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Functional interactions between the p75 neurotrophin receptor and TrkA

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ABSTRACT

The $p75^{NTR}$ neurotrophin receptor has previously been shown to increase the responsiveness of TrkA to NGF, the preferred ligand of TrkA. We performed structure/function studies to determine the domains of $p75^{NTR}$ involved in this functional interaction. Co-expression of $p75^{NTR}$ in 293T cells enhances NGF-mediated TrkA autophosphorylation while the intracellular domain of $p75^{NTR}$ is not required for this function. Indirect evidence has previously suggested that $p75^{NTR}$ may also play a role in reducing non-preferred ligand activation of TrkA. We tested this directly and found that $p75^{NTR}$ reduces both basal and NT-3/NT-4-mediated TrkA autophosphorylation. In addition, we show that the intracellular domain of $p75^{NTR}$ can by itself reduce basal TrkA activity, suggesting the involvement of intracellular signaling. These data imply that the $p75^{NTR}$ receptor can functionally interact with the TrkA receptor through two distinct domains. We hypothesize that these interactions function in combination to help the TrkA receptor discriminate between preferred and non-preferred ligands, therefore increasing the specificity of activation of the TrkA receptor.

RESUMÉ

Le récepteur aux neurotrophines $p75^{NTR}$ potentialise la réponse du récepteur TrkA au NGF, lequel constitue le ligand préférentiel de TrkA. Au cours de ce travail, nous avons réalisé des études de structure/fonction afin de déterminer les domaines de $p75^{NTR}$ impliqués dans cette interaction fonctionnelle. La co-expression de $p75^{NTR}$ et TrkA dans les cellules 293T augmente le niveau d'autophosphorylation de TrkA induite par le NGF; cependant, le domaine intracellulaire de $p75^{NTR}$ n'est pas nécessaire à l'induction de cet effet. Par ailleurs, un rôle de $p75^{NTR}$ dans la réduction de l'activation de TrkA par ses ligands de plus faible affinité a été également suggéré sur la base d'arguments indirects. Nous avons vérifié cette hypothèse et démontré que $p75^{NTR}$ diminue à la fois le niveau basal d'autophosphorylation de TrkA et celui lié à la liaison de NT-3/NT-4. De plus, nos résultats indiquent que le domaine intracellulaire de $p75^{NTR}$ peut à lui seul réduire l'activité basale de TrkA, ce qui suggère la participation de signaux de transduction cellulaire à ce phénomène. L'ensemble de ces données montre que le récepteur $p75^{NTR}$ possède deux domaines distincts d'interaction fonctionnelle avec le récepteur TrkA. Nous suggérons l'existence d'un effet coopératif entre ces domaines d'interaction augmentant la capacité du récepteur TrkA à distinguer ses ligands de haute et faible affinité, et ainsi la spécificité de l'activation dudit récepteur.

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AChE	Acetylcholinesterase
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CGRP	Calcitonin gene-related peptide
ChAT	Cholineacetyltransferase
CNS	Central nervous system
CRNF	Cysteine-rich neurotrophic factor
DMEM	Dulbecco's modified Eagle's medium
DRG	Dorsal root ganglion
eGFP	Enhanced green fluorescent protein
ERK	Extracellular signal regulated kinase
FGF	Fibroblast growth factor
ICD	Intracellular domain
Ip	Immunoprecipitate
JNK	Jun amino-terminal kinase
LRM	Leucine-rich motif
MAPK	Mitogen-activated proteine kinase
mICD	Myristoylated intracellular domain
N-CAM	Neural cell adhesion molecule
NFkB	Nuclear factor kappa-B
NGF	Nerve growth factor
NGF^{3T}	Nerve growth factor triple mutant
NPG	Nodose/petrosal ganglion
NT-3	Neurotrophin-3
NT-4	Neurotrophin-4
NT-6	Neurotrophin-6
NT-7	Neurotrophin-7
NTR	Neurotrophin receptor
PC12	Pheochromocytoma cells
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PI-3 kinase	Phosphatidylinositol-3 kinase
PLC-γ	Phospholipase C- γ
PNS	Peripheral nervous system
pY	Phosphotyrosine
RT-PCR	Reverse transcriptase-PCR
SCG	Superior cervical ganglion
SDS	Sodium dodecyl sulfate
SP	Substance P
TNF-α	Tumor necrosis factor-α
TrkA	Tyrosine kinase A
TrkB	Tyrosine kinase B
TrkC	Tyrosine kinase C

1. INTRODUCTION

The neurotrophins are a family of proteins involved in many aspects of the maintenance and development of the nervous system. The family contains nerve growth factor (NGF), brain-derived nerve growth factor (BDNF), neurotrophin-4/5 (NT-4/5), and neurotrophin-3 (NT-3). These proteins, or deficits in their function, have been suggested to play some role in neurological diseases such as Alzheimer's (Higgins and Mufson 1989;Mufson et al. 1989;Phillips et al. 1991;Strada et al. 1992), Parkinson's (Hyman et al. 1991;Spina et al. 1992), and epilepsy (Funabashim et al. 1988;Gall and Isackson 1989;Isackson et al. 1991). Although these proteins have traditionally been studied for their involvement in neuronal development, they have also been implicated in tumor progression (Kim et al. 1991;Dobrowsky et al. 1994;Dobrowsky et al. 1995). Thus, understanding the mechanisms underlying neurotrophin function may help develop future therapies which could impact on a variety of human diseases.

1.1 The Neurotrophins

1.1.1 Discovery of Nerve Growth Factor

Work on the neurotrophins began about 45 years ago with the discovery of a nerve growth factor in 1953 (Levi-Montalcini and Hamburger 1953). While studying the effect of mouse sarcoma cells upon embryonic chick tissue, it was noted that increased sympathetic and spinal sensory ganglia development had taken place in and around the graft. Further analysis revealed that this effect was the result of a diffusible factor released by the tumor tissue, which was later called nerve growth factor (NGF).

It was subsequently discovered that the mouse submaxillary gland, the mammalian homologue of the reptilian venom gland, produced high levels of NGF (Cohen and Levi-Montalcini 1956). This finding played an important role in advancing research on NGF since it permitted the purification of relatively high amounts of NGF which resulted in the cloning of the gene (Scott et al. 1983;Ulrich et al. 1983;Ebendal et al. 1986;Meier et al. 1986).

1.1.2 Structure of NGF

NGF is initially synthesized as a precursor protein that is enzymatically processed to its bioactive form (Berger and Shooter 1977). Mature NGF consists of a homodimer, composed of noncovalently bound 118 amino acid chains (Angletti and Bradshaw 1971;Angletti et al. 1971). Growth-promoting activity is displayed by dimeric NGF which is the principal physiological form (Frazier et al. 1973). Each NGF monomer is dominated by two pairs of anti-parallel β-strands which are connected by three loops (McDonald et al. 1991;Holland et al. 1994). In addition, the NGF monomer contains six cysteine residues, leading to a tertiary structure which includes three interchain disulfide bonds (Thoenen and Barde 1980).

1.1.3 Expression of NGF

1.1.3.1 Peripheral Nervous System

Northern blot analysis and in situ hybridization studies have revealed the presence of a high level of NGF mRNA in peripheral tissue innervated by sympathetic and sensory neurons. These include the vas deferans, submaxillary gland, heart, iris, skin, sciatic nerve and the splenic capsule. Also, the NGF mRNA is found in lower amounts in the adrenal glands, testis, liver, and skeletal muscle (Heumann et al. 1984;Shelton and Reichardt

1984). In the iris, the cell types found to express NGF mRNA include smooth muscle cells, epithelial cells, fibroblasts, and Schwann cells (Bandtlow et al. 1987).

1.1.3.2 Central Nervous System

In the CNS, NGF mRNA was detected in all regions tested (Shelton and Reichardt 1986), but levels varied over a range of 40-fold. In general, cortical areas displayed the highest levels of NGF transcripts. Densitometry of autoradiograms revealed that levels of NGF mRNA were highest in the hippocampus, followed, in decreasing order, by piriform-entorhinal cortex, and neocortex. Intermediate NGF mRNA levels were found in the pons, medulla, diencephalon, and olfactory bulb. Low levels were detected in the spinal cord and striatum and the lowest amounts were seen in the midbrain, septal area, and cerebellum (Shelton and Reichardt 1986).

1.1.4 Other neurotrophins

1.1.4.1 Brain-Derived Nerve Growth Factor (BDNF)

After the discovery of NGF, it took approximately 25 years before another neurotrophin was discovered. This novel neurotrophin was originally identified in glioma-conditioned media because it induced the extension of processes from the immature sensory ganglia of embryonic dorsal root ganglia (Barde et al. 1978;Barde et al. 1980). The protein was cloned in 1989 and named brain-derived neurotrophic factor (BDNF) (Leibrock et al. 1989). The BDNF gene has now been cloned in three different species including porcine, murine, and human and in all three species, the gene encodes a 252 amino acid precursor which is cleaved to a 119 amino acid mature form (Leibrock et al. 1989;Hofer et al. 1990;Rosenthal et al. 1991).

1.1.4.2 Neurotrophin-3 (NT-3) and Neurotrophin-4 (NT-4)

NGF and BDNF were found to possess a high degree of sequence homology which led to the rapid isolation and cloning of the other neurotrophins. Oligonucleotide primers devised by analyzing the regions of high sequence homology between NGF and BDNF were used to amplify genomic templates via polymerase chain reaction (PCR). These amplified segments of DNA were then used to screen genomic libraries. Neurotrophin-3 (NT-3) was isolated from many species including human, rat, and mouse (Ernfors et al. 1990;Hohn et al. 1990;Jones and Reichardt 1990;Maisonpierre et al. 1990;Rosenthal et al. 1990). The sequence encoded a precursor protein containing 258 amino acids which, similarly to NGF and BDNF, was enzymatically cleaved to a shorter (119 amino acid) bioactive form. Finally, neurotrophin-4 (NT-4) was also cloned, originally from Xenopus and Viper (Hallbook et al. 1991) and later from rat and human sources (Ip et al. 1992) using the same principle as NT-3. The sequence encodes a precursor protein of 236 amino acids which is cleaved to a mature protein of 123 amino acids. In addition, Neurotrophin-5 (NT-5) was isolated from human and rat sequences (Berkemeier et al. 1991) but further analysis of the sequence revealed that it was identical to the mammalian NT-4 sequence (Ip et al. 1992) This is why NT-4 is sometimes referred to as NT-4/5 or NT-5.

1.1.4.3 New members of the neurotrophin family

Two other neurotrophins have recently been identified, but only in fish. The two factors consists of neurotrophin-6 (NT-6) isolated from the teleost Xiphophorus (Gotz et al. 1994) and neurotrophin-7 cloned from the Cyprinus carpio (carp) and from the zebrafish Danio rerio (Lai et al. 1998;Nilsson et al. 1998). Both of these neurotrophic

factors have highest sequence homology to NGF and induce similar biological responses, albeit with lower potency (Gotz et al. 1994;Lai et al. 1998;Nilsson et al. 1998). For instance, NT-6 promotes the survival of sympathetic and DRG neurons similarly to NGF but with lower specific activity, and NT-7 is able to bind to human $p75^{NTR}$ and to induce rat TrkA tyrosine phosphorylation but with less efficiency than rat NGF. A protein called CRNF for Cysteine-Rich Neurotrophic Factor may someday also be added to the list of neurotrophins (Fainzilber et al. 1996). This 13.1 kd protein recently cloned from the mollusk *Lymnaea stagnalis*, binds $p75^{NTR}$ with nanomolar affinity but displays no sequence similarity with the neurotrophins, or other known proteins. CRNF may represent a new family of $p75^{NTR}$ ligands.

1.2 The Neurotrophin Receptors

Elucidation of the molecular mechanisms by which the neurotrophins exert their biological effects was rendered possible by the discovery of the neurotrophin receptors. To date, two types of receptors which mediate neurotrophin action have been identified.

1.2.1 Trk tyrosine kinase family

The first neurotrophin receptor discovered, a tyrosine kinase receptor, was described as the product of the Trk oncogene, a chimeric oncoprotein found in a human colon carcinoma (Martin-Zanca et al. 1986;Martin-Zanca et al. 1989). Although Trk was cloned earlier, it was not until 1991 that it was found that its physiological function was to serve as the signaling receptor for NGF (Hempstead et al. 1991;Kaplan et al. 1991a;Klein et al. 1991a;Kaplan et al. 1991b). In addition, it was later discovered that Trk was part of a family of receptors highly related to each other including two other members, TrkB (Klein et al. 1989;Klein et al. 1990a;Middlemas et al. 1991) and TrkC (Lamballe et al. 1991).

Each member displays a high degree of specificity for neurotrophin binding. Trk, now called TrkA, serves as the signaling receptor for NGF, TrkB is the mediator of the biological effects exerted by both BDNF (Soppet et al. 1991;Squinto et al. 1991;Klein et al. 1991b) and NT-4 (Berkemeier et al. 1991;Ip et al. 1992;Klein et al. 1992) and TrkC mediates NT-3 responses (Lamballe et al. 1991). In addition, a novel Drosophila tyrosine kinase receptor was recently discovered and designated as Dnrk (Drosophila neurospecific receptor kinase) since it was found to be expressed mainly in the nervous system (Oishi et al. 1997). This putative tyrosine kinase receptor is highly related to the Trk and Ror families of receptor tyrosine kinases and may represent a new neurotrophin receptor.

1.2.1.1 Common structural features of the Trk family members

The extracellular domains of the trk receptors exhibits two distinct subsets of cell adhesion-related motifs (Schneider and Schweiger 1991). The N-terminal moiety is composed of an array of three tandem leucine-rich motifs (LRM) of 24 amino acid residues flanked by two distinct cysteine clusters. The LRM sequences are found in a diverse family of proteins believed to be involved in protein-protein interactions. Examples include Von Willebrand factor, and some cell-adhesion proteins and extracellular matrix components. Next to cysteine cluster II, on the carboxy-terminal side, are two immunoglobulin-like domains (IgI and IgII) of the C_2 type. These motifs are similar to those found on neuronal cell adhesion molecules such as N-CAM and tyrosine kinase receptors such as the PDGF and FGF receptors. One other specific feature of the members of the trk tyrosine kinase receptor family is a carboxy-terminal tail of 15 amino acid residues, outside of the core kinase domain, which includes a conserved tyrosine residue.

