalk between two signaling pathways and the final decision could vary depending on the cell types and condition of cells.

The work in our lab indicates that proNGF induces death of CNS neurons both by activating p75NTR-mediated apoptotic signaling and suppressing Trk-mediated survival signaling. The phosphatase PTEN plays a key role in suppressing the PI3K survival pathway (Fig. 26). In addition to the interaction of Akt and PTEN, there may be more interaction points. Studying these signaling pathway interactions will help to fully address the mechanisms of proneurotrophin-induced cell death. Since proneurotrophins and p75NTR-sortilin receptor complex have been shown to induce cell death under pathological conditions, the further understanding of the relationship between proneurotrophin and mature neurotrophin-induced cellular effects at molecular level could facilitate the development of potential treatments of neuronal diseases such as Alzheimer’s disease.

VII. Figures and Legends:

Figure 9. **The Trk receptors and p75NTR expression are highly overlapped in the basal forebrain in vivo.** The basal forebrain brain sections from adult rat were stained with TrkA, p75NTR (A), TrkB, p75NTR (B), or TrkC and p75NTR (C). Note that there is co-localization between all three Trk receptors and p75NTR.

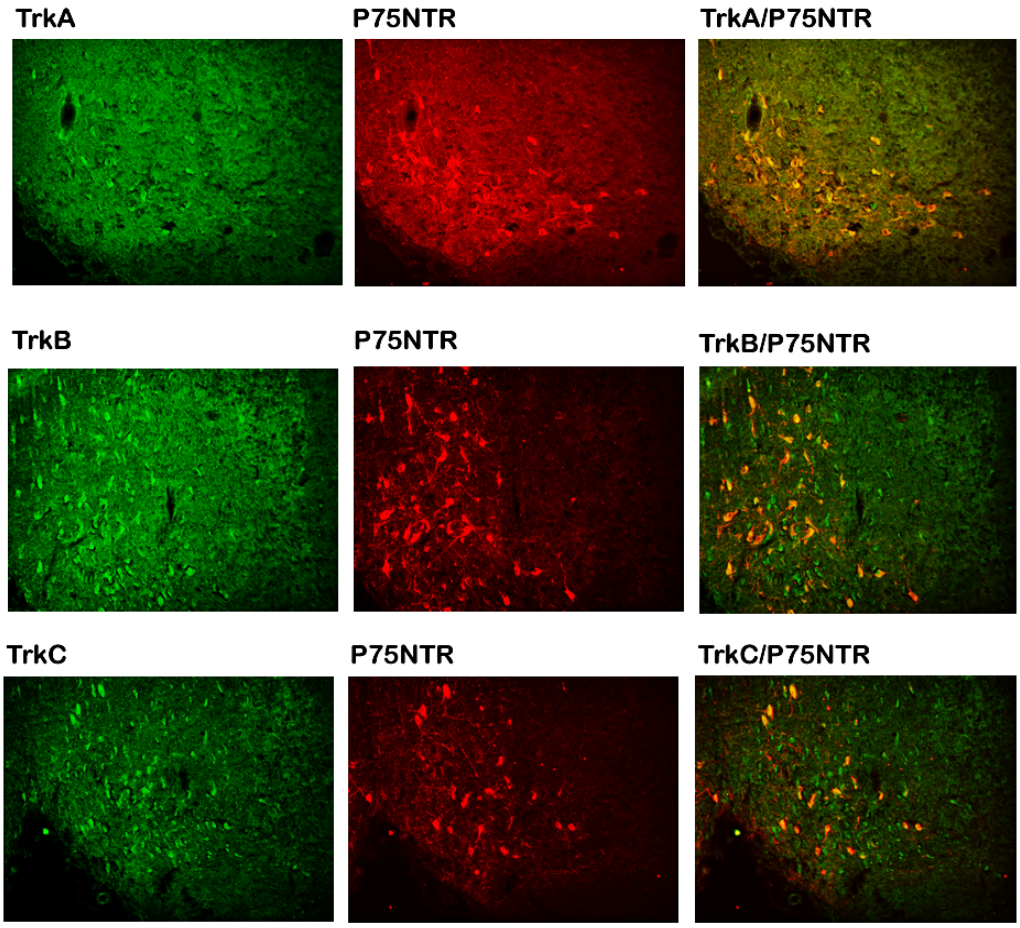

TrkA
P75NTR
TrkA/P75NTR
TrkB
P75NTR
TrkB/P75NTR
TrkC
P75NTR
TrkC/P75NTR

Figure 10. **ProNGF elicits death of basal forebrain neurons via p75NTR.** E16 BF neurons were cultured for 5 days before being treated with either vehicle (imidazole), mature NGF (100ng/ml), proNGF (1ng/ml), or proNGF (1ng/ml) with blocking antibodies to either NGF (Sigma) or p75NTR (9651) or the antibodies alone. The asterisk indicates significant difference from control ($p<0.05$).

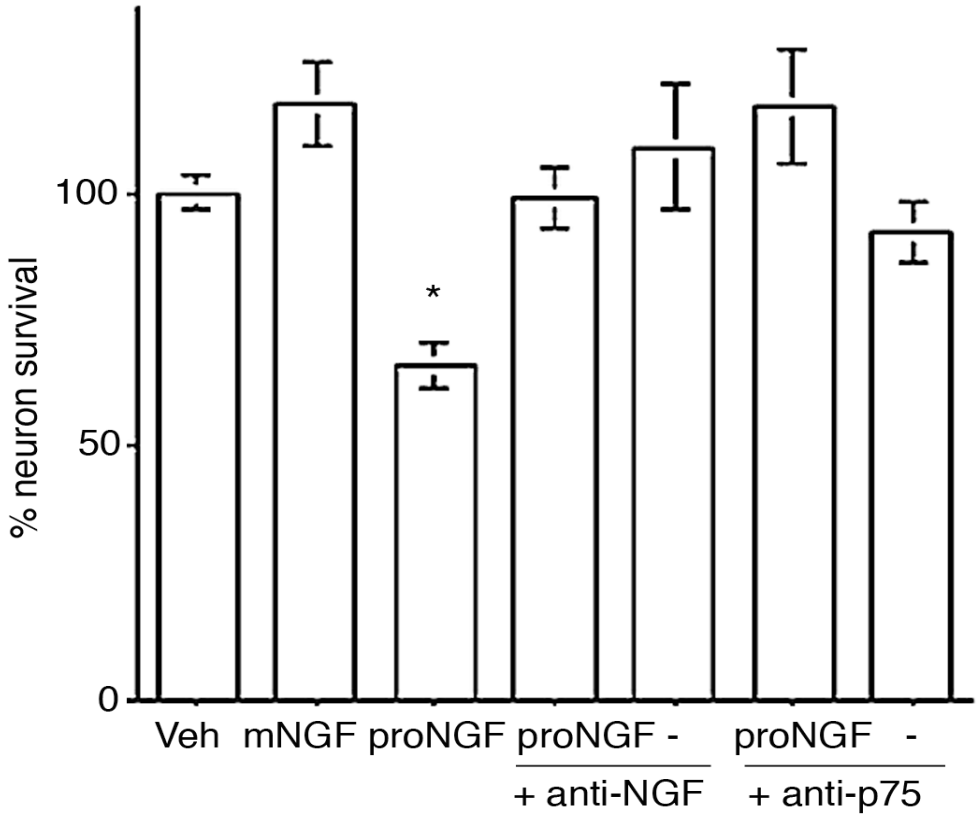

% neuron survival
100
50
0
*
Veh
mNGF
proNGF
proNGF -
+ anti-NGF
proNGF -
+ anti-p75

Figure 11. **Sortilin is necessary for death induced by proNGF.** E16 BF neurons were maintained for 5 days in culture in the presence of BDNF (10ng/ml).

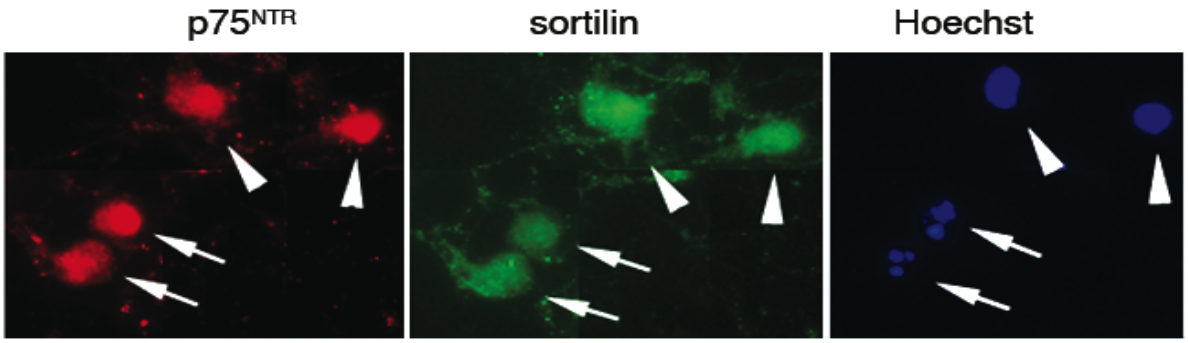
A. Cells were treated with proNGF (1ng/ml) for 30 min, then fixed and immunostained for p75NTR, sortilin and labeled with Hoechst. The antibodies were diluted 1:500 in PBS.

B. Cultured E16 BF neurons were treated overnight with proNGF (1ng/ml) or proBDNF (1ng/ml) in the presence or absence of blocking antibodies to p75NTR or sortilin. Cells were lysed and nuclei were counted.

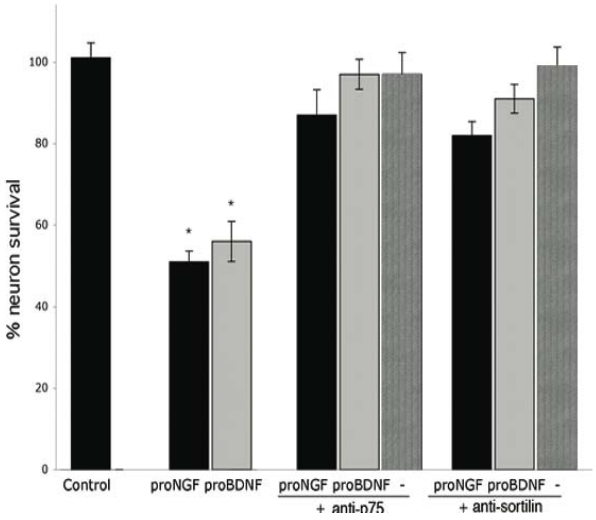
C. Cultured BF neurons were treated overnight with proNGF (1ng/ml) or proBDNF (1ng/ml) in the presence or absence of 4 or 40μM neurotensin. Cells were lysed and nuclei were counted to determine the survival.

(The arrows in A indicate p75/sortilin positive apoptotic neurons, and the arrowheads indicate p75/sortilin positive healthy neurons. The asterisks in B and C indicate significant difference from all other treatments, $p<0.05$).

A.


p75NTR
sortilin
Hoechst

B.


% neuron survival
100
80
60
40
20
0
Control
proNGF proBDNF
proNGF proBDNF -
+ anti-p75
proNGF proBDNF -
+ anti-sortilin

C.

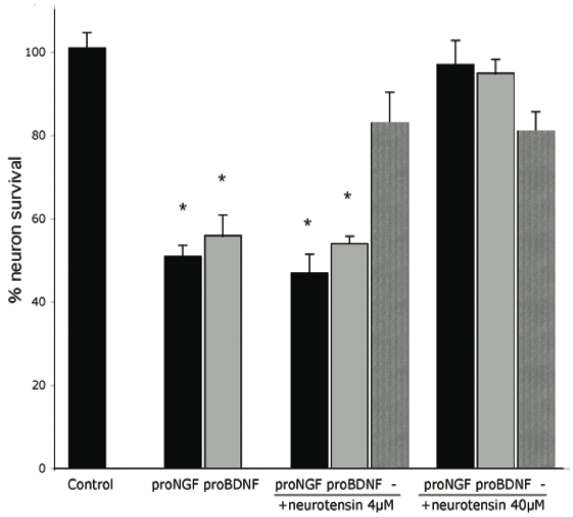

% neuron survival
100
80
60
40
20
0
Control
proNGF proBDNF
proNGF proBDNF -
+neurotensin 4μM
proNGF proBDNF -
+neurotensin 40μM

Figure 12. **ProNGF induces death in BF neurons in the presence of BDNF.** E16 BF neurons were cultured for 5 days in the presence of BDNF (10ng/ml) before being treated overnight with either mature NGF or proNGF alone or in the presence of blocking antibodies to NGF or p75NTR. The number of apoptotic cells was counted and indicated as percentage of control. The asterisk indicates the significant difference from all other treatments ($p<0.05$).

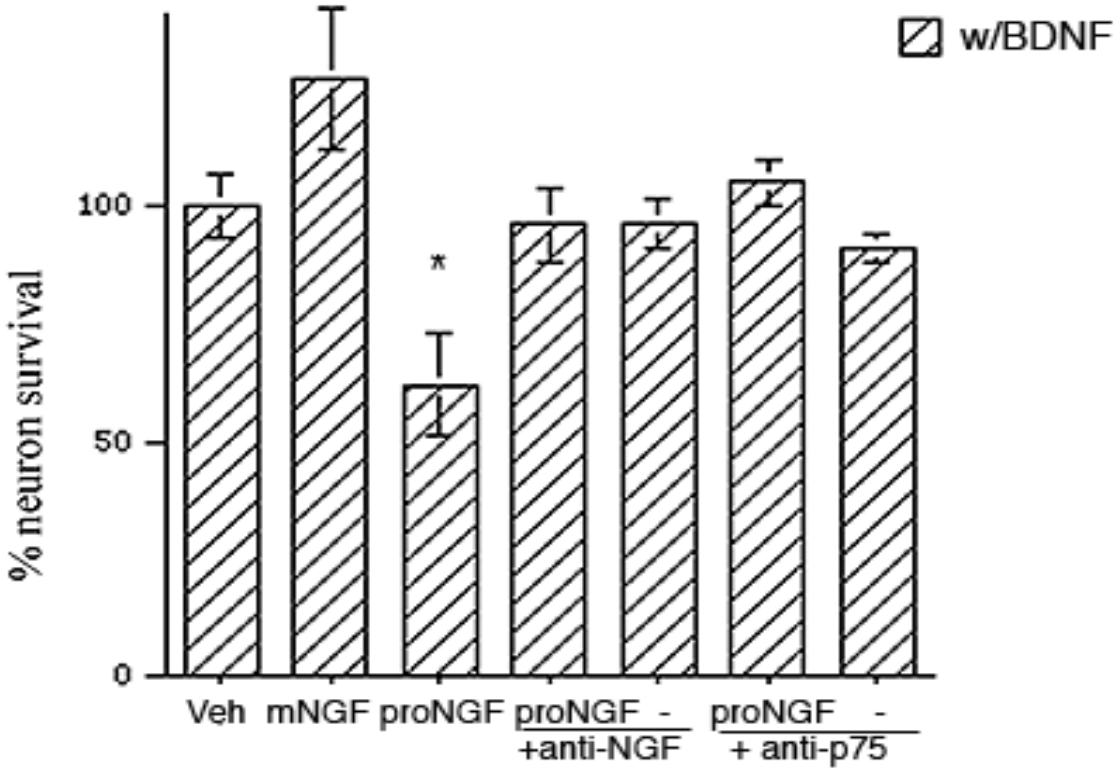

w/BDNF
% neuron survival
100
50
0
*
Veh
mNGF
proNGF
proNGF -
+anti-NGF
proNGF -
+ anti-p75

Figure 13. **ProNGF induces neuronal death in neurons with phosphorylated Trk receptor.** E16 BF neurons were cultured on slide wells for 5 days in the presence of BDNF (10ng/ml) and treated with BDNF and proNGF. Neurons were double stained for (**a**) full length TrkB ($TrkB_{in}$) or (**b**) p-Trk and p75NTR. The nuclei were stained with Hoechst to determine apoptotic cells.

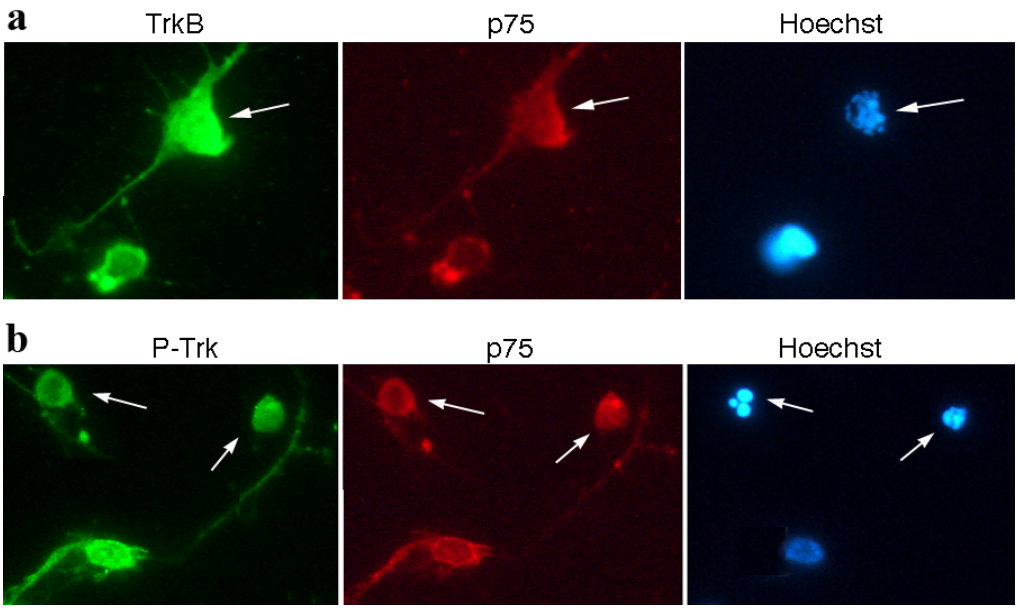

a
TrkB
p75
Hoechst
b
P-Trk
p75
Hoechst

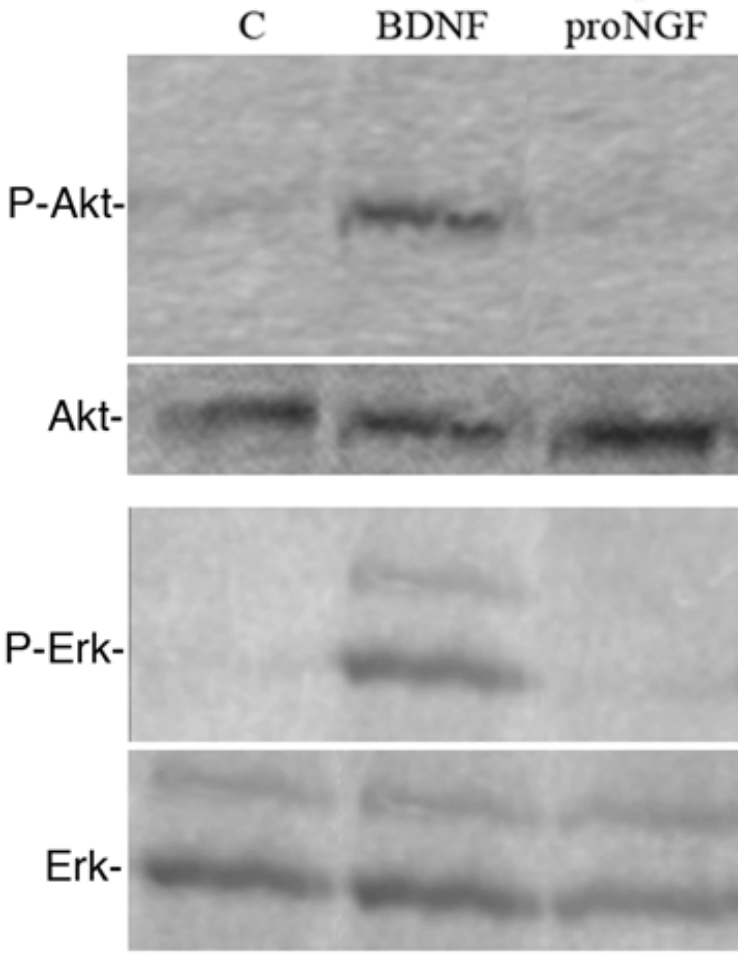
Figure 14. **BDNF and proNGF induce distinct signaling pathways in BF neurons.**

A. E16 BF neurons were cultured for 5 days before treatment with BDNF or proNGF for 30 min. Cells were harvested for western blot analysis. P-Akt and p-Erk were detected. Blots were stripped and reprobed for Akt or Erk.