1.2.1.2 Structural features of the TrkA receptor

The trk proto-oncogene encodes two tyrosine kinase receptor isoforms of 790 and 796 amino acid residues, designated as TrkAI and TrkAII, respectively (Barker et al. 1993). These two receptor isoforms contain a signal peptide, followed by an ectodomain responsible for the binding of NGF, a single transmembrane domain, and a cytoplasmic domain which encompasses a tyrosine kinase catalytic domain. They differ in the presence of 6 amino acid residues present in the ectodomain near the transmembrane domain. The two TrkA isoforms also differ in their pattern of expression since the 796 amino acid protein is mainly found in neuronal cells, while the 790 amino acid protein is expressed mostly in non-neuronal cells (Barker et al. 1993;Horigome et al. 1993). Upon NGF-dependent activation, the two TrkA isoforms induce comparable biological responses when expressed in $PC12^{nnr5}$, and fibroblasts (Barker et al. 1993;Clary and Reichardt 1994). However, TrkAII the isoform containing the variable exon, displays higher activation by the non-preferred ligand NT-3 when co-expressed with $p75^{NTR}$ (Clary and Reichardt 1994).

1.2.1.3 Structural features of the TrkB receptor

The TrkB locus encodes eight transcripts ranging in size from .7kb to 9.0kb, although only three TrkB proteins have been found in mammals so far. The TrkB receptor (sometime referred to as the $gp145^{TrkB}$ or the $TrkB^{TK-}$ receptor) is a heavily glycosylated protein made up of 821 amino acid residues which possesses the same features as the TrkA receptor (described above). Sequence comparison between the rat TrkA and TrkB have shown that the extracellular domain of the TrkB receptor exhibits 57% homology (38% identity) with TrkA. Furthermore, the TrkA and TrkB sequence which encode the

kinase domain of the receptors display a high degree of homology (88%) which suggest an important evolutionary role for the sequence contained within this region (Klein et al. 1989;Klein et al. 1990a;Middlemas et al. 1991).

A second class of TrkB receptors contains truncated versions of the receptor. They possess the same extracellular and transmembrane domain as the first class of TrkB receptors, but their cytoplasmic domains are much smaller and do not contain a tyrosine kinase domain (Klein et al. 1990a;Middlemas et al. 1991). To date, two isoforms of the truncated TrkB have been characterized. The first consists of the TrkB.T1 receptor, isolated from mouse and rat cDNA libraries which has a 23 amino acid cytoplasmic domain with the 11 amino acid residues, at the carboxy terminal, unrelated to other known sequences (Klein et al. 1990a). The TrkB.T1 protein has been shown to be expressed, in the adult mouse brain (4 weeks old), at levels similar to the full length TrkB receptor suggesting that it may play an important role in the brain (Klein et al. 1990a). In the brain, the TrkB.T1 has been observed in the ependymal linings of the cerebral ventricules as well as in the choroid plexus (Klein et al. 1990a). The other isoform, the TrkB.T2 protein, was isolated from a rat cerebellar cDNA library. This protein, is not observed in humans but in rat has a 21 amino acid cytoplasmic domain of which the last 9 amino acids are unrelated to the sequence of the TrkB.T1 receptor and to other known sequences (Middlemas et al. 1991).

1.2.1.4 Structural features of the TrkC receptor

The TrkC locus also encodes two receptor classes. The TrkC.k1 protein (also known as $gp145^{TrkC}$) was first discovered in 1991 as the product of a cDNA clone isolated from a porcine brain cDNA library and found to possess the same structural features as the

related full length TrkA and TrkB tyrosine kinase receptors. The overall homology of the TrkC.k1 protein compared with the other members of the Trk family of tyrosine kinase is 67% (54% in the extracellular domain and 87% in the kinase region) for TrkA and 68% (53% in the extracellular domain and 87% in the kinase region) for TrkB. The other members of this class differ in the presence of 14 (TrkC K14), 25 (TrkC K25), and 39 (TrkC K39) amino acid residues inserted distal to the conserved kinase domain sequence YSTDYYR which contains a putative autophosphorylation site of the TrkC receptor (Lamballe et al. 1991;Lamballe et al. 1993;Tsoulfas et al. 1993;Valenzuela et al. 1993). NT-3 binds to and activates TrkC K1, TrkC K14, and TrkC K25 with similar affinity and kinetics (Lamballe et al. 1993;Tsoulfas et al. 1993;Valenzuela et al. 1993), but only the K1 isoform seems to activate PLC-γ and PI-3 kinase. In spite of this fact, TrkC K14 and TrkC K25 have been shown to initiate DNA synthesis in fibroblasts upon activation by NT-3 (Lamballe et al. 1993). However, their biological function is restricted since neither TrkC K14 or TrkC K25 can induce survival of NIH3T3 cells in the absence of serum nor can they induce morphological transformations under normal culture conditions. Furthermore, TrkC K14 and TrkC K25 do not display the ability to mediate neuronal-like differentiation of PC12 cells (Lamballe et al. 1993;Tsoulfas et al. 1993;Valenzuela et al. 1993).

Truncated forms of the TrkC receptors are also produced. Thus, the truncated TrkC receptors possess the same extracellular and transmembrane domain as the full length TrkC receptors, however they do not have a kinase domain. To date, four isomers of this family have been discovered (Tsoulfas et al. 1993;Valenzuela et al. 1993). The physiological relevance of the truncated TrkB and TrkC receptors is presently not known

and is under investigation. One possible hypothesis is that they may act as dominant negative receptors interfering with normal biological function of full length receptors.

1.2.1.5 Trk signaling

Most of what is known about the signal transduction pathways used by TrkA to promote the differentiation of neural cells has been elucidated using PC12 cells (Kaplan and Miller 1997). Upon NGF treatment, PC12 cells undergo mitotic arrest, grow neurites and exhibit somatic hypertrophy. In addition, NGF-treated PC12 cells become dependent on the constant supply of NGF as a survival factor in serum-free medium and, once completely differentiated, these cells display properties of sympathetic neurons

The Trk family of receptors induces biological responses through the ligand-dependant activation of the tyrosine kinase domain. This step is the start of a signal cascade which occurs through the direct association with, and subsequent phosphorylation of, selective cellular substrates such as phospholipase PLC–γ1 (phospholipase C-γ1), or the adapter protein Shc (Nakamura et al. 1996), and the phosphotyrosine phosphatase SHP-1 (SH2-containing tyrosine phosphatase) (Greene and Kaplan 1995). These proteins in turn activate downstream effector proteins which lead to the activation of several signaling pathways. For instance, Shc activation by TrkA results in the rapid activation of several proteins including Ras, the serine/threonine kinases B-Raf and mitogen-activated protein kinase (MAPK) as well as the phosphatidylinositol 3'-kinase (Kaplan and Stephens 1994;Greene and Kaplan 1995;Baxter et al. 1995).

Mutagenesis studies have revealed two separable pathways which can account for most of the observable phenotypic effects seen in differentiating PC12 cells. The combination of the Shc pathway with PLC-γ1 is sufficient to regulate growth and

maintenance of neurites and cell survival (Stephens et al. 1994;Obermeier et al. 1994). Their ablation does not inhibit NGF-induced initiation of neurite elongation and somatic hypertrophy suggesting the action of another pathway (Obermeier et al. 1994;Peng et al. 1995). The deletion of a site present on the TrkA receptor which mediates the tyrosine phosphorylation of the nuclear protein SNT (Suc1-associated neurotrophic factor target) can suppress theses events (Peng et al. 1995). Thus, the Trk family members regulate their effects through more than one signal transduction pathway.

1.2.1.6 Pattern of expression of Trk members in the Mouse

1.2.1.6.1 TrkA

The onset of the TrkA gene expression occurs at early stages of neurogenesis, around E9.5 (Martin-Zanca et al. 1990). In the peripheral nervous system (PNS), TrkA transcripts are detected in small size neurons of the dorsal root ganglia at E13.5, as well as in cranial sensory ganglia including the trigeminal (V), superior (sup IX), and jugular (sup X) ganglia. This pattern of expression is highly specific since other neighboring cranial ganglia do not exhibit detectable TrkA transcripts (Martin-Zanca et al. 1990). At a later age (E17.5), the analysis of embryos indicates that the TrkA gene is also expressed in sympathetic ganglia (Martin-Zanca et al. 1990;Tessarollo et al. 1993).

The TrkA proto-oncogene is also expressed in distinct neuronal populations of the central nervous system (CNS). These include the cholinergic neurons of the basal forebrain (medial septal nucleus and the diagonal band of Broca), and the striatum (Vazquez and Ebendal 1991;Holtzman et al. 1992;Merlio et al. 1992;Steininger et al. 1993), as well as two densely packed populations of noncholinergic thalamic neurons

(Venero and Hefti 1993), and magnocellular neurons of several brainstem nuclei (Merlio et al. 1992).

Outside the nervous system, TrkA mRNA as been detected in activated $CD4^+$ T lymphocytes, and in monocytes, as well as in the lungs and kidneys (Barker et al. 1993;Ehrhard et al. 1993a;Ehrhard et al. 1993b).

1.2.1.6.2 TrkB

The TrkB gene is expressed extensively throughout the CNS and PNS (Klein et al. 1990a;Klein et al. 1990b). The expression pattern of the TrkB gene is more complex than the TrkA gene, due to the various splice variants that are produced at this locus. The TrkB transcript is first detected in embryos at age E8.5 in many rudimentary structures within the developing nervous system. These include the forebrain, caudal midbrain, hindbrain, spinal cord, the trigeminal ganglion, and the differentiating neural crest cells (Klein et al. 1990b). In the midgestation period (embryos of age E13.5), most regions of the developing CNS and PNS express the TrkB gene (Table 1). For example, there are high levels of TrkB transcripts detected in the spinal cord, the olfactory lobe, the ependymal layer of the fourth ventricle, and others which are described in Table 1. DRGs also express TrkB, but the expression here is limited to a small subset of neurons (Carroll et al. 1992).

In the adult mouse the highest levels of TrkB expression are found in the brain and spinal cord (Klein et al. 1990a;Merlio et al. 1992). Using *in situ* hybridization techniques, the TrkB transcripts are detected in the majority of cerebrum structures including the cortical layers, the thalamus, and the hippocampus. The areas of strongest expression are the granular layer of the dentate gyrus as well as the pyramidal cell layer in the

Table 1: Expression of Trk Genes in the Murine Nervous System.

Structure	TrkA	TrkB	TrkC	Structure	TrkA	TrkB	TrkC
CENTRAL NERVOUS SYSTEM				PERIPHERAL NERVOUS SYSTEM			
Forebrain				Cranial Ganglia			
Cerebral Cortex	—	++	++	V	++	+	+
Corpus Callosum	—	—	ND	VII	—	++	++
Hippocampus	—	++	++	VIII	—	++	++
Fimbria Hippocampus	—	—	ND	IX/X Superior	++	++	++
Basal Forbrain	++	++	++	IX/X Inferior	—	++	++
Striatum	+	++	+				
				Dorsal Root Ganglia			
Diencephalon				Small-size neurons	++	—	—
Thalamus	+	++	+	Medium-size neurons	—	+	—
Hypothalamus	—	++	++	Large-size neurons	—	—	+
Medial Habenular Nucleus	—	++	+	Other Structures			
Substantia Nigra	—	+	ND	Sympathetic Ganglia	++	+	+
Interpenduncular				Inner Ear	—	+	ND
Olfactory Epithelium	—	+	+				
Nucleus	—	+	+	ENTERIC NERVOUS SYSTEM			
				Enteric ganglia	—	—	++
Midbrain							
Red Nucleus	—	+	ND	NON-NERVOUS SYSTEM			
Superior Colliculus	—	+	+	Arteries	—	—	++
				Diaphragm	—	—	++
Brainstem				Multilocular			
Purkinje Cells	—	++	—	Adipose tissue	—	—	+
Granular cell layer	—	—	++	Mesophenic and			
Pontine nuclei	—	+	+	Urogenital Ducts	—	—	+
Nucleus Ambiguous	—	++	ND	Palate, Tongue,			
				and Tooth buds	—	+	+
Other Structures				Snout	—	+	+
Spinal Cord	—	++	++	Submaxillary Gland	—	+	++
Choroid Plexus	—	++	—				
Ependymal Layer of Ventricules	—	++	—				
Retina	—	++	ND				
Ophthalmic Nerves	—	++	ND				

Taken from Barbacid, 1994. ND: not determined

hippocampus, the medial habenular nucleus, the hypothalamic nuclei, the choroid plexus, and the ependymal lining of the ventricules (Table 1). In the midbrain, moderate expression levels of the TrkB receptor are found in most of the large and medium-size neurons of the substantia nigra, and in the superior colliculus, interpeduncular nuclei, and red nucleus. TrkB transcripts are also detected in the brainstem and the cerebellum. In the brainstem, abundant TrkB transcripts are found in the nucleus ambiguous and in some of the precerebellar nuclei such as the pontine. In the cerebellum, TrkB expression appears to be more restricted. High levels of TrkB transcripts are detected in the Purkinje cell layer, and the caudal peduncle while moderate levels are found in other neurons and glial cells present in the cerebellum (Klein et al. 1990a). Some non-neuronal cells which exhibit TrkB expression include the tongue, the whisker pad mesenchyme, and the outer dermal papilla (Klein et al. 1990a).

TrkB transcripts are not equally expressed in mouse neural tissues. *In situ* hybridization experiments have revealed that the expression of the various TrkB transcripts displays a highly specialized pattern. To generalize, the kinase active TrkB is preferentially expressed in neurons, while the kinase dead truncated TrkB is particularly found in non-neuronal cells such as the astrocytes, oligodendrocytes, and Schwann cells (Frisen et al. 1993).

1.2.1.6.3 TrkC

The pattern of TrkC gene expression is more complex than the other members of the trk receptor family. As noted above, the TrkC locus possibly encodes as many as eight receptors, some of which do not possess a tyrosine kinase catalytic domain. To date, all studies regarding gene expression of the TrkC receptor have been generated using probes

which do not differentiate between the various isoforms of the receptor. Nonetheless, this information still is very helpful in determining the role the receptor may play in the developing and adult nervous system.

The TrkC transcripts can be first observed, as a weak signal at E7.5 in the early neuroectoderm (Tessarollo et al. 1993). At E9.5, the expression of the receptor is seen within the forming DRGs, the telencephalon, and the spinal cord. At E11.5, TrkC mRNA is detected throughout the CNS such as the telencephalon, the rhombencephalon, the diencephalon, the mesencephalon, and the neural tube. In the PNS, the DRGs exhibit TrkC mRNA production. Finally during late embryogenesis, when most of the nervous system has been generated, the TrkC expression is seen throughout the neuraxis (Tessarollo et al. 1993;Lamballe et al. 1994). In contrast to TrkA and TrkB, TrkC transcripts have been detected in the muscular layers of the gut wall which eventually form the ganglia of the enteric nervous system (Tessarollo et al. 1993;Lamballe et al. 1994).

During embryogenesis, TrkC transcripts can also be detected in various structures outside of the nervous system. For example, in facial structures such as the vibrissae of the snout, the dental papillae, the posterior tongue, sublingual glands, and the olfactory epithelium as well as in the body cavity including the acini of the submandibular gland, the subendothelial mesenchyme of arteries, the mesenchyme surrounding mesophenic and urogenital ducts, the diaphram, the adrenal gland, and in multilocular adipose tissue (Tessarollo et al. 1993;Lamballe et al. 1994).