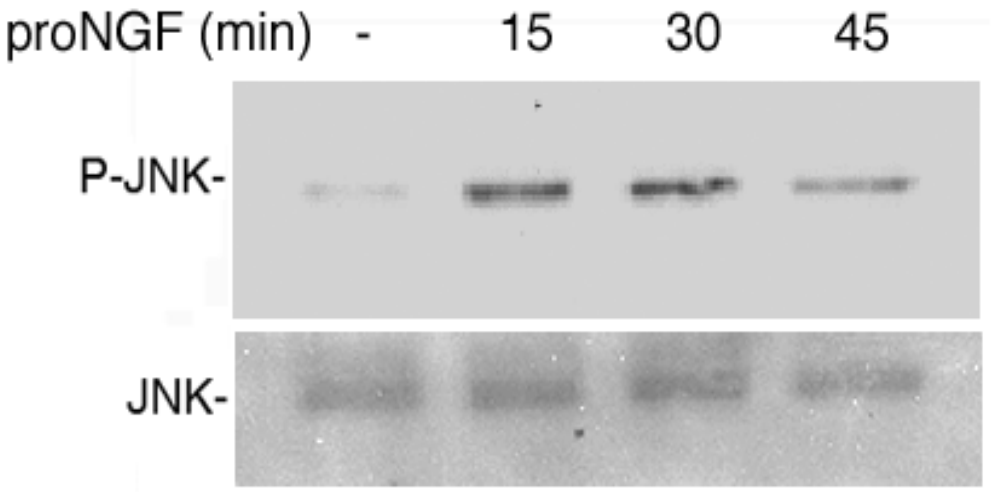
B. Cultured BF neurons were treated with proNGF at different time points as indicated. Cells were lysed for western blot and probed for p-JNK. The blot was stripped and reprobed for total JNK.

C. Cultured BF neurons were treated with vehicle, mature NGF and proNGF for 4 hours. Lysed samples were used for western blot analysis and probed for cleaved caspase 3 and 6. The blot was stripped and reprobed for tubulin.

A.



B.



C.

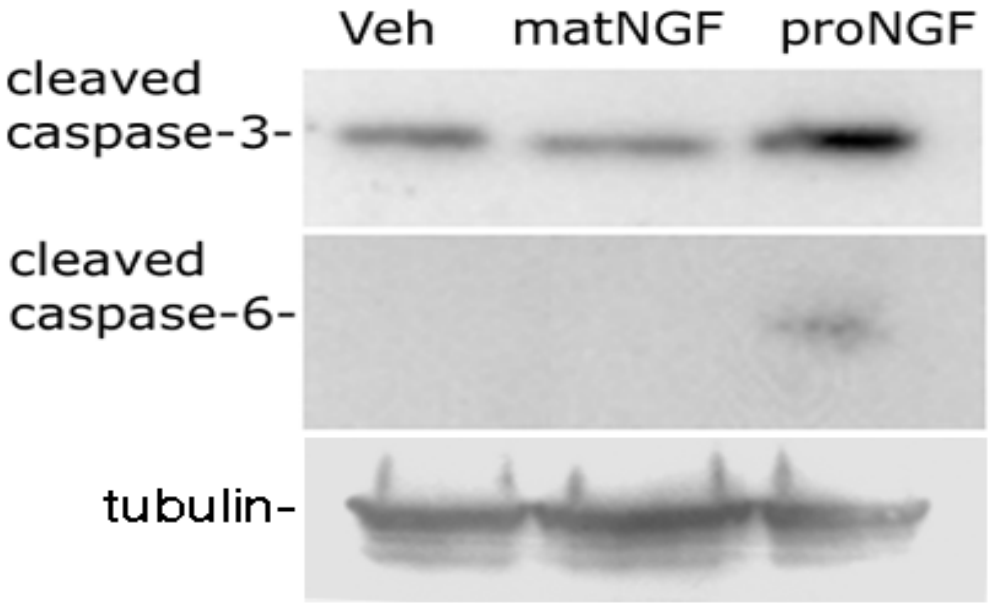

Veh
matNGF
proNGF
cleaved
caspase-3-
cleaved
caspase-6-
tubulin-

Figure 15. **Proneurotrophins induce the activation of caspase 3 in the presence of BDNF.** E16 neurons were cultured for 5 days in the presence of BDNF. Cells were then treated with BDNF, proNGF or BDNF plus proNGF for 4 hours. Cleaved caspase 3 was detected by western blot analysis. The blot was stripped and reprobed for tubulin.

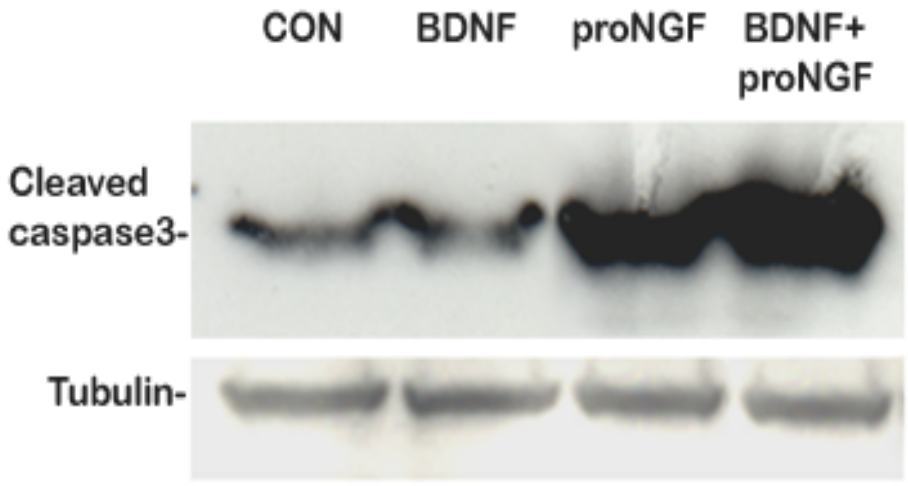

CON
BDNF
proNGF
BDNF+
proNGF
Cleaved
caspase3-
Tubulin-

Figure 16. **ProNGF causes apoptosis of BF neurons expressing p-Trk but not expressing p-Akt or p-Erk in the presence of BDNF.** E16 BF neurons were cultured with BDNF for 5 days. BDNF was added again to activate TrkB signaling 15 min prior to treatment with proNGF. Cultures were labeled with Hoechst to identify apoptotic neurons and double-immunostained for:

Top: P-Trk and P-Akt; Trk receptor can be activated in both healthy and apoptotic cells. P-Akt is only activated in healthy cells.
Middle: P-Akt and P-Erk; both p-Akt and p-Erk can be induced only in healthy cells.
Bottom: Cleaved caspase-3 and p-Akt; Cleaved caspase 3 can only be seen in apoptotic cells, p-Akt is only in healthy cells.