In the adult mice, TrkC is expressed extensively throughout the CNS. For example, TrkC transcripts have been detected in certain areas of the forebrain, including the cerebral cortex, hippocampus, thalamus, and hypothalamus (Table 1). In the cerebral cortex, TrkC

is highly expressed in layers 2, 3, and 6. In the hippocampus, strong TrkC hybridization is seen in the pyramidal cells of Ammon's horn in CA1, CA2, CA3, and the hilus. In addition, the granule cells of the dentate gyrus also express high levels of TrkC. In the diencephalon, high amounts of mRNA are detected in the centromedian area of the thalamus and in the habenular nuclei. In the hypothalamus, TrkC mRNA can be detected in the dorso- and ventromedial nuclei found near the third ventricle as well as in the medium eminence.

TrkC transcripts are also present in the midbrain, brainstem, and spinal cord. In the midbrain, TrkC hybridization is observed in the substantia nigra, superior colliculus, and central grey, as well as the neurons of the pretectal, interpeduncular, and trochlear nuclei. In the brain stem, strong TrkC signals are found in the nucleus ambiguous, dorsal motor nucleus, and the nucleus of the solitary tract, as well as in the orafacial motor nuclei, the sensory trigeminal nuclei, and the vestibular nuclei (Table 1). Finally, all large motor neurons of the spinal cord express high amounts of TrkC (Merlio et al. 1992;Tessarollo et al. 1993;Lamballe et al. 1994).

1.2.2 The $p75^{NTR}$ Receptor

The second type of neurotrophin receptor is designated as the p75 neurotrophin receptor ($p75^{NTR}$) and although it was the first neurotrophin receptor to be discovered (Johnson et al. 1986), its precise function still remains relatively poorly defined. $p75^{NTR}$ is a member of a superfamily of related proteins which includes the tumor necrosis factor (TNF) receptors, TNFRI and TNFRII, the Fas antigen receptor, CD40, and OX40 as well as other related proteins (Smith et al. 1994). Although the $p75^{NTR}$ receptor was originally identified as the NGF receptor, it was later shown that $p75^{NTR}$ could bind all neurotrophins

with similar affinities, but distinct kinetics (Rodriguez-Tebar et al. 1990;Rodriguez-Tebar et al. 1992). CRNF, a cysteine-rich neurotrophic factor cloned from the mollusk *Lymnaea stagnalis* also binds $p75^{NTR}$ (Fainzilber et al. 1996).

1.2.2.1 Structural features of the p75 neurotrophin receptor.

The cloning of the $p75^{NTR}$ neurotrophin receptor was rendered possible because of the availability of monoclonal antibodies against the human and rat $p75^{NTR}$ receptor and library screening techniques (Johnson et al. 1986;Radeke et al. 1987). The molecular weight of the $p75^{NTR}$ receptor in humans is roughly around 75kd hence the name $p75^{NTR}$ (Johnson et al. 1986). The 3.8kb $p75^{NTR}$ mRNA transcript encodes a single peptide chain of roughly 400 amino acid residues that includes a 28 amino acid signal peptide, an extracellular domain containing four tandem cysteine-rich motifs followed by a region rich in serine/threonine residues, a single membrane-spanning domain, and a shorter cytoplasmic tail (Johnson et al. 1986). The cysteine-rich motif present in the extracellular domain of the receptor is the hallmark of all members of the TNF receptor superfamily.

A comparison between human, rat and chick $p75^{NTR}$ amino acid sequences reveal significant homology in the four cysteine-rich regions, the juxtamembrane and transmembrane domains, as well as in the C-terminus of the cytoplasmic terminal (Large et al. 1989), suggesting that these regions may be important in the function of the receptor. Indeed, the cysteine-rich repeats have been shown to be essential to the binding of NGF (Welcher et al. 1991;Yan and Chao 1991;Baldwin et al. 1992). The conserved C-terminal region contains a mastoparan-like sequence which resembles the consensus domain for the recruitment of G proteins (Feinstein and Larhammar 1990). A separate isoform of the $p75^{NTR}$ receptor has been detected in conditioned media of cells (PC12, Schwann cells,

and SCGs) which express full length $p75^{NTR}$ and in various biological fluids (Distefano and Johnson 1988). This protein is a soluble, truncated version of the $p75^{NTR}$ receptor which still binds NGF and migrates at around 50kd. This truncated protein may be a splice variant of the full length $p75^{NTR}$ or rather the result of proteolytic cleavage which could indicate a mechanism by which $p75^{NTR}$ induces its biological functions.

The binding properties of the $p75^{NTR}$ receptor were measured by expressing the receptor in L-cells. The receptor has a K_d of 10^{-9} M for all the neurotrophins but shows distinct binding characteristics for each of them such as fast on/fast off for NGF (Sutter 1979). In addition, BDNF and NT-3 display positively cooperative binding to $p75^{NTR}$ (Rodriguez-Tebar et al. 1990;Rodriguez-Tebar et al. 1992).

1.2.2 Potential functions of the $p75^{NTR}$ receptor

1.2.2.1 $p75^{NTR}$ enhances NGF-mediated TrkA activation

Although defining the precise function(s) of $p75^{NTR}$ remains a challenge, recent studies have provided some insights on various potential functions of the receptor. In PC12 cells, the rate of association of NGF to the TrkA receptor increases by 25 fold in the presence of $p75^{NTR}$ suggesting a potential role of $p75^{NTR}$ in regulating NGF-dependant activation of TrkA (Mahadeo et al. 1994). Consistent with this, disruption of NGF binding to the $p75^{NTR}$ receptor results in a decrease in NGF binding to the TrkA receptor coupled with a reduction in tyrosine phosphorylation. However, disruption of NGF binding to the $p75^{NTR}$ receptor does not affect NGF-dependent TrkA activation when high concentrations of NGF are used (Barker and Shooter 1994). Similar results were obtained using an immortalized sympathoadrenal progenitor cell line (MAH cells; (Verdi et al. 1994)); co-expression of $p75^{NTR}$ in MAH cells enhances the NGF-induced tyrosine

autophosphorylation of the TrkA receptor as well as the NGF-dependent TrkA-induced biological responses. Comparable results were also obtained in a study which demonstrated that $p75^{NTR}$-deficient trigeminal sensory neurons needed 3 to 4 fold higher concentrations of NGF, compared to wild type trigeminal neurons, in order to promote half-maximal survival suggesting that the loss of $p75^{NTR}$ had decrease the sensitivity of the TrkA receptor to NGF (Davies et al. 1993). Together, these results provide direct evidence that $p75^{NTR}$ can enhance NGF-mediated TrkA activation.

1.2.2.2.2 Indirect evidence that $p75^{NTR}$ may reduce the response of TrkA to its non-preferred ligands NT-3 and NT-4

The TrkA receptor has also been shown to be activated by NT-3 and NT-4 (Cordon-Cardo et al. 1991;Chao 1992;Belliveau et al. 1997) and recent studies have proposed that $p75^{NTR}$ may selectively reduce the response of TrkA to these neurotrophins. For instance, preincubation of NTA_I and NTA_{II} variant $PC12^{nnr5}$ cell lines expressing the two TrkA isoforms I and II, with the polyclonal anti-$p75^{NTR}$ antibody REX causes an increase in the NT3-mediated TrkA autophosphorylation of both cell lines (Clary and Reichardt 1994). In addition, a PC12 variant cell line (mR) engineered to over-express a truncated form of $p75^{NTR}$ lacking the extracellular domain has very little or no full length $p75^{NTR}$ and displays enhanced neuritic outgrowth in response to neurotrophin-3 (Benedetti et al. 1993). Although these results can be explained by other means, they suggest a potential role for $p75^{NTR}$ in decreasing the responsiveness of TrkA to non-preferred ligands (NT-3 and NT-4).

1.2.2.2.3 $p75^{NTR}$ may mediate apoptosis

The fact that $p75^{NTR}$ is a member of the TNF receptor superfamily and that some of the members of this family have been shown to mediate cell death (Dealtry et al. 1987;Larrick and Wright 1990;Itoh et al. 1991) led to investigations of whether $p75^{NTR}$ could regulate programmed cell death. Several studies have emerged recently that have suggested that $p75^{NTR}$ may induce cell death both *in vitro* and *in vivo*. In E4 chick embryonic retinal neurons, $p75^{NTR}$-expressing cells undergo apoptosis in a NGF-dependent manner in the absence of TrkA, suggesting that NGF mediates developmentally regulated cell death of these neurons through the $p75^{NTR}$ receptor (Frade et al. 1996). Moreover, genetic evidence that $p75^{NTR}$ mediates cell death processes in the developing mouse retina and spinal cord was also established. During axonal tract elongation, embryos carrying deletions in the NGF and $p75^{NTR}$ genes display an increased number of neurons in the optic cup and in the mantle zone of the spinal cord, neurons which normally express $p75^{NTR}$ (Frade and Barde 1999).

Among cholinergic neurons in the basal forebrain that normally express both $p75^{NTR}$ and TrkA in the adult, a subpopulation of neurons (~25%), which do not express TrkA, undergo naturally occurring cell death during postnatal day 6 and 15 in contrast to the $p75^{NTR}$ null mice where the same does not occur (Van der Zee et al. 1996). Overexpression of the intracellular domain of $p75^{NTR}$ in transgenic mice resulted in profound reductions in numbers of sympathetic and peripheral sensory neurons, two cell types which normally express $p75^{NTR}$, and of central neurons of the neocortex where little or no $p75^{NTR}$ is found (Majdan et al. 1997).

Other studies have also demonstrated *in vitro* that $p75^{NTR}$ can mediate apoptosis. Expression of the $p75^{NTR}$ receptor in immortalized neuronal cells results in a faster rate of apoptosis after serum withdrawal (Rabizadeh et al. 1993). In rat terminally differentiated primary oligodendrocytes that do not express TrkA, NGF binding to $p75^{NTR}$ induces apoptosis, accompanied by an increase in ceramide levels and JNK activity (Casaccia-Bonnefil et al. 1996). Sympathetic neurons sustained by low quantities of NGF, which express $p75^{NTR}$, TrkA, and little TrkC, undergo apoptosis when exposed to BDNF (Bamji et al. 1998).

The available evidence indicates that decision between survival or death in cells which express $p75^{NTR}$ and TrkA may be dependent on the level of activity, or the molar ratio of the two receptors. $p75^{NTR}$-mediated cell death occurs when no TrkA is present or when TrkA levels are lowered. Some studies have shown that in the periphery, the ratio of $p75^{NTR}$ to TrkA varies depending on the developmental stage (Wyatt and Davies 1993;Wyatt and Davies 1995). Therefore when TrkA activity is high, $p75^{NTR}$ death signaling is repressed.

Although $p75^{NTR}$ has been shown to mediate apoptosis in rat neonatal oligodendrocytes (Casaccia-Bonnefil et al. 1996), we have recently shown that $p75^{NTR}$ may not induce the same response in human oligodendrocytes (Ladiwala et al. 1998). Adult human oligodendrocytes grown in three different culture conditions express $p75^{NTR}$ but no TrkA but do not undergo apoptosis upon addition of NGF (Ladiwala et al. 1998). These latest contrasting results could reflect differences in gene expression between species, and also suggest that factors other than NGF, $p75^{NTR}$ and TrkA, may play a role in apoptotic $p75^{NTR}$ signaling.

1.2.2.3 Signaling and $p75^{NTR}$

Although little is known about the mechanisms of $p75^{NTR}$ function, $p75^{NTR}$ can signal independently of other neurotrophin receptors. How $p75^{NTR}$ elicits these signaling events still remains undefined. However, recent evidence has emerged providing clues as to some of the molecules which may be implicated in $p75^{NTR}$ signaling. The findings that the cytokine TNF-α is able to activate the sphingomyelin cycle (Kim et al. 1991) and that the $p75^{NTR}$ and TNF-α receptors display some homology led to the hypothesis that $p75^{NTR}$ could potentially signal through the sphingomyelin cycle. Indeed, in Rat T9 anaplastic glioblastoma cells and NIH 3T3 fibroblasts, $p75^{NTR}$ activation by NGF results in the hydrolysis of sphingomyelin coupled to the accumulation of ceramide (Dobrowsky et al. 1994). Subsequently, a study by the same group demonstrated that all neurotrophins could induce sphingomyelin hydrolysis (Dobrowsky et al. 1995) through $p75^{NTR}$ activation. Ceramide is a bioactive metabolite which has been implicated in the mediation of anti-mitogenic pathways leading to cessation of cell growth, cell differentiation, and apoptosis (Pushkareva and Hannun 1994). Interestingly, TrkA can suppress $p75^{NTR}$-induced sphingomyelin hydrolysis since in PC12 cells, NGF mediates sphingomyelin hydrolysis only when TrkA tyrosine kinase is down-regulated by K252A.

Sphingomyelin hydrolysis resulting in ceramide production may allow the $p75^{NTR}$ receptor to modulate TrkA activation. In fact, short term treatment of PC12 cells with ceramide analogues results in reduced NGF-induced TrkA activation (MacPhee and Barker 1997). Furthermore, ceramide treatment of PC12 cells increases TrkA receptor phosphoserine content, suggesting a potential selective phosphorylation event that occurs as a function of ceramide production (MacPhee and Barker 1997). This type of receptor

modulation mechanism has been previously shown in other systems, notably with TNF affecting the IRS-1 receptor phosphoserine content (Kanety et al. 1995), and with the modification of the phosphorylation state of the epidermal growth factor receptor (Countaway et al. 1989;Olson and Pledger 1990;Northwood and Davis 1990;Theroux et al. 1992).

In addition to ceramide signaling, $p75^{NTR}$ may signal through a NFkB-dependent pathway. Primary Schwann cells (extracted from the rat sciatic nerve) were cultured overnight, treated with 100 ng/ml of NGF for various periods of time and then immunostained with an anti p65 (also called RelA: a subunit of NFkB) antibody which recognizes only the activated form of NFkB (Carter et al. 1996). NGF induction of $p75^{NTR}$ resulted in strong immunostaining after 30 minutes which lasted up to at least 4 hours. Furthermore, only NGF displayed this effect which could not have been caused by TrkA since the Schwann cells did not express any TrkA protein; confirmed by RT-PCR (Carter et al. 1996).

Although these results indicate that $p75^{NTR}$ is able to induce NFkB, our own investigations of whether $p75^{NTR}$ can activate NFkB have given different results. We have shown that concentrations of up to 500 ng/ml of NGF, BDNF, and NT-3 do not produce any significant NFkB activation in PCNAs, 293HEKs, Hela cells, NIH 3T3 fibroblasts, and A875 melanoma cells under normal conditions, but that when PCNA cells are kept in severely stressful conditions, we do see a modest NFkB activation (Bhakar et al. 1999).

Activation of NFkB has been shown to block apoptotic cell death to many different insults, such as TNF-α (Beg and Baltimore 1996;Liu et al. 1996;Van Antwerp et al. 1996). We have shown that activation of $p75^{NTR}$ by NGF in adult human oligodendrocytes, which

do not express TrkA, does not cause apoptosis, and that nuclear translocation of NFkB is detected in these cells suggesting a role for NFkB in preventing cell death (Ladiwala et al. 1998).

Proteins which directly interact with $p75^{NTR}$ to mediate signaling have not been identified but a recently published study has demonstrated that TNF receptor superfamily members share common downstream effectors, notably, the TRAF proteins (McKean et al. 1995). In addition, it has also been reported that possible converging signals between different receptors are able to amplify ligand-induced NFkB activation (McKean et al. 1995). Since $p75^{NTR}$ is part of the TNF receptor superfamily, it would not be surprising to find that $p75^{NTR}$ signaling occurs through similar pathways as other members of this family. In fact, we have shown in 293HEK cells transiently transfected with $p75^{NTR}$ that exposure to TNF and NGF for two hours results in a decrease in NFkB activation compared to TNF alone suggesting that ligand signaling through $p75^{NTR}$ can affect signaling of other related receptors. Furthermore this response is modified upon longer exposure to TNF and NGF. After 10 hours of NGF and TNF treatment, a robust increase in NFkB activation is seen compared to TNF alone. None of these effects are obtained with NGF alone (Bhakar et al, 1999).

1.3 Gene Ablation

The initial work on the neurotrophins by Levi-Montalcini and colleagues, demonstrated that NGF was required by specific subsets of peripheral neurons during the period of target tissue innervation and that the proportion of NGF-dependant neurons surviving during that period was determined by the amount of NGF produced by the target tissue (Levi-Montalcini 1987). The 'neurotrophin hypothesis', formulated from this

early work explained the phenomenon of programmed cell death that occurs in the developing nervous system (Purves 1988;Oppenheim 1991). The neurons which make a connection with the target tissue and establish a continuous supply of neurotrophins survive the period of neuronal cell death. Observations supporting this hypothesis were subsequently found. For instance, a strong correlation was observed in the PNS between the density of sympathetic innervation and the levels of NGF mRNA, and in the CNS between the density of basal forebrain cholinergic neuron innervation and regional brain NGF mRNA quantities (Korshing and Thoenen 1983;Korshing et al. 1985).

In order to confirm what was originally thought to be the role of the neurotrophins as well as to determine new roles the neurotrophins might play in the developing and adult nervous system, mice lacking each neurotrophin and their receptors were generated.

1.3.1 NGF and TrkA knockouts (Table 2)

Results from the NGF and TrkA knockouts are very similar confirming the role of TrkA as the receptor for NGF. The knockouts also establish the importance of NGF for the survival of sensory and sympathetic neurons (Crowley et al. 1994;Smeyne et al. 1994). The behaviour of theses animals are similar to the behaviors of wild type animals who have been prenatally exposed to NGF antisera (Johnson et al. 1980;Pearson et al. 1983) which are viable at birth but when compared to their wild type littermates, do not gain weight. They develop sores and signs of self-mutilations and most of them do not live past the first month postnatally (Johnson et al. 1980;Pearson et al. 1983). Further analysis of the mice shows a lack of responsiveness to nociceptive and thermoceptive stimuli. This is explained by an approximate 70% neuronal loss in the DRGs as well as an almost complete ablation of calcitonin gene-related peptide (CGRP)-positive cells, and a

Table 2: Characteristics of mice with NGF, and TrkA gene ablations

	NGF (-/-)	TrkA (-/-)
Phenotype	**die within 1 month; nociceptive loss; and ptosis**	**similar to NGF**
	% neuronal loss	
DRG	**70%**	**70-90%**
Trigeminal	**ND**	**70-90%**
SCG	**ganglia volume reduced (not quantified)**	**severely shrunken**
Motor neurons	**no loss**	**no loss**
CNS	**decreased AChE staining in basal forebrain**	**decreased ChAT staining in basal forebrain**

Taken from Conover and Yancopoulos, 1997; ND: not determined; AChE: acetylcholinesterase ChAT: cholineacetyltransferase

substantial reduction in the numbers of substance P-positive (SP) cells (Crowley et al. 1994;Smeyne et al. 1994;Minichiello et al. 1995). Other sensory neurons of the TrkA(-/-) and NGF(-/-) mice are also affected. For instance, a reduction of 70-90% (Smeyne et al. 1994) and 28% (Crowley et al. 1994) is seen in trigeminal neurons of TrkA(-/-) mice and in volume of nodose ganglion of NGF(-/-) mice, respectively.

In the case of sympathetic neurons, an 82-90% loss of superior cervical ganglion (SCG) volume occurs in NGF(-/-) mice and in TrkA(-/-) mice the SCGs have severely reduced sizes (Crowley et al. 1994;Smeyne et al. 1994). These neuronal losses results in ptosis and lack of iris dilation in both TrkA(-/-) and NGF(-/-) mice.

Other neurons believed to be NGF-dependent include basal forebrain cholinergic neurons but in TrkA(-/-) and NGF(-/-) mice, cholinergic neurons appeared normal. Closer examination showed that the NGF(-/-) mice exhibited reduced synthesis of the transmitter enzyme, choline acetyltransferase. In addition, cortical target fields of TrkA(-/-) mice showed a reduction in the activity of the axonal cholinesterase enzyme suggesting a potential role for NGF in mediating cholinergic neurotransmission (Crowley et al. 1994;Smeyne et al. 1994).

1.3.2 BDNF, NT-4, and TrkB knockouts (Table 3)

The TrkB(-/-) animals die within the first 24-48 postpartum hours but the BDNF(-/-) and NT-4(-/-) have less severe phenotype; for BDNF(-/-), the animals die within several weeks postnatally and for NT-4(-/-), the animals show no obvious behavioural deficit and have a normal lifespan. Further analysis reveals that mice lacking BDNF or NT-4 display a reduction in specific sensory neurons whereas motor and basal forebrain neurons are not affected (Jones et al. 1994;Ernfors et al. 1994a;Conover et al. 1995;Liu et al. 1995).

Table 3: Characteristics of mice with BDNF, NT-4, and TrkB gene ablations

	BDNF (-/-)	NT-4 (-/-)	TrkB (-/-)
Phenotype	**die within 2-3 weeks;viable balance & coordination deficits; breathing abnormalities**		**die within 2 weeks; balance & coordination deficits**
		% neuronal loss	
DRG	**30%**	**no loss**	**25-35%**
Trigeminal	**27-45%**	**no loss**	**60%**
NPG	**43-64%**	**56%**	**ND**
Vestibular	**82-98%**	**no loss**	**56%**
Cochlear	**7%**	**ND**	**15%**
Geniculate	**48%**	**50%**	**ND**
SCG	**no loss**	**ND**	**ND**
Motor neurons	**no loss**	**no loss**	**no loss**
CNS	**appears normal; alterations in neuropeptide levels**	**appears normal**	**appears normal**

Taken from Conover and Yancopoulos, 1997; ND: not determined

Interestingly, TrkB(-/-) mice display deficits similar to mice lacking both BDNF and NT-4, supporting the notion that BDNF and NT-4 are the principle ligands of TrkB. Although TrkB(-/-) mice were originally reported to display a 70% reduction of facial and spinal cord motor neurons (Klein et al. 1993), subsequent analysis revealed normal cell numbers in both neuron populations (Conover and Yancopoulos 1997). The vestibular system is important for sensing motion and for balance and BDNF(-/-) mice display abnormal vestibular system function resulting from a loss of more than 80% of the neurons in the vestibular ganglia (Jones et al. 1994;Ernfors et al. 1994b).

The nodose/petrosal ganglion complex (NPG) contains neurons that relay sensory inputs essential for the regulation of respiration, heart rate, and blood pressure to nuclei within the brain stem. The TrkB mRNA is detected in petrosal and nodose neurons (Ernfors et al. 1992;Dechant et al. 1993) and the mRNA for BDNF and NT-4 is seen in target tissues from fetal, newborn, and adult mice (Maisonpierre et al. 1990;Ibanez et al. 1993;Timmusk et al. 1993). In the BDNF(-/-) or the NT-4(-/-) mice, a neuronal loss of at least 50% is reported in the NPG complex (Jones et al. 1994;Ernfors et al. 1994a;Conover et al. 1995;Liu et al. 1995). However, when the same populations of cells are examined in the TrkB(-/-) or the BDNF/NT-4(-/-) a greater loss is seen (90%) (Conover et al. 1995;Erickson et al. 1996). The latter findings suggest that most of the neurons from the NPG complex, are either BDNF or NT-4 dependent for survival and that normal expression of both BDNF and NT-4 is essential for a normal complement of these neurons (Conover et al. 1995;Liu et al. 1995;Erickson et al. 1996). Consistent with this is the fact that mice which are BDNF(-/-) display a loss of a subpopulation of dopaminergic NPG neurons that innervate the carotid body and these neurons are not lost in NT-4(-/-) mice.

Due to the nature of the function of the carotid body, which is to sense changes in arterial O_2 and CO_2 levels and provide excitatory drive to ventilation, a loss of these neurons may explain why postnatal death is seen in the BDNF(-/-) and not in the NT-4(-/-) (Erickson et al. 1996). BDNF(-/-) and TrkB(-/-) mice also display a significant but incomplete loss of neurons in trigeminal and dorsal root ganglions (Klein et al. 1993).

Finally, investigations of the BDNF(-/-) and NT-4(-/-) mice suggest that BDNF and NT-4 do not mediate neuronal survival of neurons in the central nervous system. However, the absence of BDNF does result in a reduction in the levels of neuropeptides and calcium-binding protein expression in certain regions of the brain (Jones et al. 1994).

Other findings from studies on BDNF(-/-) mice demonstrate that the amount of neuronal survival in the NPG complex and vestibular ganglia is dependent on the number of functioning BDNF alleles (+/+, +/-, or -/-). This suggests that BDNF may be present in limiting quantities in the target tissues of these neurons which provides additional experimental support for the neurotrophin hypothesis (Erickson et al. 1996). The same cannot be said about NT-4 since only one allele supports survival of all NT-4 dependent NPG neurons (Erickson et al. 1996).

1.3.3 NT-3 and TrkC knockouts (Table 4)

Disruption of the NT-3 gene results in death within several weeks of birth with mice showing severe sensory and sympathetic neuronal loss. For instance, NT-3(-/-) display abnormal limb movement and placement suggesting disrupted proprioceptive function (Farinas et al. 1994;Tessarollo et al. 1994;Ernfors et al. 1994a). In the TrkC(-/-) mice, similar loss is detected although not as severe. These mice still die however within several weeks of birth (Klein et al. 1993). In contrast, motor neurons and major brain regions

Table 4: Characteristics of mice with NT-3, and TrkC gene ablations

	NT-3 (-/-)	TrkC (-/-)
Phenotype	**die within 1 month; abnormal movements and postures; loss of proprioception**	**die around 3 weeks; loss of proprioception**
	% neuronal loss	
DRG	**55-80%**	**19%**
Trigeminal	**60%**	**ND**
NPG	**50%**	**ND**
Vestibular	**23-34%**	**16%**
Cochlear	**85%**	**51%**
Geniculate	**25%**	**ND**
Superior jugular	**18%**	**ND**
SCG	**50%**	**ND**
Motor neurons	**no loss**	**no loss**
CNS	**appears normal**	**appears normal**

Taken from Conover and Yancopoulos, 1997; ND: not determined

appear unaffected since they develop normally in the absence of NT-3 (Farinas et al. 1994;Tessarollo et al. 1994;Ernfors et al. 1994a).

Mice lacking NT-3, display a loss of 55-78% of DRG neurons involved in proprioceptive functions (Farinas et al. 1994;Ernfors et al. 1994a). These include neurons which project to the primary endings of muscle spindles and golgi-tendon organs, lost in the absence of either TrkC or NT-3 (Klein et al. 1993;Farinas et al. 1994;Ernfors et al. 1994a;Kucera et al. 1995), as well as two subsets of cutaneous afferents, D-hair receptors and slowly adapting mechanoreceptors lost in the NT-3(-/-) mice (Airaksinen et al. 1996). In contrast, the TrkC(-/-) mice reveal a loss of only 19% of all DRG neurons (Klein et al. 1993). The larger ratio of neuronal loss in the NT-3(-/-) mice compared to the TrkC(-/-) mice indicates that NT-3 may be supporting the survival of other neurons which are not TrkC-positive.

Other sensory neuronal loss occurs in the cochlear (85%), trigeminal (61-65%), and nodose/petrosal (30%) ganglia. P10 to P15 mice lacking NT-3 display a loss of approximately 85% of cochlear ganglion neurons (Farinas et al. 1994;Ernfors et al. 1995) which contrasts with P1 mice lacking TrkC that exhibit a 51% loss (Klein et al. 1993). Whether this discrepancy in the percentage of lost neurons is due to the difference in the developmental stage or to NT-3 binding to other receptors, possibly TrkA, is not known. However, there is further evidence which supports that NT-3 may affect survival through other receptors since mice lacking NT-3 and TrkC display a loss of 23-34% and 16% of vestibular ganglia neurons, respectively (Farinas et al. 1994;Minichiello et al. 1995;Ernfors et al. 1995) demonstrating that a complete NT-3 ablation affects more neurons then the

TrkC ablation. Taken together, these results suggest a role for NT-3 in the development of a population of neurons of the vestibular and auditory systems.

Intriguingly, mice lacking both BDNF and NT-3 or both TrkB and TrkC are born with a complete loss of all vestibular and cochlear neurons (Ernfors et al. 1995;Fritzsch et al. 1995) suggesting that BDNF and NT-3 are important for inner ear development with each neurotrophin acting on distinct populations of neurons. However this does not rule out the possibility of neurotrophin having overlapping effects on more than one receptor.

In the case of sympathetic neurons, mice lacking NT-3 show an approximate 50% loss in SCG neurons (Farinas et al. 1994). Recent analysis of the NT-3(-/-) has demonstrated that the loss of sympathetic neuroblasts occurs prenatally and plateaus at around E17.5. This period corresponds to the suggested period of a switch from NT-3 to NGF-dependency (Dechant et al. 1993;Birren et al. 1993;DiCicco-Bloom et al. 1993).