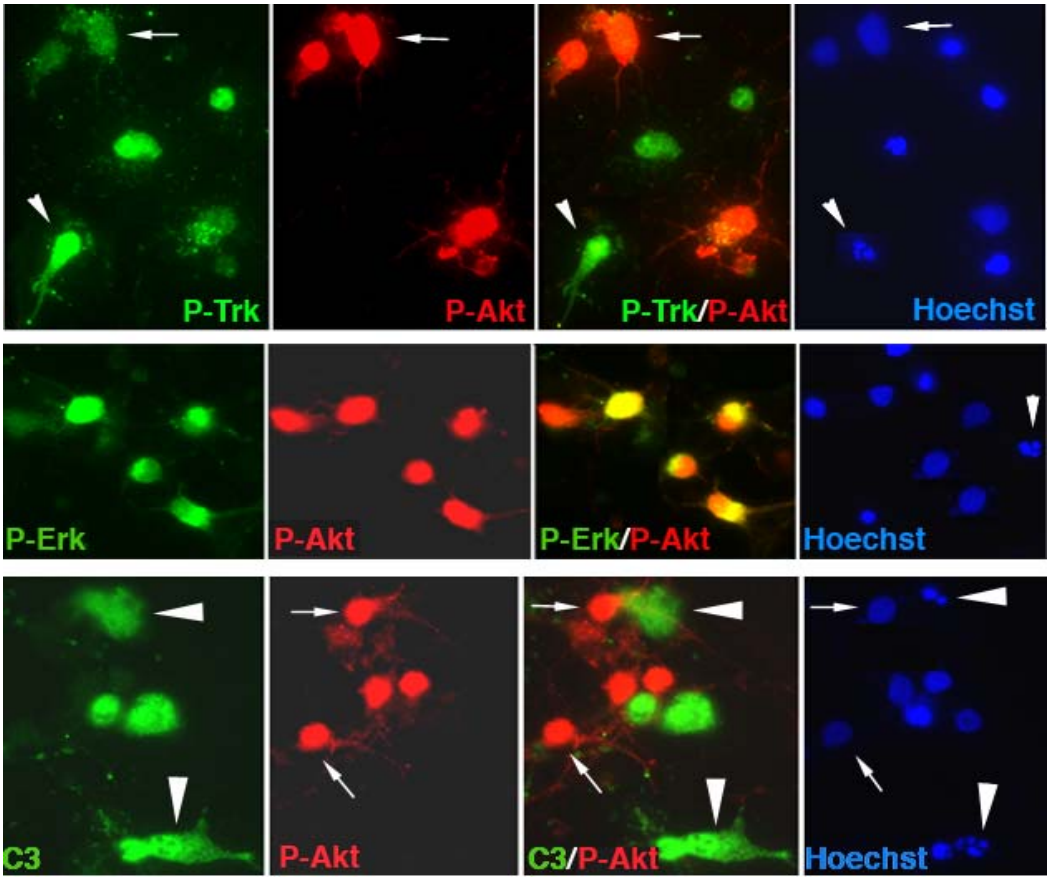
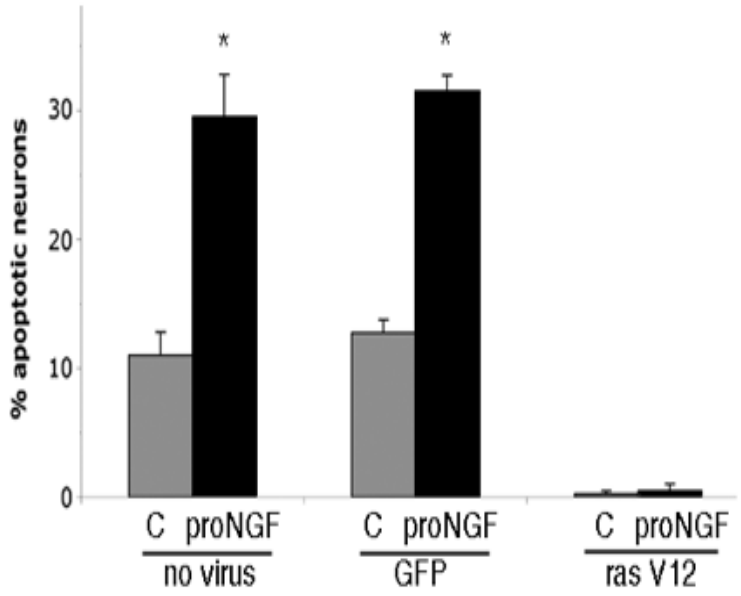

P-Trk
P-Akt
P-Trk/P-Akt
Hoechst
P-Erk
P-Akt
P-Erk/P-Akt
Hoechst
C3
P-Akt
C3/P-Akt
Hoechst

Figure 17. **Activation of PI3 kinase and MAP/Erk kinase pathways protects BF neurons from death caused by proNGF.** Cultured BF neurons were infected with 50 MOI of adenovirus expressing either GFP or myc-tagged constitutively activated ras (ras V12) for 36 hours. ProNGF was used to treat both uninfected and infected neurons for 5 hours. Cells were labeled with Hoechst to identify apoptotic nuclei. (A) The number of apoptotic cells was counted and expressed as the percentage of total cells. The asterisk indicates the significant difference from all other groups ($p<0.05$). (B) Cultured cells were immunostained for p-Akt and myc. The cells were also labeled with Hoechst to identify the apoptotic nuclei.

A.


% apoptotic neurons
30
20
10
0
*
*
C proNGF
no virus
C proNGF
GFP
C proNGF
ras V12

B.

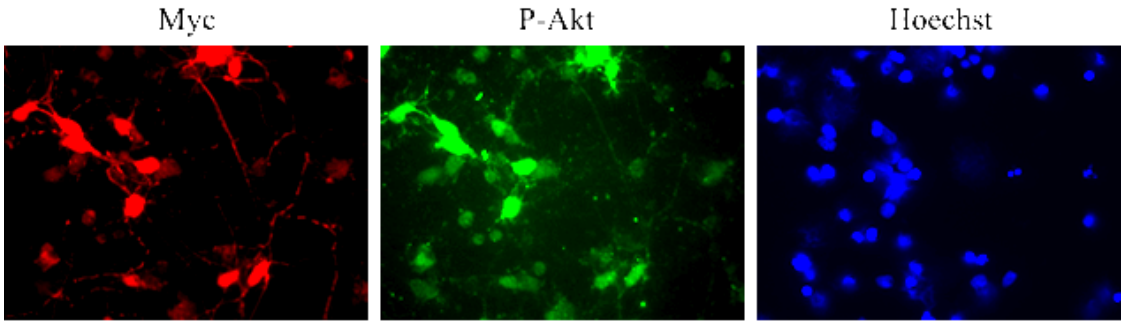

Myc
P-Akt
Hoechst

Figure 18. **Akt is more crucial for the protection of BF neurons from proNGF-induced death than Erk.** Cultured BF neurons were treated overnight with PI3 inhibitor LY294002 (LY) (50μM) or Mek1 inhibitor PD98059 (PD) (10μM) alone or with proNGF (1ng/ml). The number of apoptotic neurons was counted and indicated as the percentage of total cells. The asterisk indicates the significant difference from control ($p<0.05$). The double asterisk indicates the significant difference from treatment with proNGF alone ($p<0.05$).

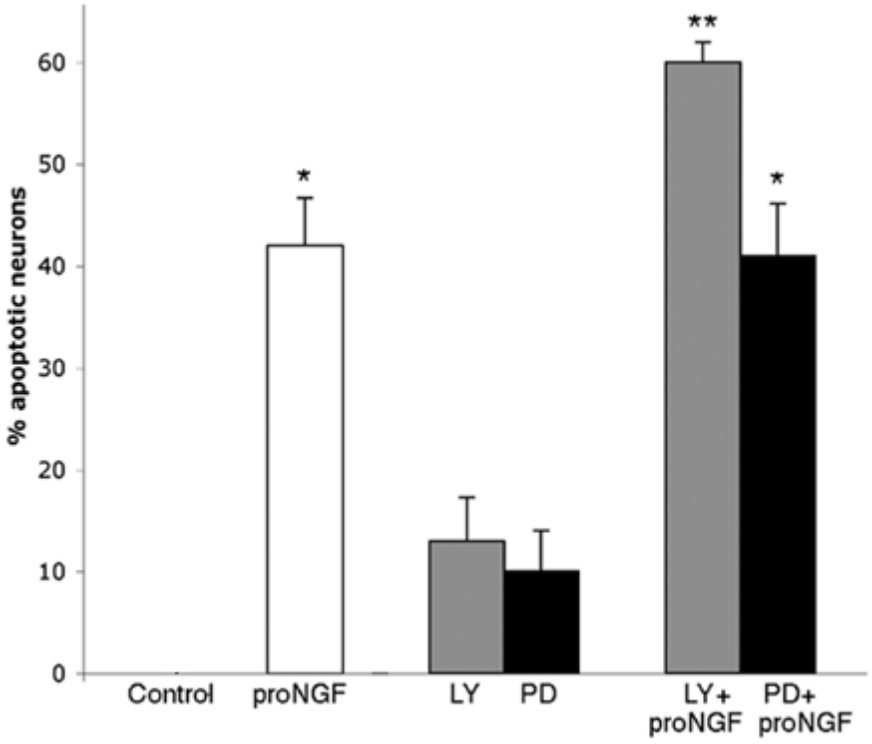
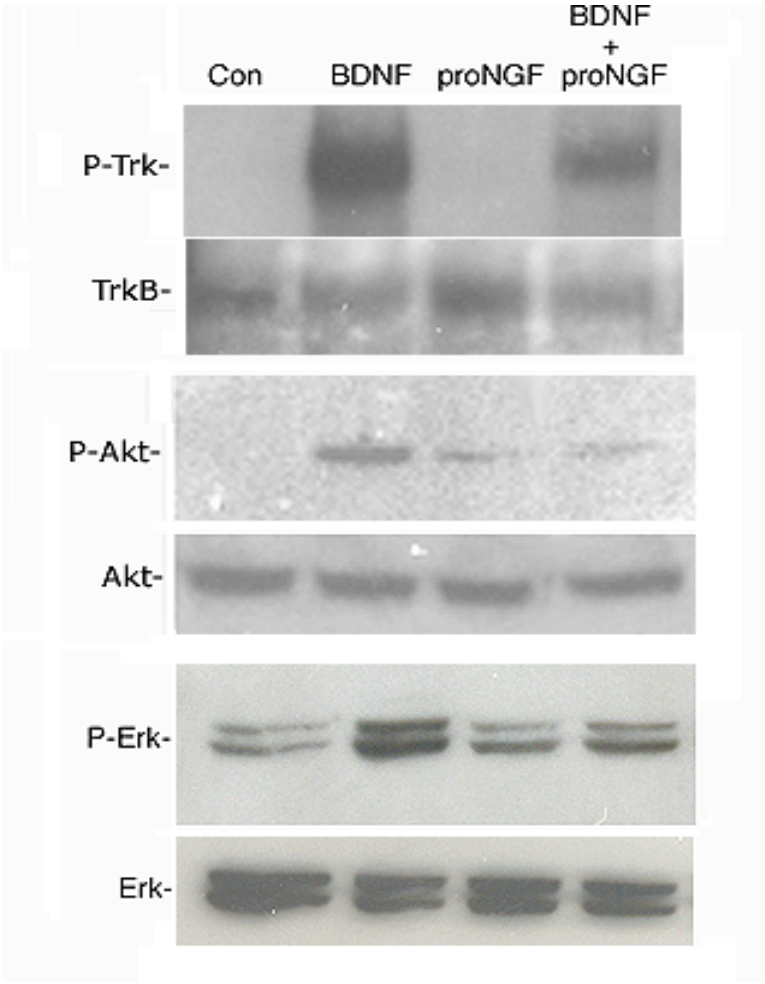

% apoptotic neurons
0
10
20
30
40
50
60
*
**
*
Control
proNGF
LY
PD
LY+ proNGF
PD+ proNGF

Figure 19. **ProNGF inhibits the phosphorylation of Akt and Erk, but not Trk**.