1.3.4 $p75^{NTR}$ knockout (Table 5)

In order to determine the physiological function of the $p75^{NTR}$ receptor, a mouse strain carrying a null mutation in the gene encoding $p75^{NTR}$ was generated (Lee et al. 1992). The animals are fertile, viable, show no impairment of any organs and generally appear normal. Additional characterization however, revealed deficits in cutaneous innervation and heat sensitivity. Immunohistochemical procedures showed an almost complete loss in the CGRP-immunoreactive fibers of the skin as well as a significant reduction in the SP-immunoreactive fibers in sections of the transgenic mice compared to sections of the wild type mice. Moreover, a substantial loss of neurons in lumbar DRG of $p75^{NTR}$-deficient mice was reported which may explain the reduced heat sensitivity (Lee et al. 1994a;Dreetz et al. 1997). In addition, the loss of small diameter nerve fibers was also observed in the

Table 5: Characteristics of mice with $p75^{NTR}$ gene ablation

	$p75^{NTR}$ (-/-)
Phenotype	**viable; loss of heat sensitivity**
	% neuronal loss
DRG	**ganglia volume reduced (not quantified**
SCG	**pineal gland – no innervation; sweat gland innervation reduced or absent in some footpads; other sympathetic innervation appears normal.**
CNS	**ND**

Taken from Conover and Yancopoulos, 1997; ND: not determined

dermis. Regarding the sympathetic system, the mutation in the $p75^{NTR}$ gene had no effect on the size of sympathetic ganglia and did not affect iris and salivary gland sympathetic innervation density.

Other studies regarding mice deficient in the $p75^{NTR}$ receptor were performed (Lee et al. 1994b;Van der Zee et al. 1996;Yeo et al. 1996). Examination of sympathetic innervation in $p75^{NTR}$-deficient mice showed a lack of innervation in the pineal gland and reduced innervation in the sweat glands in particular footpads (Lee et al. 1994b). Among cholinergic neurons of the basal forebrain, a subpopulation of neurons which do not express TrkA and normally undergo naturally occurring cell death between postnatal day 6 and 15 do not go through this process in $p75^{NTR}$-deficient mice (Van der Zee et al. 1996). In addition, mice carrying a null mutation for $p75^{NTR}$ display an increase in cholinergic neuron size and hippocampal target innervation as well as an increase in the activity of the cholinergic neurotransmitter synthetic enzyme choline acetyltransferase (Yeo et al. 1996). Together, these results suggests that $p75^{NTR}$ may have different functions depending on the cellular context in which it is expressed. It can mediate apoptosis of a population of cholinergic neurons while facilitating the development of a population of sympathetic neurons.

Although these mice were revealing concerning potential functions of $p75^{NTR}$, additional studies demonstrated that other isoforms of the receptor were still expressed in those mice (Dechant and Barde 1997). Hence, caution has to be taken when considering these results since they do not arise from the complete loss of $p75^{NTR}$.

1.4 Rationale of Studies and Prososed Investigations

The neurotrophins are molecules crucial to the development and survival of neurons. Their importance in neuronal development is especially evident in the knockout mice where distinct cell populations are killed upon ablation of neurotrophin function either through the removal of the ligand or the receptor which mediates the biological responses of the ligand. For example, in both the NGF and TrkA knockout mice, a lack of responsiveness to nociceptive and thermoceptive stimuli is observed and explained by a loss of DRGs, calcitonin gene-related peptide-positive cells, and Substance P-positive cells (Crowley et al. 1994;Smeyne et al. 1994;Minichiello et al. 1995). The importance of the neurotrophins in the proper maintenance of the nervous system has been established. Hence, understanding the mechanisms by which the neurotrophins induce their biological responses may lead to the discovery of new therapies against neurological disorders.

We and others have previously shown that the $p75^{NTR}$ receptor increases the sensitivity of TrkA to NGF (Davies et al. 1993;Barker and Shooter 1994;Verdi et al. 1994). NGF is the preferred ligand for TrkA and $p75^{NTR}$ thus plays a role in facilitating the biological responses conferred by the preferred ligand through its cognate Trk receptor. Furthermore, this role of $p75^{NTR}$ may also be important for the other Trk receptors, TrkB and TrkC (Hantzopoulos et al. 1994). In contrast, other studies suggests that $p75^{NTR}$ may also play a role in decreasing the sensitivity of the Trk receptors to their non-preferred ligands (Benedetti et al. 1993;Ip et al. 1993;Clary and Reichardt 1994). However, this evidence is correlative and has not been shown directly. For instance, experiments performed in $PC12^{nnr5}$ cells expressing TrkA showed that blocking of $p75^{NTR}$ function using the polyclonal anti-$p75^{NTR}$ antibody REX caused an increase in NT3-mediated TrkA

autophosphorylation (Clary and Reichardt 1994) while in another study, a PC12 variant cell line (mR) expressing a truncated form of $p75^{NTR}$ lacking the extracellular domain and very little or no full length $p75^{NTR}$, displayed enhanced neuritic outgrowth in response to neurotrophin-3 (Benedetti et al. 1993). Although these results suggest a potential role for $p75^{NTR}$ in reducing non-preferred ligand-induced TrkA responsiveness, they do so indirectly and other mechanisms not involving $p75^{NTR}$ could account for these results.

In order to contribute to the field of neurotrophins, I have tried to determine whether or not $p75^{NTR}$ can alter Trk receptor activation. Using a direct method, the following questions were asked:

1. $p75^{NTR}$ has been shown to enhance NGF-mediated TrkA activation. Can this be reproduced in my cell system (in 293T cells)?
2. What are the domains of $p75^{NTR}$ which are essential to this functional interaction?
3. The role of $p75^{NTR}$ in reducing non-preferred ligand-mediated TrkA activation has been suggested (shown indirectly). Can $p75^{NTR}$ directly reduce NT-3 or NT-4 dependent activation of TrkA?

2. MATERIALS AND METHODS

Cell cultures: 293T fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% bovine calf serum in 5% CO_2 at 37 °C.

Calcium phosphate transient transfections of 293T cells: 293T cells were plated on 100 mm plates to a density of 1 x 10^6 cells/plate 24hr prior to transfection. The cell media was replaced 4 hrs prior the addition of the $CaHPO_4$ precipitate to the cells. The precipitate containing the plasmid DNA was formed by mixing solution 2 (250µl) to an equal amount of 2x HBS (pH 7.05; 283mM NaCl, 23mM Hepes, 1.5 mM Na_2HPO_4) while bubbling air via the use of an automatic pipettor. Solution 2 (for 1 plate of 293T cells) is prepared by adding in this order to H_2O (the amount of H_2O needed at this step is calculated in function of a total volume of 205µl of solution 2), 5µl of $CaCl_2$ (2M), 45µl of solution 1, 5µg of a $p75^{NTR}$ or vector control plasmid DNA, and 26µl of $CaCl_2$ (2M). This last step must be performed while bubbling air via the use of an automatic pipettor. Solution 1 (for 1 plate of 293T cells) is prepared by adding in any order the following reagents: 5µg of placental DNA (carrier DNA), 1µg to 5µg of TrkA plasmid DNA depending on the experimental goals, 0.1µg of eGFP plasmid DNA as an indicator of transfection efficiency, and H_2O to a total volume of 45µl. Once solution 2 is added to the HBS, a white precipitate should start to form. A waiting period of 10-20 min is needed to complete the precipitation step. Then 500µl of the precipitate solution can be added to each 100mm plate while slightly rotating the plate. Finally, the media on the cells should be replaced with fresh media approximately 18 hrs after addition.

Induction of 293T cells and Immunoprecipitation of TrkA: Approximately 48 hrs after transfection, the cells were washed once with 5 mls of DMEB (10 μM L-Glutamine, DMEM, 2.5 ml penicillin/streptomycin; Biowhittaker, 0.1% Bovine Serum Albumin; ICN). Then the washing media was removed and replace by 5 mls of induction media composed of DMEB with the appropriate concentrations of neurotrophins. Due to the importance of timing in these experiments, all washes and inductions were performed directly in the 37°C incubator. Neurotrophins were left on cells for exactly 10 min then the media was removed and the cells were washed immediately with ice cold tris-buffered saline [TBS: 10 mM Tris (pH 8.0), 150 mM NaCl] and placed on ice. Then 1ml of NP-40 lysis buffer [10 mM Tris (pH 8.0), 150 mM NaCl, 1% NP40; CalBiochem, 10 % Glycerol; Bioshop,] supplemented with sodium vanadate (1mM), aproptinin (1μg/ml), leupeptin (1μg/ml), phenanthroline (5mM), and phenyl methyl sulfonyl fluoride (PMSF; 1mM), was added to each plate and incubated with shaking at 4°C for 20 min. Next, the lysate was scraped off the plates using a rubber policeman and transferred to eppendorf tubes, vortexed, and spun down at 13000 rpm for 5 min. Nuclei pellets were discarded and 50μl of lysate was taken at this step, transferred to new tubes and designated as lysate. An equal amount of 2x Laemmli loading buffer [4% SDS, 100mM Dithiothreitol, 120mM Tris (pH 6.8), a sparkle of Bromophenol Blue, 10% Glycerol] was added to the lysates tubes. The tubes were vortexed and boiled for 5 min and then kept at -20°C for storage. Next, 4μl of the PAN-trk 203E polyclonal antibody (supplied by D.R. Kaplan), directed against the intracellular domain common to all trks, was added to the original tubes which were incubated on a rotator at 4°C for 2hrs. Following this incubation, 45μl

of protein-A sepharose (sigma: prepared as directed by manufacturer's specifications) was added and an additional incubation of 1hr was performed. Then the supernatant was removed and the sepharose beads were washed 3 times with 1ml of NP-40 lysis buffer. Following the washing step, the beads were re-suspended into 60 µl of 2x Laemmli loading buffer , boiled for 5 min and spun down. The sample buffer now containing the proteins eluted from the beads was transferred to new eppendorf tubes which were designated as Ip (immunoprecipitates). Tubes were kept at -20°C for storage.

Standard curve. One extra 100mm plate of 293T fibroblast was transfected with TrkA in order to be used as a TrkA pool for the production of a protein standard curve. The immunoprecipitation was performed the same way as the other plates but the protein-A sepharose beads were re-suspended into 90µl of 2x Laemmli loading buffer instead of 60µl. Then a serial dilution of this sample was performed by taking 60µl of the sample and transferring it to a new tube containing 24µl of Laemmli loading buffer, vortexing and repeating this step with 7 other tubes resulting in a total of 9 tubes each with a dilution factor of .71 compared to the previous one. 20µl of each samples were separated on acrylamide gels (see western blots) and the intensity of each TrkA bands was estimated by scanning of the image and analysis with the program Scion Image. Then a graph demonstrating the relationship between band intensity and relative protein concentration was plotted in order to estimate the relative differences in TrkA protein between each experimental samples. These were then normalized as a function of relative differences in TrkA protein and analyzed by western blot first for TrkA tyrosine autophosphorylation, and second for TrkA content (see western blots).

Detection of phosphotyrosine on TrkA Proteins from the Immunoprecipitated (Ip) samples were separated on 8% SDS-acrylamide gels and transferred to nitrocellulose. Then phosphotyrosine blots were blocked in TBST [10mM tris (pH 8.0), 150mM NaCl, and 0.2 % Tween 20] containing 2% BSA for 1hr and then rinsed 3 times and replaced with TBST containing an anti-phosphotyrosine monoclonal antibody (4G10: 1/1000; UBI). After an overnight incubation with shaking at 4°C the blots were washed 3 times and incubated, for approximately 1hr, with a horseradish peroxidase-conjugated goat anti-mouse secondary antibody, in blotto [10mM tris (pH 8.0), 150mM NaCl, 5% dry skim milk powder, 0.2% Tween 20]. Then all reactive bands were detected using enhanced chemiluminescence according to protocols provided by the manufacturer (Amersham).

Detection of the TrkA receptor. Following detection of TrkA phosphotyrosine levels, the membranes were washed from all antibodies by incubating them with agitation in stripping buffer (0.1mM β-mercaptoethanol, 2% SDS, 62.5mM Tris-HCl pH 6.8) for 30mins at approximately 70°C. Then the membranes were blocked in blotto for 1hr and re-probed with the polyclonal antibody RTA (a gift from Louis Reichardt, UCSF; 1/3000) directed against the TrkA extracellular. After an overnight incubation with shaking at 4°C the blots were washed 3 times in TBST and incubated with a horseradish peroxidase-conjugated protein-A secondary antibody for approximately 1 hr. All reactive bands were then detected using enhanced chemiluminescence according to protocols provided by the manufacturer (Amersham).

Detection of $p75^{NTR}$. The cell lysates were separated on 10% SDS-acrylamide gels and transferred to nitrocellulose membranes and then blocked in blotto for approximately 1 hr. Then the blocking solution was removed and replaced with blotto containing a polyclonal

anti-p75 antibody and incubated overnight with shaking at 4°C. For the detection of either the full length $p75^{NTR}$ receptor, or the intracellular domain of the receptor an antibody directed against the intracellular domain of $p75^{NTR}$ was used (Buster). For the detection of the extracellular domain of the $p75^{NTR}$ receptor an antibody (9651) raised against the extracellular domain of $p75^{NTR}$ was used instead. The next day, the antibody solution was washed three times and incubated with a horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody, in blotto, for approximately 1 hr. All reactive bands were detected using enhanced chemiluminescence according to protocols provided by the manufacturer (Amersham). The p75NTR constructs used in these studies encoded the full length receptor, containing the

P75NTR FL - full length receptor – complete 152 amino acid intracellular domain

P75NTR ΔDD - receptor lacking distal 43 amino acids of the intracellular domain

P75NTR ΔICD - receptor lacking distal 150 amino acids of the intracellular domain

P75NTR ICD - distal 150 amino acids of intracellular domain, preceded by a iMET

P75NTR mICD - distal 150 amino acids of intracellular domain, preceded by a iMET and a myristoylation sequence derived from blk, a member of the src tyrosine kinase family.

3. RESULTS

3.1 $p75^{NTR}$ reduces basal TrkA tyrosine autophosphorylation

In order to investigate the functional interactions between $p75^{NTR}$ and TrkA, it was essential to develop an accessible cellular system. The 293T human kidney fibroblasts were chosen because they express the SV40 T-antigen, which transforms the cells into high expressers of genes which are controlled by viral promoters such as the CMV promoter, because they do not express any of the Trk receptors, because they do not produce high amounts of neurotrophins that could cause erroneous readings, and because transfected Trk receptors can be activated by physiological concentrations of neurotrophins (data not shown). Finally, these cells divide very rapidly and are very easy to maintain in culture.

We first attempted to investigate the effects exerted by $p75^{NTR}$ on TrkA by examining changes in basal TrkA tyrosine phosphorylation resulting from the co-expression of $p75^{NTR}$. We transiently transfected identical amounts of TrkA plasmid into each plate and varied the amounts of $p75^{NTR}$ plasmid. 293T fibroblasts were transfected with TrkAII, the neuronal isoform that contains the extra 18bp sequence (Barker et al. 1993) and with either 0, 1, or 5 ug of $p75^{NTR}$, harvested two days later in NP-40 lysis buffer and immunoprecipitated with an anti-TrkA antibody (203B: supplied by David Kaplan). For each individual sample, lysate was collected for direct analysis prior to immunoprecipitation. The levels of TrkA tyrosine phosphorylation (the initial response to the activation of the receptor: see introduction) was monitored using western blots; proteins of immunoprecipitated samples were separated using 8% SDS polyacrylamide gels, transferred to nitrocellulose and immunoblotted with an antiphosphotyrosine

antibody 4G10. Trk protein levels were subsequently obtained by stripping, and re-probing the membrane with an anti-TrkA antibody RTA, directed against the extracellular domain. In addition, protein levels of $p75^{NTR}$ were analyzed from lysates using $p75^{NTR}$ specific antibodies by western blots, (see Materials and Methods). Analysis of TrkA phosphotyrosine revealed that the expression of $p75^{NTR}$ in 293T cells causes a dramatic reduction in basal TrkA tyrosine phosphorylation (Figure 1). Furthermore, as p75NTR levels climbed, basal TrkA activity was reduced. This experiment was repeated three times with similar results. Thus, the $p75^{NTR}$ receptor reduces levels of basal TrkA activity in a dose-dependent manner.

3.2 $p75^{NTR}$ enhances NGF-mediated TrkA tyrosine autophosphorylation in 293T human fibroblasts

We have shown that $p75^{NTR}$ enhances NGF-mediated TrkA activation in PC12 cells (Barker and Shooter 1994). In order to determine whether $p75^{NTR}$ could do the same in 293T fibroblasts, we transfected the cells with either TrkA, or TrkA and $p75^{NTR}$ and two days later induced the cells with 10ng/ml of NGF for 10 mins. The cell lysates were then harvested and TrkA immunoprecipitated. Although the analysis of TrkA phosphotyrosine levels indicated that $p75^{NTR}$ could decrease basal TrkA activity, in this experiment, we did not detect any significant potentiating effect of p75 on TrkA at 10ng/ml of NGF in 293T fibroblasts (Figure2: compare lane 2 with lane 4).

Because we have shown that the $p75^{NTR}$ receptor can modulate NGF-mediated TrkA activity at low concentrations of NGF (Barker and Shooter 1994) and we have seen no increase in TrkA's response to NGF at 10ng/ml of NGF (Figure 2), we set out to determine if the stoichiometry of the receptors and the concentration of NGF used in our

experiments could affect the modulatory effects of $p75^{NTR}$ on TrkA. In order to answer these separate questions, 293T cells were transfected with 5ug of TrkA and with either 0, 1, or 5ug of $p75^{NTR}$ plasmid. The cells were then treated two days later with either 1ng/ml of NGF (considered to be the suboptimal concentration for NGF-dependent survival) or 10 ng/ml of NGF (optimal concentration) for 10 min. We found that the activation of TrkA by NGF was potentiated to the same extent by the presence of $p75^{NTR}$ whether expressed at low or high levels (Figure3: compare column 2 with 3 and column 5 with 6). In contrast, $p75^{NTR}$ exerts a stronger effect on NGF-dependent TrkA activity when the concentration of NGF is in the suboptimal range (Figure3: compare column 1 and 2 with column 4 and 5 or column 1 and 3 with column 4 and 6). This last observation could explain why the previous figure (Figure2) did not reveal an enhancement in TrkA activity upon $p75^{NTR}$ expression in the presence of 10ng/ml of NGF. In addition, this may also explain why a previous study investigating the effects of $p75^{NTR}$ on TrkA was unable to show a p75NTR-dependent modulation of TrkA activation since in this study, a concentration of 50ng/ml , five times the optimal concentration observed in our experiments, was used (Clary and Reichardt 1994).

3.3 Structure/function studies reveals domains of $p75^{NTR}$ important for the modulation of TrkA activity

In order to hypothesize on potential mechanisms by which $p75^{NTR}$ modulates TrkA activity, we performed experiments to ask which domains of $p75^{NTR}$ are involved in modulating TrkA activity. We transfected the 293T fibroblasts with various $p75^{NTR}$ mutants in combination with TrkA and asked whether they could regulate TrkA activity by

examining phosphotyrosine blots. The cloning of the $p75^{NTR}$ deletion mutants was done previously in the lab.

We examined the response of trkA to NGF in the presence and absence of either the full length or mutant $p75^{NTR}$ receptors. In the first experiment we examined the role of $p75^{NTR}$ mutants that lacked either a portion of the cytoplasmic tail containing the death domain, or most of the intracellular domain of the $p75^{NTR}$ receptor; these constructs were designated ΔDD and ΔICD, respectively. This was done to see if the intracellular domain of $p75^{NTR}$ is involved in the facilitation of trkA activation by NGF. Thus 293T fibroblasts were transfected with TrkA and the various $p75^{NTR}$ DNA constructs and two days later the cells were washed in DMEB and induced for 10 minutes with 10 ng/ml of NGF. Then samples were collected and TrkA immunoprecipitated using the anti-Trk antibody 203B. The results demonstrate that the presence of full length $p75^{NTR}$ facilitates NGF-mediated activation of TrkA (Figure4: comparing the phosphotyrosine band of column 4 with column 2). Interestingly, when the $p75^{NTR}$ mutants, ΔICD and ΔDD, are used in place of the full length receptor, the enhancement of the response of TrkA to NGF can still be observed (Figure4). These latest results suggest that the intracellular domain of $p75^{NTR}$ lacking in the ΔICD-$p75^{NTR}$ mutant is not essential in order for $p75^{NTR}$ to enhance NGF-mediated TrkA activity. In this experiment, we have used 10ng/ml of NGF and the results clearly show an enhancement in TrkA activity dependent on the presence of $p75^{NTR}$. These results vary from previous observations (Figure 2 and Figure 3) and this variability could be explained in two ways. First, there are likely to be experiment to experiment variations in transfection procedures. Second, different aliquots of neurotrophins may have slightly different bioactivity and the ability of the 293 cells to support this effect could

change with increasing passage number. It would appear that slight changes in the concentration of receptor and/or bioactive ligand can have dramatic effects on the receptors functional collaboration.

In the next experiment, we tested the role of the extracellular domain of the $p75^{NTR}$ receptor in modulating the TrkA response to NGF. In order to answer this question, we used two $p75^{NTR}$ mutants lacking the extracellular and transmembrane domain of the $p75^{NTR}$ receptor. The first mutant, called the ICD (for intracellular domain) consists simply of the intracellular domain of $p75^{NTR}$. The second $p75^{NTR}$ mutant designated as the mICD (for myristoylated intracellular domain) consists of the same protein attached to a myristoylation tag which targets it to the plasma membrane. Thus, the ICD is expressed freely in the cytoplasm while the mICD is attached to the cytoplasmic membrane. The mICD was used in this experiment to see if the protein had to be localized to the membrane in order to function.

293T fibroblasts were transfected with TrkA and with either the vector alone or the plasmid constructs corresponding to the full length, ICD, or mICD $p75^{NTR}$ receptors. The cells were later treated with either nothing, or 10 ng/ml of NGF for 10 min. and harvested immediately after. TrkA was immunoprecipitated and phosphotyrosine levels of normalized TrkA protein were examined. The NGF induction of the TrkA receptor was, as expected, potentiated by the presence of the full length $p75^{NTR}$ (Figure5: compare the increase in tyrosine phosphorylation between column 4 and column 2). However, neither the ICD or the mICD had any affect on the TrkA response to NGF. These results indicates that the protein motifs encompassed by the extracellular domain, or transmembrane domain, or both domains of the $p75^{NTR}$ receptor are essential for $p75^{NTR}$

modulation of TrkA's response to NGF. The question of whether the extracellular domain, or the transmembrane domain can, on their own, elicit the increase in NGF-mediated TrkA activity still remains.

Interestingly, the ICD and mICD, which lack the extracellular and transmembrane domain of $p75^{NTR}$, were able to elicit a reduction in basal TrkA activity (Figure5: compare column 1 with column 3,5, and 7), although the ICD displays more potency than the mICD in this regard. This suggests that the intracellular domain of $p75^{NTR}$ may be responsible for the reduction in basal TrkA tyrosine phosphorylation seen when the full length $p75^{NTR}$ is expressed.

3.4 $p75^{NTR}$ reduces NT-3/NT-4-mediated TrkA tyrosine autophosphorylation

Previous studies have suggested indirectly that the $p75^{NTR}$ receptor may down-regulate TrkA activity induced by non-preferred ligands (Benedetti et al. 1993;Ip et al. 1993;Clary and Reichardt 1994). In order to investigate directly whether $p75^{NTR}$ can modulate the activation of TrkA by non-preferred ligands, we have used the same experimental model described previously in this study to answer this question; transient transfections in 293T cells.

293T cells were transfected with TrkA and with or without $p75^{NTR}$ and two days later induced with either nothing or with 100 ng/ml of NT-4. Comparison of the phosphotyrosine levels of each samples confirms that NT-4 induction of TrkA can be reduced by the expression of $p75^{NTR}$ (Figure 6). Interestingly, the levels of tyrosine phosphorylation of TrkA when $p75^{NTR}$ is expressed, are approximately equivalent to basal TrkA tyrosine phosphorylation (seen by comparing column 1 and 3 in Figure6). Taken

together, these results suggests that $p75^{NTR}$ can almost completely repress NT-4-mediated TrkA activation.

Next, we looked at NT-3 induction of TrkA in the presence and absence of $p75^{NTR}$. 293T fibroblasts were transfected with either TrkA, or TrkA + p75 and later, treated with nothing or 100ng/ml of NT-3 for a time period of 10 min. NT-3 was able to induce TrkA activation but a down-regulation of NT-3-mediated TrkA tyrosine phosphorylation resulted from the expression of $p75^{NTR}$. Taken together, the results suggest that a potential function for $p75^{NTR}$ may be to induce the formation of a TrkA receptor which is more sensitive to its primary ligand and less sensitive to its non-preferred, secondary ligands.

4. DISCUSSION

The neurotrophins regulate the maintenance and development of the nervous system. The identification of the neurotrophin cell surface receptors has greatly increased our knowledge of the signal transduction events initiated by the neurotrophins. Although the vast majority of events initiated by the neurotrophins arise from the activation of one or more of the members of the Trk tyrosine kinase family, emerging evidence has revealed that the $p75^{NTR}$ receptor may exhibit important functions as a modulator of the members of this family.

Whereas the cellular responses mediated by the Trk family of receptors are fairly well understood, the biological functions of the $p75^{NTR}$ receptor still remain poorly defined. However, evidence has emerged recently that suggests $p75^{NTR}$ functionally collaborates with the TrkA receptor. I have examined this partnership in regard to the $p75^{NTR}$ modulation of ligand-dependent TrkA activity. We have previously shown that in PC12 cells, reducing the amount of NGF binding to $p75^{NTR}$ or increasing the affinity of NGF for $p75^{NTR}$ results in a lowered response of TrkA to NGF (Barker and Shooter 1994). Similarly, a second study showed that co-expression of $p75^{NTR}$ in MAH cells enhanced the NGF-dependent tyrosine autophosphorylation of the TrkA receptor and that the biological responses of the TrkA receptor were affected similarly (Verdi et al. 1994). These results provide direct evidence that $p75^{NTR}$ can enhance NGF-mediated TrkA activation. Our data further supports this conclusion since we show that $p75^{NTR}$ can potentiate the TrkA response to NGF in 293T cells particularly at lower concentrations of NGF. This indicates that $p75^{NTR}$ can mediate the same type of modulatory effect on the TrkA

receptor whether expressed in a neuronal or non-neuronal cell context. It seems very likely then that whatever results we have obtained in fibroblasts will reveal significant in neuronal cell types as well.

4.1 A mutant $p75^{NTR}$ receptor lacking most of the intracellular domain can still potentiate NGF-mediated TrkA activation

We have taken the structure/function analysis approach in order to determine the domains of $p75^{NTR}$ involved in modulating TrkA activity. 293T cells were transfected with $p75^{NTR}$ mutants that contain deletions from the full length receptor. We have shown that the expression of a $p75^{NTR}$ mutant receptor lacking most of the intracellular domain can affect NGF-dependent TrkA activation similarly to the wild type $p75^{NTR}$ receptor. Interestingly, the level of TrkA activity is even slightly higher in cells expressing this mutant compared to cells expressing the full length $p75^{NTR}$, which could suggest that the intracellular domain of $p75^{NTR}$ contains a regulatory domain which suppress this effects. This hypothesis is supported by a study which showed that a truncated form of $p75^{NTR}$ lacking most of the intracellular domain displayed a much greater ability to potentiate the functional response of the Trk family members to the neurotrophins (Hantzopoulos et al. 1994) then its wild type counterpart. Furthermore, we show that the intracellular domain alone, or containing a myristoylation tag sequence, does not produce any effects on the initial response of TrkA to NGF. The myristoylation was added to the intracellular domain in order to target this protein to the cytoplasmic membrane and see if this truncated form of the receptor could induces effects when in closer proximity of TrkA. These results demonstrate that the intracellular domain of the $p75^{NTR}$ receptor does not play a role in mediating this effect whether free in the cytoplasm or joined to the cell

membrane. Furthermore, this suggests that the mechanisms by which $p75^{NTR}$ alters TrkA activity in 293T cells are not intracellular, but rather arise from interactions with the TrkA protein from either the transmembrane or extracellular domains.

With these types of structure/function analyses we can make hypotheses on various mechanisms of functions. One mechanism which could account for the observations described above and which agrees with the binding properties of both the $p75^{NTR}$ and TrkA receptor is one that we have previously stated (Barker and Shooter 1994). The $p75^{NTR}$ receptor may act to increase the local concentration of NGF in the microenvironment surrounding the TrkA neurotrophin receptor. The binding kinetics of NGF with the $p75^{NTR}$ receptor show rapid association and dissociation at 37°C (Sutter 1979). This fact and the fact that the internalization mediated by $p75^{NTR}$ is slow and unaffected by NGF binding, creates a stable pool of $p75^{NTR}$ receptors which can rapidly exchange NGF. This rapid exchange of NGF may contribute to raise the local NGF concentration relative to the overall NGF concentration. In contrast, the TrkA receptor displays slow rates of association and dissociation with NGF when expressed by itself (Hartman et al. 1992;Meakin et al. 1992). In addition, in conditions which are considered physiological TrkA has been shown to be rapidly internalized upon binding to NGF (Jing et al. 1992). Therefore, it seems very likely that the activation of the TrkA receptor is dependent on the rate at which NGF can associate to the TrkA receptor. This may be why $p75^{NTR}$ can enhance the NGF-mediated TrkA response, by increasing the local concentration of NGF, which augments the likelihood of a stable TrkA-NGF association. Whether or not the two receptors have to directly associate with one another in order for $p75^{NTR}$ to be able to modulate TrkA activity still remains undetermined. However some

recently published studies have revealed a possible physical interaction between the two receptors. Fluorescence recovery after photobleaching has revealed that in PC12^{nnr5} and Sf9 insect cells, the expression of the TrkA receptor slows the lateral mobility of the p75^{NTR} protein along the cytoplasmic membrane suggesting the possible formation of some type of complex (Wolf et al. 1995). Immunocytochemical analyses of Sf9 cells infected with p75^{NTR} and TrkA baculovirus has shown that the two receptors copatch together (Ross et al. 1996). Furthermore, evidence which supports our function/structure analyses is the fact that the extracellular domain of p75^{NTR} was found to be sufficient in order to get copatching of the two receptors (Ross et al. 1996). A physical interaction between the two receptors was also shown directly by co-immunoprecipitation and cross-linking experiments (Huber and Chao 1995;Gargano et al. 1997;Bibel et al. 1999).