A. E16 BF neurons were grown in culture for 5 days, and treated with BDNF, proNGF or both. Cells were lysed and analyzed by Western blot for activation of different signaling proteins: p-Trk (top), p-Akt (middle) and p-Erk (bottom). Blots were stripped and reprobed for total TrkB, Akt or Erk.

B. The amount of p-Trk, p-Akt and p-Erk relative to total TrkB, Akt and Erk, respectively was quantified. Data came from three independent experiments. The asterisks represent the significance difference from control ($p<0.05$).

A.


Con
BDNF
proNGF
BDNF + proNGF
P-Trk-
TrkB-
P-Akt-
Akt-
P-Erk-
Erk-

B.

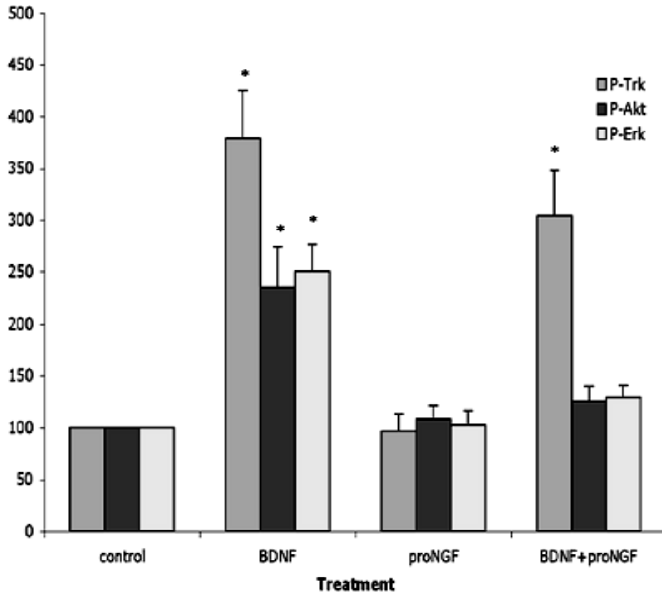
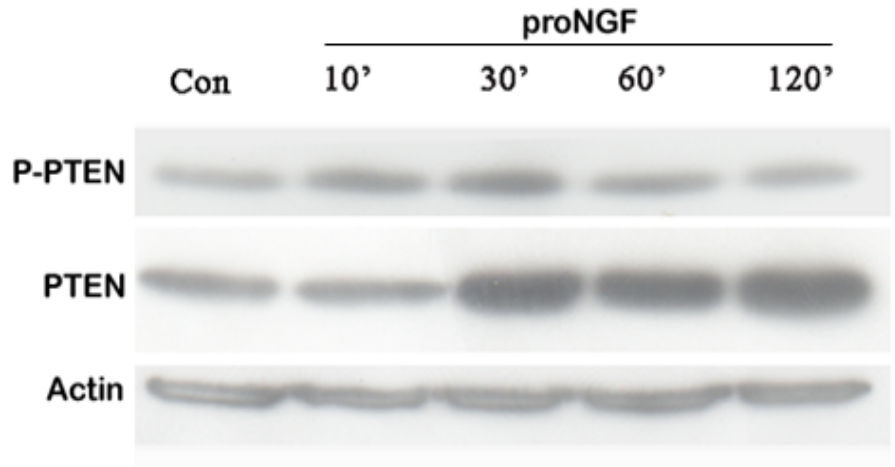

500
450
400
350
300
250
200
150
100
50
0
P-Trk
P-Akt
P-Erk
control
BDNF
proNGF
BDNF+proNGF
Treatment

Figure 20. **ProNGF increases the level of PTEN in BF neurons in the presence and absence of BDNF.**

A. E16 BF neurons were cultured for 5 days and then treated with proNGF for different time points as indicated. Cells were lysed for western blot to probe for p-PTEN and PTEN. Blots were stripped and reprobed for actin.

B. Cultured BF neurons were treated with BDNF alone, proNGF alone, BDNF then proNGF or proNGF then BDNF for 30 minutes as indicated. Western blot analysis was conducted to probe for PTEN. The blots were then stripped and reprobed for total Erk.

A.


proNGF
Con
10'
30'
60'
120'
P-PTEN
PTEN
Actin

B

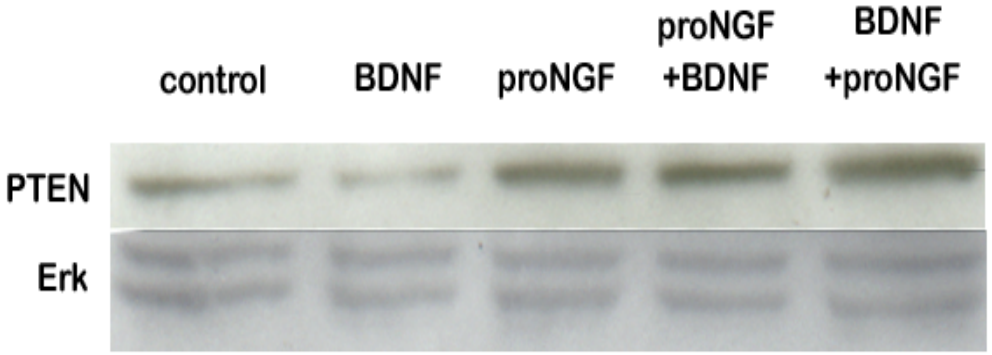
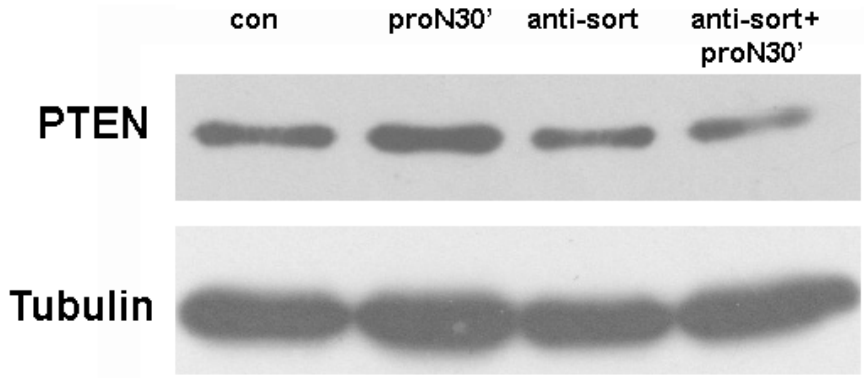

control
BDNF
proNGF
proNGF +BDNF
BDNF +proNGF
PTEN
Erk

Figure 21. **ProNGF induces PTEN via p75NTR-sortilin receptor complex.** BF neurons were treated with proNGF (1ng/ml, 30 minutes), anti-sortilin antibody (A, 60 minutes), anti-p75NTR antibody (B, 60 minutes) or one of the antibodies plus proNGF (60min+30min). The level of PTEN protein was measured by western blot analysis. The blots were stripped and reprobed for tubulin

A.


con
proN30'
anti-sort
anti-sort+
proN30'
PTEN
Tubulin

B.

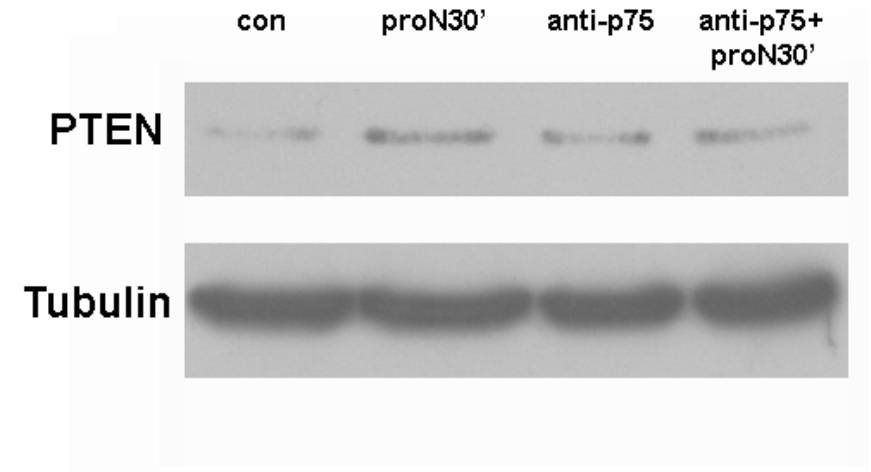
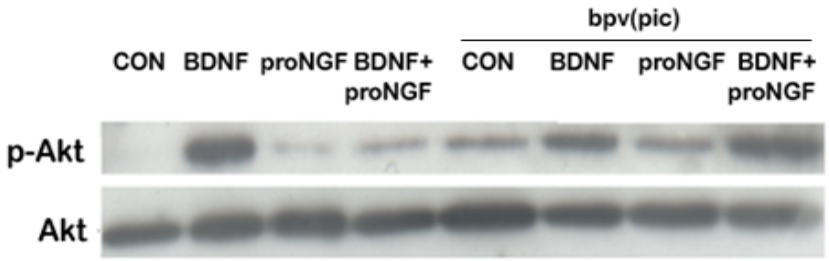

con
proN30'
anti-p75
anti-p75+
proN30'
PTEN
Tubulin

Figure 22. **PTEN inhibitor reverses the inhibition of Akt phosphorylation by proNGF and blocks the effect of proNGF in BF neurons in the presence of BDNF.**

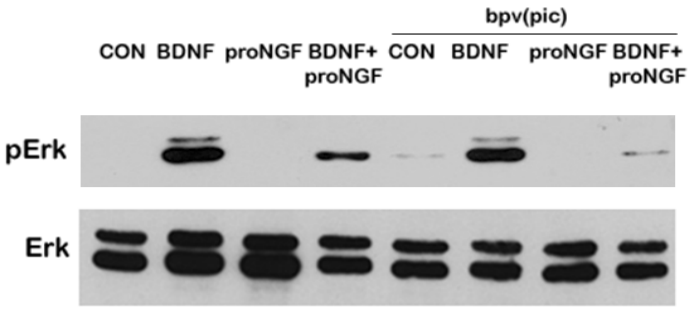
A. E16 BF neurons were cultured in the presence of BDNF for 5 days. BDNF was re-added prior to further treatment to assure that TrkB receptors were activated. The cells were then treated with BDNF (10ng/ml), proNGF (1ng/ml) or both in the presence or absence of PTEN inhibitor (bpv(pic), 5μm). Western blot analysis was performed to probe for p-Akt . Blots were then stripped and reprobed for total Akt.