A second mechanism by which p75^{NTR} could enhance the response of TrkA to NGF may be by altering the conformation of the TrkA receptor. This change in conformation could allow faster binding of NGF to TrkA resulting in a true high affinity TrkA receptor for NGF. The kinetics of binding of this new high affinity NGF receptor would result in more NGF binding to TrkA at lower NGF concentrations. Both mechanisms described above would be very effective at low NGF concentrations but would become inoperative at higher NGF concentrations. The idea that p75^{NTR} can alter TrkA affinity has been the center of considerable debate. Originally, the TrkA receptor alone was thought to produce a NGF high-affinity binding site. A small amount of high affinity sites were reported, by equilibrium binding analyses, in cells expressing only TrkA receptors (Klein et al. 1991a). In addition, co-expression of p75^{NTR} in COS cells did not increase the amount of high affinity binding site per cell (Jing et al. 1992). In contrast, it has been proposed

that both $p75^{NTR}$ and TrkA may be required for the formation of a high affinity binding site. Membrane isolates from cells expressing both receptor have been reported to show some high affinity NGF-binding site while these high affinity sites were not detected in cells expressing only TrkA (Hempstead et al. 1991). When $p75^{NTR}$ was introduced into fibroblasts expressing TrkA using recombinant herpes simplex virus (HSV-1), a new binding site was unmasked, consistent with the high-affinity NGF binding site (Battleman et al. 1993).

The key to the ability of $p75^{NTR}$ to generate high-affinity was uncovered recently in a series of experiments (Mahadeo et al. 1994). By examining the binding kinetics of NGF to the two receptors in PC12 cells which overexpressed the receptors alone, or in combination, it was demonstrated that co-expression of $p75^{NTR}$ and TrkA increased the rate of association of NGF to TrkA by 25-fold (Mahadeo et al. 1994). Thus, by combining the fast association rate of NGF to TrkA (resulting from $p75^{NTR}$ expression) and fast internalization rate of TrkA (typical response of TrkA), we get the alleged high affinity NGF binding site.

A similar mechanism of action was proposed recently for the TNF receptors (Tartaglia et al. 1993). Disruption of TNF binding to the TNF-R2 receptor has been shown to results in less binding of TNF to the TNF-R1 receptor and decreased signaling by the TNF-R1 receptor. Furthermore, TNF-R1 and TNF-R2 display similar kinetic trends to TrkA and $p75^{NTR}$ respectively. The TNF-R1 receptor exhibits slow association and dissociation rates to TNF, whereas TNF-R2 manifests rapid association and dissociation rates. The modulatory effects exerted on the TNF-dependent activation of TNF-R1 by TNF-R2 were additionally supported by the generation of mice deficient in TNF-R2

(Erickson et al. 1994). These mice exhibit increase resistance to TNF-induced death, a biological response normally characteristic of TNF binding to TNF-R1.

4.2 The $p75^{NTR}$ receptor influences the specificity of ligand dependent phosphorylation of TrkA

As previously stated, the $p75^{NTR}$ receptor enhances NGF-mediated TrkA activation. In adddition, we show using a direct approach that co-expression of the $p75^{NTR}$ receptor in 293T cells attenuates NT-3/NT-4-dependent activation of TrkA to levels similar to basal TrkA activation. Thus, the $p75^{NTR}$ receptor can increase the specificity of ligand-dependent TrkA activation. As such, $p75^{NTR}$ can facilitate the activation of TrkA by the preferred ligand NGF and simultaneously inhibit the interactions of TrkA with the non-preferred ligands, NT-3 and NT-4. Of special relevance are previous experiments which suggests that $p75^{NTR}$ increases ligand specificity. For instance, sympathetic neurons originating from $p75^{NTR}$(-/-) mice display a greater sensitivity to NT-3 than their counterpart cells in the wild type animals (Lee et al. 1994a) and PC12 cells which express lower levels of the full length $p75^{NTR}$ receptor show a greater responsiveness to NT-3 (Benedetti et al. 1993). More importantly, a recent report has demonstrated directly that $p75^{NTR}$ could down-regulate NT-4/NT-3-mediated TrkB activation while BDNF induction of TrkB was not affected (Bibel et al. 1999). Excluding our results, this latest study is the only published report which shows a direct link between the expression of $p75^{NTR}$ and a decrease in non-preferred ligand-mediated activation of a Trk tyrosine kinase family member.

4.3 $p75^{NTR}$ reduces Basal TrkA activation

We have shown that $p75^{NTR}$ reduces basal TrkA tyrosine phosphorylation. In addition, we show by structure/function analysis that the intracellular domain of the $p75^{NTR}$ receptor may be capable, on its own, of eliciting this reduction in TrkA basal phosphorylation. This suggest that potential mechanisms by which $p75^{NTR}$ reduces basal TrkA phosphorylation may come from interactions of TrkA with the $p75^{NTR}$ intracellular domain alone, or in association with intracellular signal transducers.

In fact, the $p75^{NTR}$ receptor has been shown to mediate intracellular signaling on its own. First, the $p75^{NTR}$ cytoplasmic domain lacks intrinsic kinase activity but can associate with certain protein such as ERK1 and ERK2, an undefined 120/104 kDa kinase, and two uncharacterized phosphoproteins (Ochmichi et al. 1991;Volonte et al. 1993;Canossa et al. 1996). Whether $p75^{NTR}$ mediates a reduction in basal TrkA activity through these proteins remains unknown. In addition, the $p75^{NTR}$ receptor also contains a mastoparan-like domain which indicates that it may interact with G-proteins (Feinstein and Larhammar 1990). Nevertheless, evidence for G-proteins playing a role in $p75^{NTR}$ signaling are lacking.

Another potential candidate for $p75^{NTR}$ intracellular signaling is ceramide, a product resulting from the hydrolysis of sphingomyelin. We know that all members of the neurotrophin family have been shown to mediate, through the $p75^{NTR}$ receptor, sphingomyelin hydrolysis resulting in ceramide accumulation in cell types such as Rat T9 anaplastic glioblastoma cells and NIH 3T3 fibroblasts (Dobrowsky et al. 1994;Dobrowsky et al. 1995). Subsequently, increases in ceramide levels resulting from $p75^{NTR}$ induction were also shown in cells of the central nervous system such as primary cultures of

mesencephalic neurons and oligodendrocytes (Casaccia-Bonnefil et al. 1996;Blochl and Sirrenberg 1996). Ceramide is known to be a potent second messenger which is implicated in the mediation of antimitogenic pathways leading to cessation of cell growth, cell differentiation, and apoptosis (Pushkareva and Hannun 1994). Therefore ceramide may be involved in $p75^{NTR}$ signal transduction resulting in a reduction in basal TrkA phosphorylation. Consistent with this hypothesis, we have previously shown that BDNF pretreatment (a non-ligand for TrkA) of PC12 cells reduces NGF^{3T}-mediated TrkA tyrosine autophosphorylation and that C_2-ceramide can also produce this effect (MacPhee and Barker 1997). However, whether or not ceramide is really involved in $p75^{NTR}$-mediated down-regulation of basal TrkA activity is not known. The $p75^{NTR}$ receptor may also signal through the NFkB and JNK pathways to down-regulate TrkA activity since evidence establishing a link between $p75^{NTR}$ and these pathways has been presented (Casaccia-Bonnefil et al. 1996;Carter et al. 1996;Ladiwala et al. 1998).

We have shown that the full length $p75^{NTR}$ receptor reduces basal TrkA activation and that the cytoplasmic domain, on its own can also elicit this effect. The fact that ceramide down-regulates TrkA activity (MacPhee and Barker 1997), and that the transfection of a chimeric receptor consisting of the EGF ligand binding domain coupled to the $p75^{NTR}$ cytoplasmic domain enables the EGF-meditated production of ceramide in cells expressing the receptor chimera (Yan et al. 1991), supports the conjecture that the activation of the $p75^{NTR}$ cytoplasmic domain results in a down-regulation of basal TrkA activity through a ceramide-dependent pathway. Moreover, another study reported that a $p75^{NTR}$ deletion mutant lacking amino acids 249-305 of the cytoplasmic domain still retained NGF binding capabilities but was incapable of inducing sphingomyelin hydrolysis in fibroblasts

(Hempstead et al. 1990). This demonstrates that the structural requirements that regulate the coupling of $p75^{NTR}$ to the production of ceramide and potentially to the reduction in TrkA basal activity, may be contained within the cytoplasmic domain of the p75 receptor.

Although structure/function analyses on $p75^{NTR}$'s involvement in the reduction of NT-3/NT-4-mediated TrkA activation were not performed, results indicating that $p75^{NTR}$ reduces both non-preferred ligand-mediated (NT-3, NT-4) and basal TrkA activity hints at the possibility that the mechanisms of functions of these separate events may occur through the same signaling pathway.

4.4 The modulatory effects of $p75^{NTR}$ on other members of the Trk tyrosine kinase family

Whether $p75^{NTR}$ is involved in tightening the specificity of ligand-dependent activation of the other Trk family members in 293T cells still remains to be determined. Nevertheless, previous studies have revealed potential modulatory effects of $p75^{NTR}$ on TrkB and TrkC. A truncated form of the $p75^{NTR}$ receptor, lacking most of the cytoplasmic domain, was able to functionally collaborate with TrkA, TrkB, and TrkC to potentiate ligand-dependent growth of NIH 3T3 fibroblasts (Hantzopoulos et al. 1994) supporting the idea that $p75^{NTR}$ could modulate all Trk family members.

Evidence designating a role for $p75^{NTR}$ in reducing the response of TrkB to its non-preferred ligands has also been reported. $PC12^{nnr5}$ cells transfected with the TrkB receptor (express both the $p75^{NTR}$ and TrkB receptor) need 100-fold higher concentrations of NT-3 to induce TrkB autophosphorylation, neurite outgrowth, and survival in serum-free conditions compared to TrkB expressing NIH 3T3 fibroblasts (Ip et al. 1993). In addition, co-expression of $p75^{NTR}$ in 293A fibroblasts results in the reduction of NT-3/NT-

4 mediated TrkB activation, whereas BDNF-dependent activation of TrkB is unaffected (Bibel et al. 1999).

As previously stated, $p75^{NTR}$ may modulates TrkA activity by physically interacting with it. If $p75^{NTR}$ functions to modulate the biological responses of all Trk members in the same way, then we could postulate that this type of physical interaction may also occur between $p75^{NTR}$ and TrkB and TrkC. Indeed a physical association has been shown between $p75^{NTR}$ and all of the members of the Trk family. The study reported the co-immunoprecipitation of $p75^{NTR}$ with TrkA, TrkB, and TrkC from 293A fibroblasts, demonstrating that not only can TrkA physically interact with $p75^{NTR}$ but TrkB and TrkC can apparently do so as well (Bibel et al. 1999).

In contrast, NGF-dependent neurons from the knockout mice display a shift, compared to wild type animals, in their survival dose-response curves which is not manifested by either BDNF or NT-3-dependent neurons suggesting that the ability of $p75^{NTR}$ to increase neurotrophin responsiveness of the Trk receptors may be limited, in vivo, to TrkA (Davies et al. 1993).

4.5 Physiological relevance of the modulation of TrkA activity by $p75^{NTR}$

In 1905, Sherrington extended Ramon y Cajal's views by showing that communication between neurons and target tissues occurred via chemical transmissions at the synaptic junctions (Sherrington 1905). To provide order in the developing nervous system, organizational input and regulatory mechanisms assuring that only proper connections are formed would be both expected and necessary. Such a system was unmasked with the discovery of the first neurotrophin, NGF (Levi-Montalcini and Hamburger 1953). Further work on NGF revealed that it was required by specific subsets of peripheral neurons

during the period of target tissue innervation and that the proportion of NGF-dependant neurons surviving during that period was determined by the amount of NGF produced by the target tissue (Hamburger et al. 1981). The 'neurotrophin hypothesis', was formulated from this early work, stating that the neurons which make a connection with the target tissue and establish a continuous supply of neurotrophins survive the period of neuronal cell death (Purves 1988). Intriguingly, studies have indicated that the levels of neurotrophins, in vivo, are found in limiting quantities which sets neurons in a state of competition for the availability of neurotrophins (Thoenen 1991). Taking this into consideration, a role for $p75^{NTR}$ as a potentiator of NGF-mediated TrkA activity would be of crucial importance physiologically, since it would enable TrkA to mediate responses to the lower concentrations of NGF. Thus cells expressing both receptors would have a crucial advantage over cells which only express TrkA during the developmental stages of the nervous system. Observations supporting this theory were made in the $p75^{NTR}$ knockout mice. These animals display a progressive loss of target innervation by sensory and sympathetic neurons suggesting that these neurons exhibit a decreased sensitivity to the low concentrations of NGF (Lee et al. 1992;Lee et al. 1994a). Consistent with this observation is the fact that embryonic trigeminal and dorsal root ganglion sensory neurons from the knockout mice need 3-4-fold higher concentrations of NGF, than the same neurons from normal mice, to elicit half-maximal survival. Moreover, at higher concentrations of NGF the survival response for the two populations of neurons is the same supporting the hypothesis that $p75^{NTR}$ can alter NGF-mediated TrkA activity only at low concentrations of NGF (Davies et al. 1993;Lee et al. 1994a).

Strikingly the neurotrophins exhibit a high degree of sequence homology. Therefore it's not surprising to observe some cross-activation between various neurotrophins and their receptors. In fact, although NT-3 and NT-4 can activate TrkA, the response is never as substantial as when TrkA is activated by NGF, suggesting that these ligands are non-preferred ligands exhibiting non-specific functional interactions with TrkA (Cordon-Cardo et al. 1991;Berkemeier et al. 1991;Ip et al. 1993). In addition, random collisions between TrkA receptors may result in spontaneous tyrosine autophosphorylation. While $p75^{NTR}$ increases the response of TrkA to it's primary ligand NGF, we show that it also has the ability to down-regulate basal and non-specific TrkA activity. Such a role for $p75^{NTR}$ would prove very important physiologically during the period of neuronal development since a decrease in non-specific survival signals would allow for a better refinement of the neural network required in the mature nervous system.