B. Cultured E16 BF neurons were treated overnight with proNGF (1ng/ml) alone, proNGF plus bpv(pic) (5μm) in the presence or absence of BDNF (10ng/ml). A survival assay was performed to count the number of healthy neurons in the culture and indicated as the percentage of total cells. The asterisk indicates values different from control at $p<0.05$.

A.


bpv(pic)
CON
BDNF
proNGF
BDNF+ proNGF
CON
BDNF
proNGF
BDNF+ proNGF
p-Akt
Akt

B.


bpv(pic)
CON
BDNF
proNGF
BDNF+ proNGF
CON
BDNF
proNGF
BDNF+ proNGF
pErk
Erk

C.

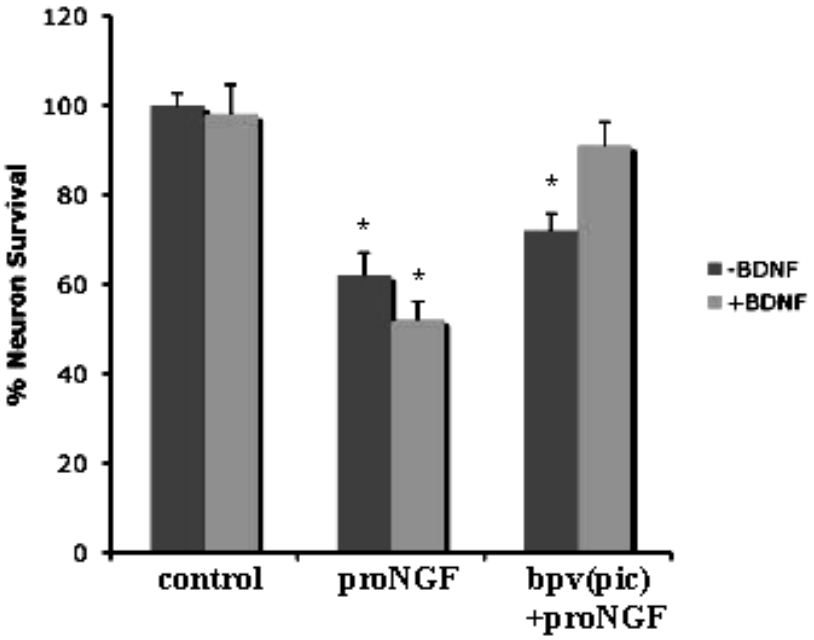
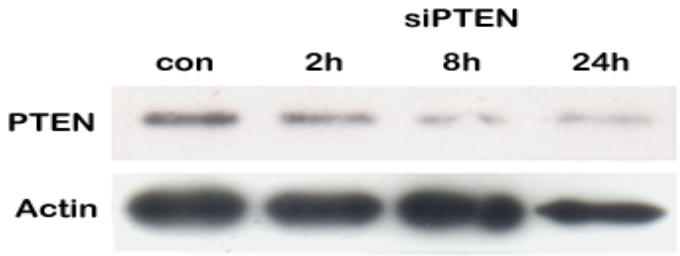

% Neuron Survival
120
100
80
60
40
20
0
*
*
*
control
proNGF
bpv(pic) +proNGF
-BDNF
+BDNF

Figure 23. **Small interfering RNA of PTEN can block the up-regulation of PTEN induced by proNGF and rescue BF neurons from proNGF-induced apoptosis in the presence of BDNF.**

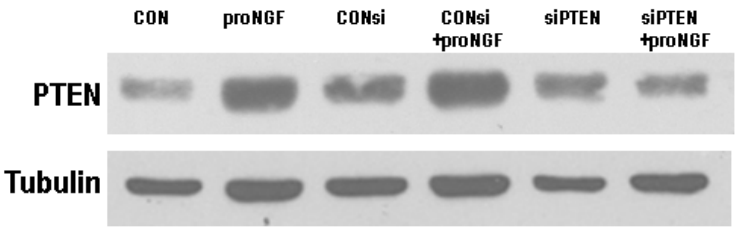
A. PTEN siRNA can inhibit the increase of PTEN induced by proNGF. BF neurons were pretreated with siRNA to PTEN for 30 min before treated with proNGF (1ng/ml) for 30 min. Western blot analysis was performed to detect PTEN protein level. The same treatments were conducted using scrambled siRNA as a control.

B, C. Cultured BF neurons were treated overnight with proNGF (1ng/ml) alone, control siRNA, siRNA to PTEN or siRNA combined with proNGF in the presence (B) or absence (C) of BDNF (10ng/ml). The number of healthy neurons was counted and indicated as the percentage of total cells. The asterisk indicates values different from control at $p<0.05$.

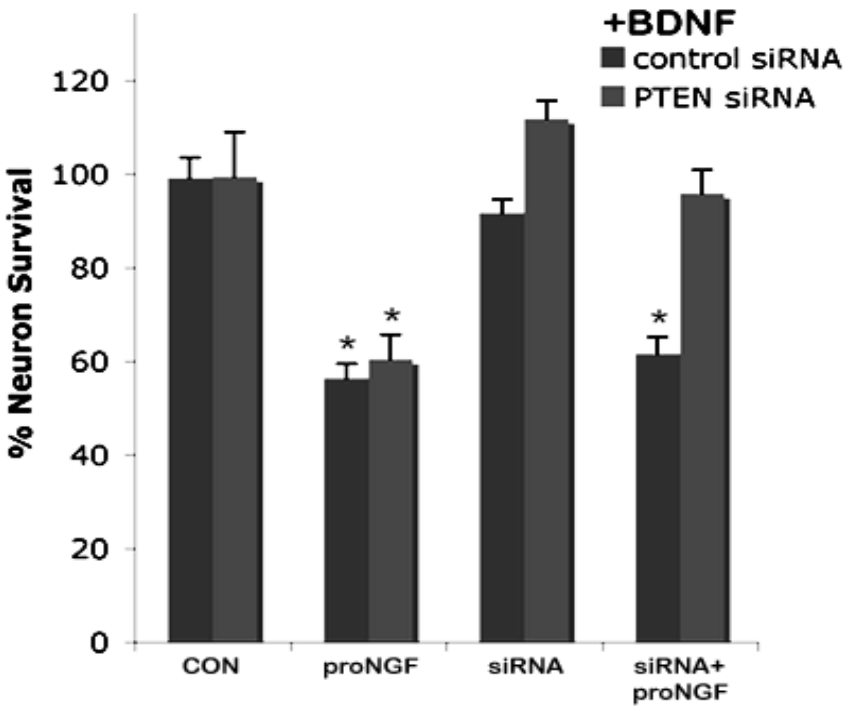
A.


siPTEN
con
2h
8h
24h
PTEN
Actin

B.


CON
proNGF
CONsi
CONsi +proNGF
siPTEN
siPTEN +proNGF
PTEN
Tubulin

C.


+BDNF
control siRNA
PTEN siRNA
% Neuron Survival
120
100
80
60
40
20
0
*
*
*
CON
proNGF
siRNA
siRNA+ proNGF

D.

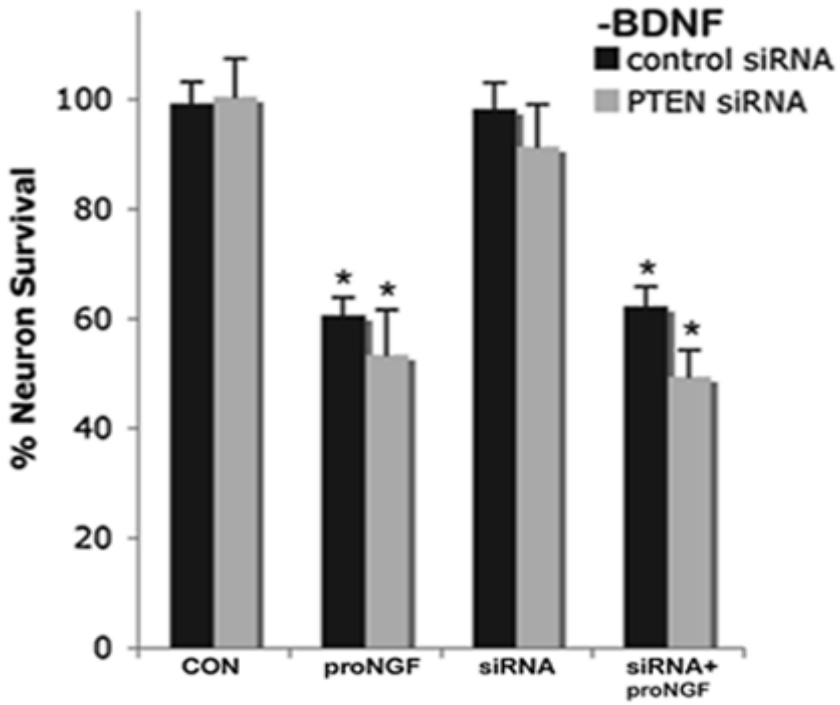
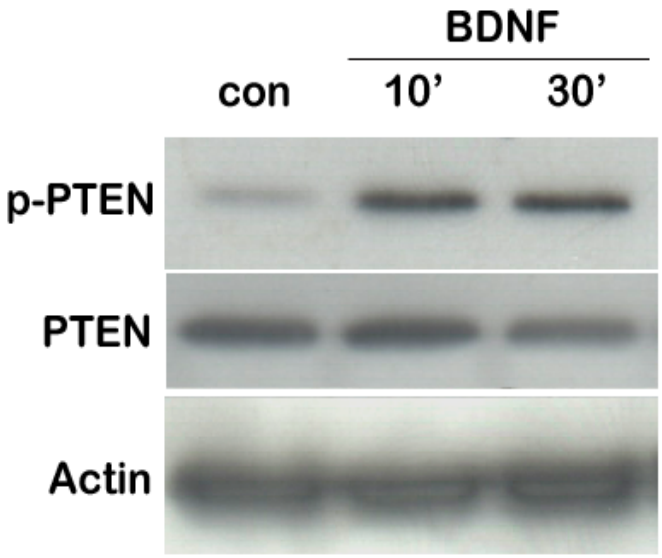

-BDNF
control siRNA
PTEN siRNA
% Neuron Survival
100
80
60
40
20
0
*
*
*
*
CON
proNGF
siRNA
siRNA+
proNGF

Figure 24. **BDNF increases the non-active form of PTEN protein in BF neurons.**

A. BDNF increases phosphorylated PTEN protein in BF neurons. E16 neurons were cultured for 5 days before treatment with BDNF (10ng/ml) at different time points as indicated. Cells were lysed for western blot analysis and probed for p-PTEN and PTEN. Blots was stripped and reprobed for actin. **B.** ProNGF inhibits BDNF-induced increase of p-PTEN. BF neurons were treated with BDNF for 10 and 30min in the presence or absence of proNGF. The level of p-PTEN and PTEN were measured using western blot analysis. The blot was stripped and reprobed for tubulin.

A.


BDNF
con
10’
30’
p-PTEN
PTEN
Actin

B.

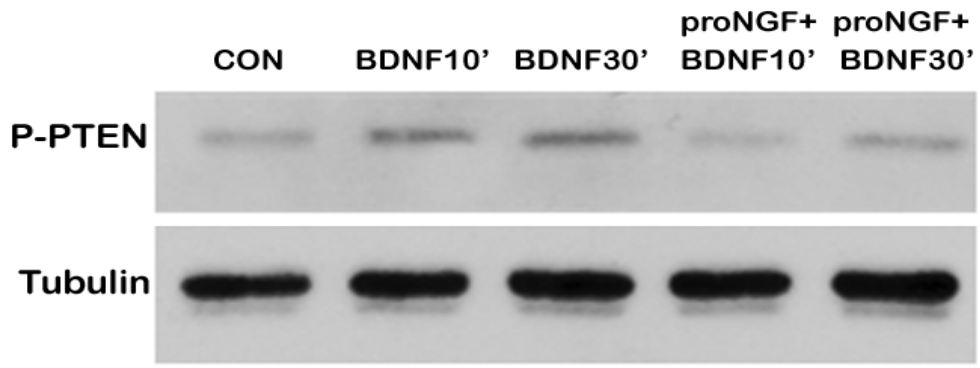
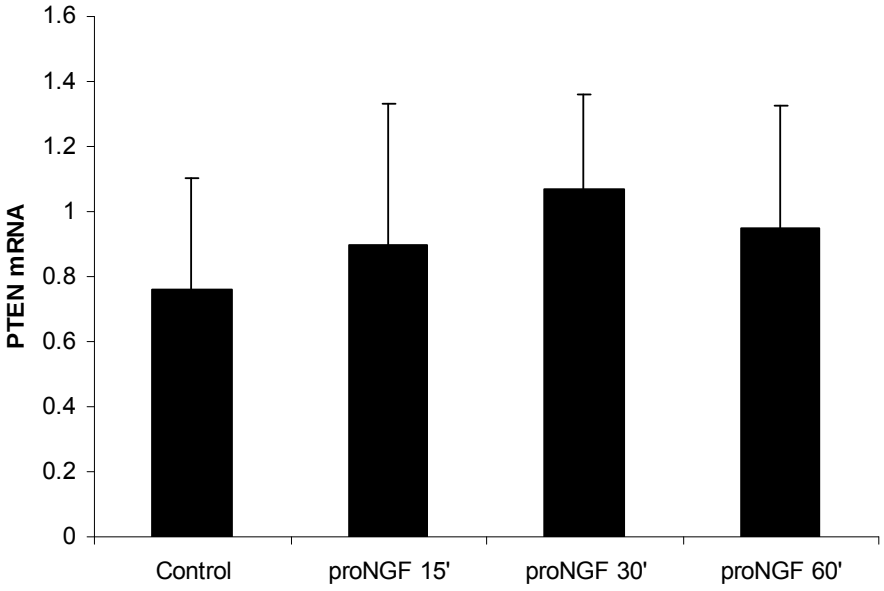

CON
BDNF10’
BDNF30’
proNGF+ BDNF10’
proNGF+ BDNF30’
P-PTEN
Tubulin

Figure 25. **New protein synthesis is required for PTEN up-regulation.**

A. BF neurons were treated with proNGF at different time points. The total RNA was extracted and qPCR was performed to detect the level of PTEN mRNA.

B. Cultured BF neurons were treated with proNGF (1ng/ml, 30min), cycloheximide (1μg/ml, 60min), actinomycin-D (60min) or both. Western blot analysis was performed to detect the level of PTEN. The blots were then stripped and reprobed for tubulin.

A.


PTEN mRNA
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
proNGF 15'
proNGF 30'
proNGF 60'

B.

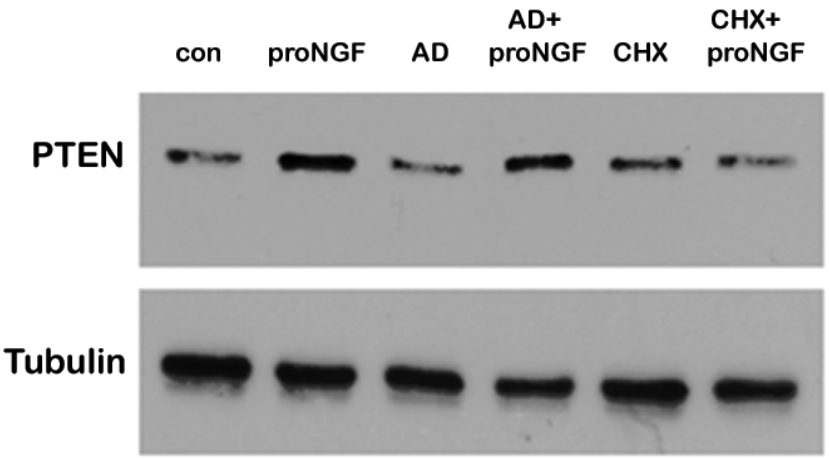
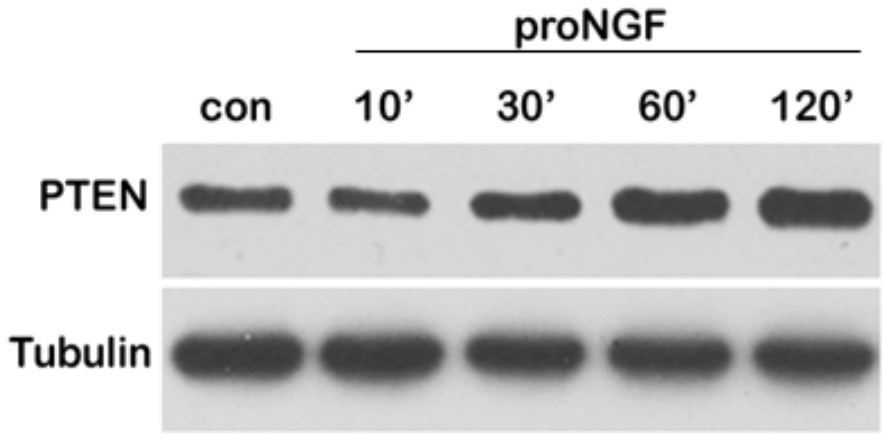

con
proNGF
AD
AD+ proNGF
CHX
CHX+ proNGF
PTEN
Tubulin

Figure 26. **ProNGF induces PTEN up-regulation to block Akt activation in cultured Hippocampal neurons**

A. E18 hippocampal neurons were cultured and treated with proNGF (1ng/ml) at different time points as indicated. Western blot analysis was performed to detect the level of PTEN protein. The blots were stripped and re-probed for tubulin.

B. E18 hippocampal cultured neurons were treated with BDNF (10ng/ml, 30min) then proNGF (1 ng/ml, 30min), or in reverse order at 5 min and 25 min, respectively. The level of p-Akt was measured by western blot analysis. The blot was stripped and re-probed for tubulin.

A.


proNGF
con
10'
30'
60'
120'
PTEN
Tubulin

B.

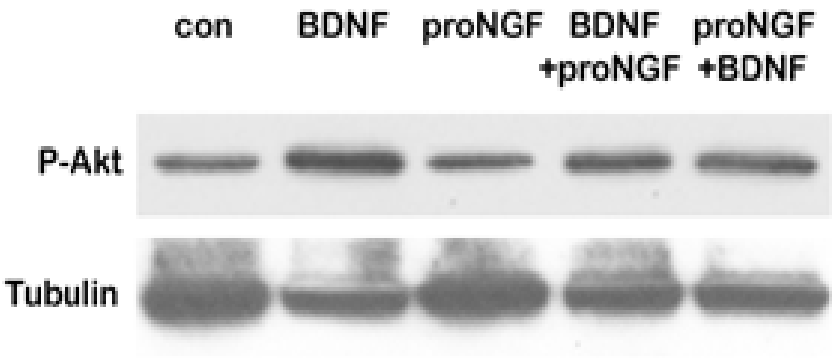
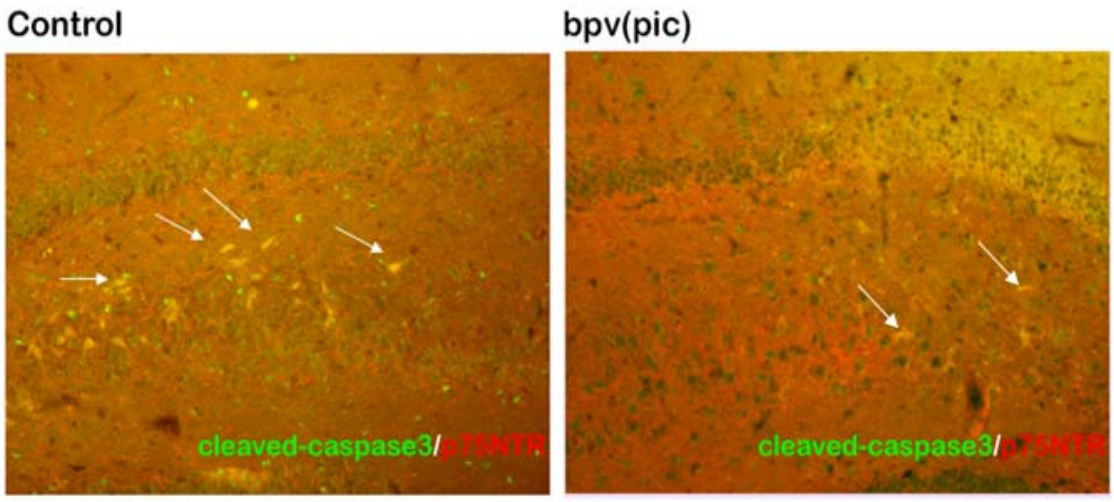

con
BDNF
proNGF
BDNF +proNGF
proNGF +BDNF
P-Akt
Tubulin

Figure 27. **PTEN inhibitor rescues neuronal loss after seizure in vivo.** Adult rats were cannulated bilaterally in the dorsal hippocampus 1 week before pilocarpine-induced seizure. After seizures, PTEN inhibitor (bpv(pic)) was infused into one side and saline into the contralateral side two times a day for three days..

A. Double immunostaining for p75NTR and cleaved caspase 3 was performed on hippocampal brain sections. There is a decrease in the number of cells expressing both p75NTR and cleaved caspase 3 on the side with the inhibitor relative to the control side in the same brain.

B. The number of cells expressing both p75NTR and cleaved caspase 3 was counted in both hippocampal hemispheres. The average number of cells coexpressing p75NTR and cleaved caspase 3 from three independent experiments was calculated. The asterisk indicates the value significantly different from control at $p<0.05$.

A.


Control
bpv(pic)
cleaved-caspase3/p75NTR
cleaved-caspase3/p75NTR

B.

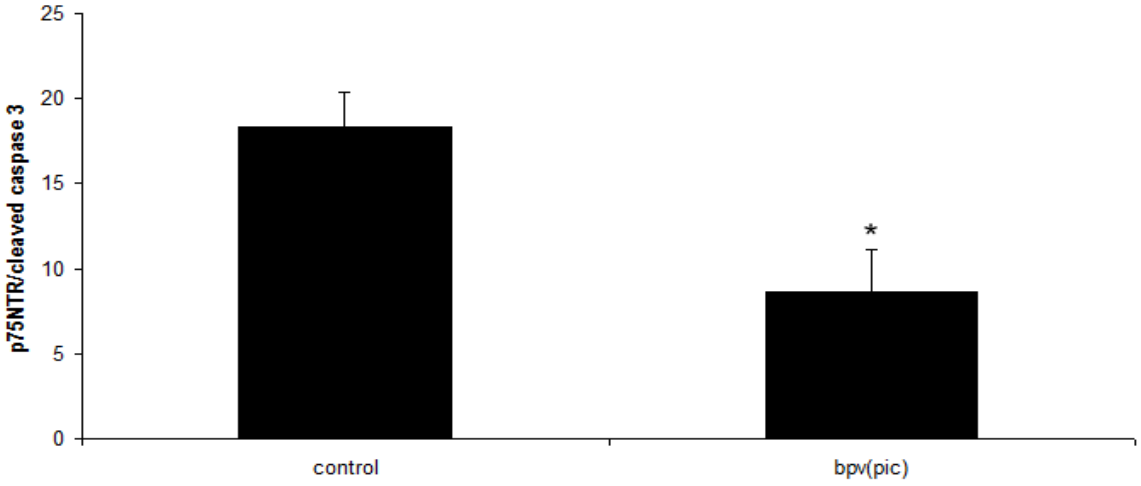

p75NTR/cleaved caspase 3
25
20
15
10
5
0
*
control
bpv(pic)

Fig 28. **Schematic of the interaction between the Trk-mediated and p75NTR-mediated signaling pathways**. PTEN can be up-regulated by proNGF via the p75NTR-sortilin receptor complex and subsequently block Trk-mediated survival signaling.

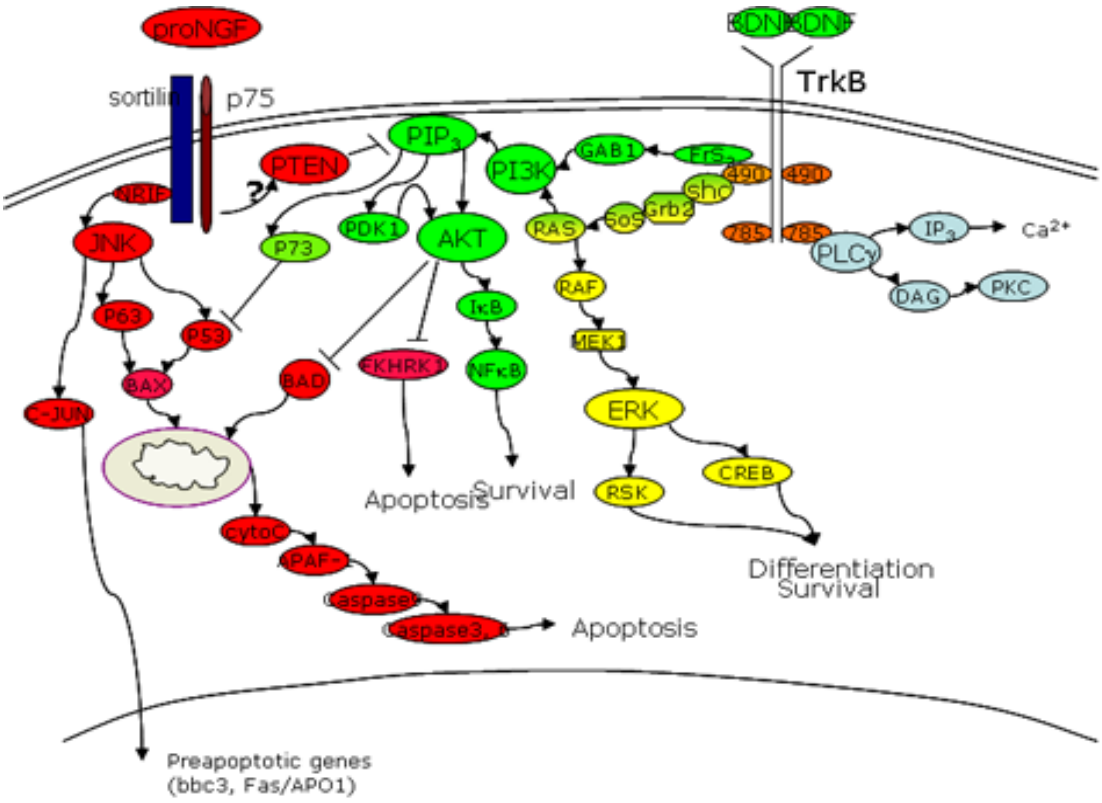


Table 1. **Co-localization between Trks and p75NTR in basal forebrain in vivo**

Double immunostaining of p75NTR and Trk A or B or C was performed on basal forebrain sections from adult rat. The number of cells expressing both Trk receptor and p75NTR in both the medial septum and diagonal band was counted and percentage of these cells against cells expressing only Trk receptor or p75NTR was calculated.

	Percentage in Trks population %	Percentage in P75NTR population %
TrkA/P75	71	91
TrkB/P75	47	95
TrkC/P75	42	95

Table 2. **ProNGF induces apoptosis of TrkB+ BF neurons.** E16 BF neurons were cultured on slide wells for 5 days in the presence of BDNF (10ng/ml) before treatment overnight with vehicle, mature NGF or proNGF. The number of neurons double labeled for TrkB and p75NTR in both healthy and apoptotic populations is indicated as percentage of total.

Pro-NGF induces apoptosis of TrkB+ basal forebrain neurons

TrkB/p75	Vehicle	NGF(4h)	Pro-NGF(4h)
% Healthy	96	92	61
% Apoptotic	4	8	39

Table 3. **ProNGF induced the death of p-Trk positive BF neurons, but not the death of neurons with p-Akt.** E16 BF neurons were cultured for 5 days in the presence of BDNF. Cells were treated with BDNF or proNGF as indicated, then double labeled with different combinations of antibodies (diluted in the medium 1:500). The number of neurons stained with both antibodies in both healthy and apoptotic populations was counted and indicated as the percentage of total cells:

P-Trk and TrkB (percentage of TrkB+ neurons that also stained for p-Trk in both healthy and apoptotic populations with the indicated treatments).

P-Akt and p-Trk (percentage of p-Trk+ neurons that also stained for p-Akt that were either healthy or apoptotic with indicated treatments).

P-Akt and cleaved caspase 3 (no cells stained for both p-Akt and cleaved caspase 3).

	Vehicle	BDNF	ProNGF	BDNF+proNGF
P-Trk/TrkB				
% healthy	65/94	97/97	54/75	72/72
% apoptotic	0/6	0/3	8/25	28/28
P-Akt /P-Trk				
% healthy	93/93	92/92	68/68	56/56
% apoptotic	0/7	0/8	0/32	0/44
P-Akt /CC3	no double labeling. 100% of the P-Akt+ cells were healthy and lacked cleaved caspase-3 (CC3) staining, and 100% of the CC3+ were apoptotic and lacked P-Akt labeling.			

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