Although the $p75^{NTR}$ receptor was initially regarded as a non-essential neurotrophin receptor which could not induce signal transduction events, it has become apparent that this receptor mediates crucial biological processes. Although some of these crucial roles are antagonistic (i.e. inducing cell death or facilitating cell survival), they are all tied to one main goal which is to help refine the neural connectivity imperative to the normal function of the nervous system. Neurotrophins, or deficits in their function, have been suggested to play some role in neurological diseases (Funabashim et al. 1988;Higgins and Mufson 1989;Mufson et al. 1989;Gall and Isackson 1989;Phillips et al. 1991;Hyman et al. 1991;Isackson et al. 1991;Strada et al. 1992;Spina et al. 1992) as well as in tumor progression (Kim et al. 1991;Dobrowsky et al. 1994;Dobrowsky et al. 1995). Therefore, understanding the mechanisms underlying neurotrophin function may help uncover future

therapies which could impact on the life of many human beings. Over the coming years, further investigations on the functional mechanisms of neurotrophins will no doubt uncover important scientific facts that will draw the "dream" of curing these diseases closer to reality.

5. FIGURES AND LEGENDS

Figure 1:

Modulation of basal TrkA activation upon $p75^{NTR}$ expression. 293T fibroblasts were transiently transfected with with TrkA and with either 0, 1, or 5ug of $p75^{NTR}$ and harvested two days later. TrkA phosphotyrosine levels were detected using western blotting and TrkA proteins levels were subsequently analysed by first stripping the phosphotyrosine blots and re-probing the blots with an anti-TrkA antibody (RTA; see Materials and Methods). TrkA pY, TrkA, $p75^{NTR}$ are used to designate TrkA phosphotyrosine levels, TrkA protein, and $p75^{NTR}$ protein, respectively. The results show a reduction in basal TrkA activation resulting from the co-expression of $p75^{NTR}$.

Figure 1

Figure 2:

Comparison of TrkA tyrosine autophosphorylation in the absence and presence of $p75^{NTR}$ when uninduced or induced with 10ng/ml of NGF. 293T cells were transfected with either nothing, TrkA, or TrkA and $p75^{NTR}$ and two days later, stimulated with NGF (10ng/ml). Immunoprecipitations and western blots were performed as described in Materials and Methods. $p75^{NTR}$ reduces basal TrkA tyrosine phosphorylation.

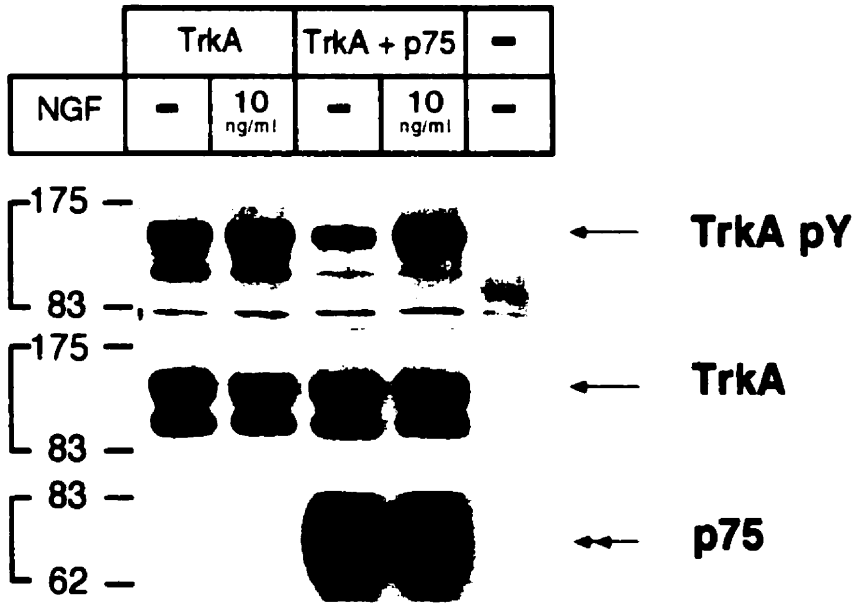
TrkA
TrkA + p75
-
NGF
-
10 ng/ml
-
10 ng/ml
-
175
83
175
83
83
62
TrkA pY
TrkA
p75

Figure 2

Figure 3:

Dose-response curve showing the activation of TrkA in response to different $p75^{NTR}$ levels and NGF concentrations. 293T fibroblasts were transfected with TrkA and varying amounts of $p75^{NTR}$, and two days later stimulated with either 1ng/ml, or 10ng/ml of NGF for 10 minutes and harvested. TrkA was immunoprecipitated and phosphotyrosine blots were used for analysis of TrkA activity. The data indicates that $p75^{NTR}$ expression potentiates the response of TrkA to NGF most effectively at lower NGF concentrations.

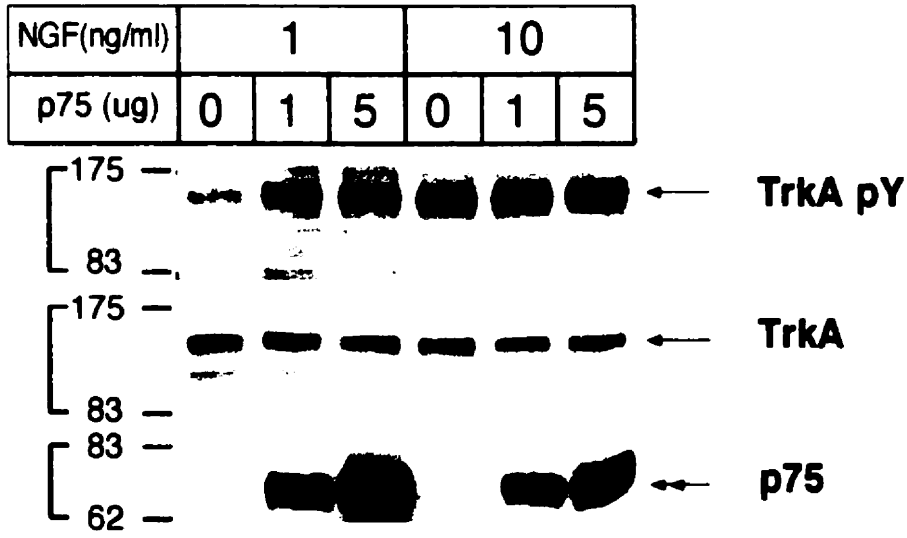

Figure 3

Figure 4:

Structure function analysis of $p75^{NTR}$ mutants lacking regions of the intracellular domains. 293T fibroblasts were transfected with TrkA, and full length $p75^{NTR}$ or $p75^{NTR}$ mutants lacking intracellular regions of the receptor and induced two days later with nothing or 10 ng/ml of NGF for 10 mins. Cells lysates were immunoprecipitated with a polyclonal antibody raised against the intracellular region that is common to all trk family members. $p75^{NTR}$ blots were performed using 293T cell lysates instead of immunoprecipitates. ΔDD and ΔICD, are used to designate, $p75^{NTR}$ mutants lacking the putative death domain and most of the intracellular domain, respectively. $p75^{NTR}$ can potentiate NGF-dependent TrkA activation, and interestingly, the $p75^{NTR}$ mutant which lacks most of the intracellular domain of the receptor still retains the functional interaction with TrkA

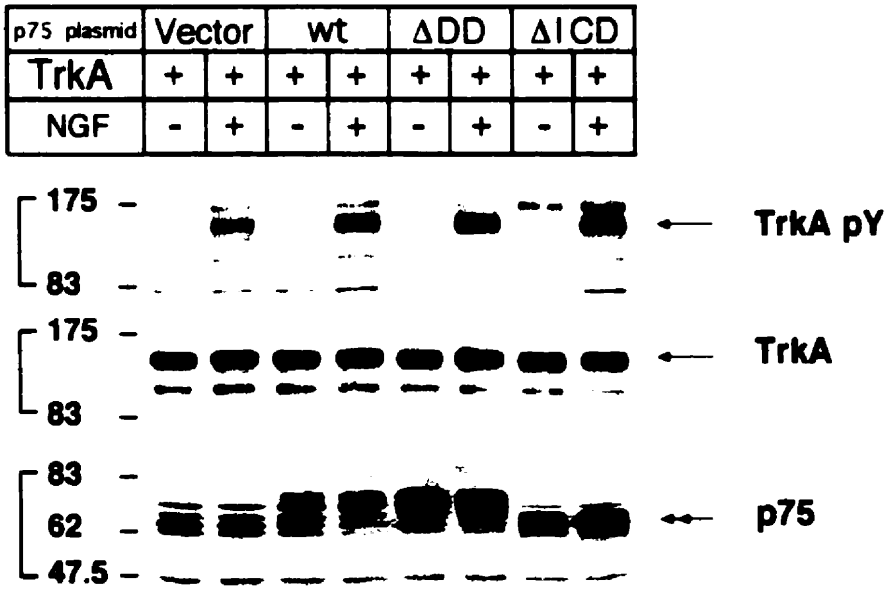
p75 plasmid
Vector
wt
ΔDD
ΔICD
TrkA
NGF
175
83
TrkA pY
175
83
TrkA
83
62
47.5
p75

Figure 4

Figure 5:

Structure function analysis of $p75^{NTR}$ mutants lacking all of the extracellular and transmembrane domains. 293T cells expressing transiently TrkA and either the full length $p75^{NTR}$ (wt) or the $p75^{NTR}$ intracellular domain alone (ICD) or the $p75^{NTR}$ intracellular domain containing a myristoylation tag (mICD), were induced with 10ng/ml of NGF. Phosphotyrosine levels of each experimental samples were visualized using a phosphotyrosine-specific antibody (4G10) and compared between each samples. Although the full length $p75^{NTR}$ receptor was able to enhance the NGF-induced TrkA autophosphorylation, the ICD and mICD could not produce the same effects. In addition, the $p75^{NTR}$ receptor caused a decrease in basal levels of TrkA phosphorylation (seen by comparing lane 1 to lane 3) and this reduction in basal TrkA activity was maintained even when $p75^{NTR}$ mutants lacking the extracellular and transmembrane domains were expressed instead of wild type $p75^{NTR}$.

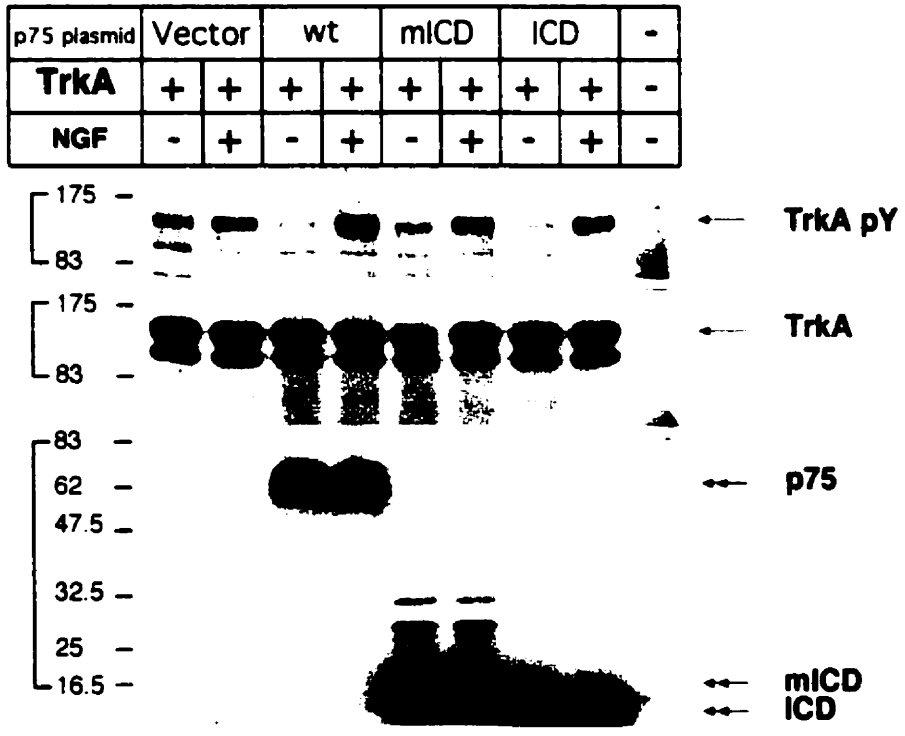
p75 plasmid
Vector
wt
mICD
ICD
-
TrkA
+ + + + + + + + -
NGF
- + - + - + - + -
175
83
175
83
83
62
47.5
32.5
25
16.5
TrkA pY
TrkA
p75
mICD
ICD

Figure 5

Figure 6:

Comparison of TrkA's response to NT-4 in the presence and absence of $p75^{NTR}$ expression. All cells were transfected with TrkA and with nothing or $p75^{NTR}$. Two days later cells were induced with 100ng/ml of NT-4 for 10 mins. Phosphotyrosine levels were visualized and compared using western blotting. Although NT-4 could activate TrkA in cells containing no $p75^{NTR}$, TrkA was not activated by NT-4 when we expressed $p75^{NTR}$ in the same cells.

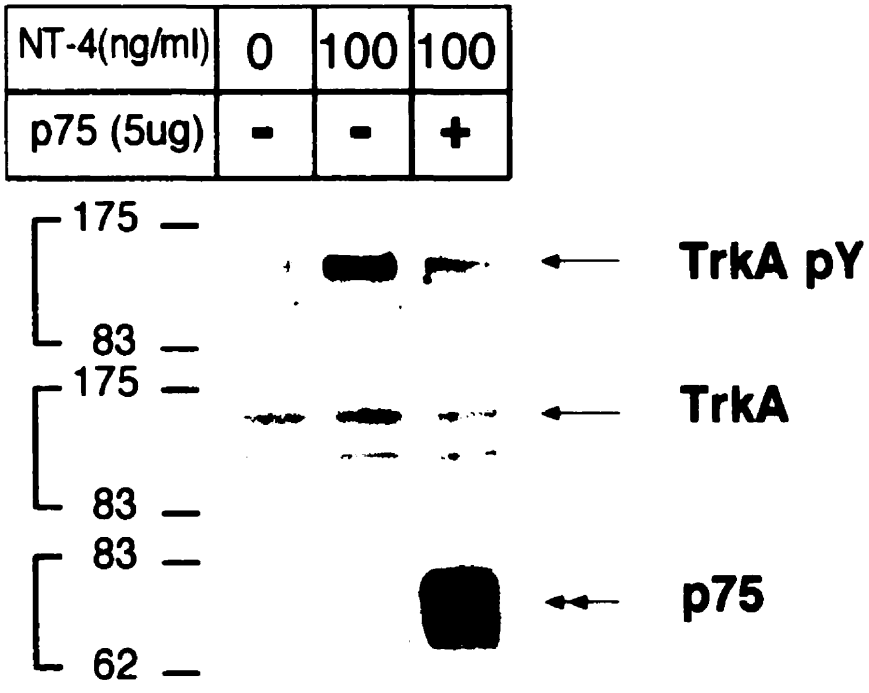

Figure 6

Figure 7:

Comparison of TrkA's response to NT-3 in the presence and absence of $p75^{NTR}$ expression. All cells were transfected with TrkA and with nothing or $p75^{NTR}$. Two days later cells were induced with 100ng/ml of NT-3 for 10 mins. $p75^{NTR}$ was able to decrease the response of TrkA to NT-3, a non-preferred ligand.

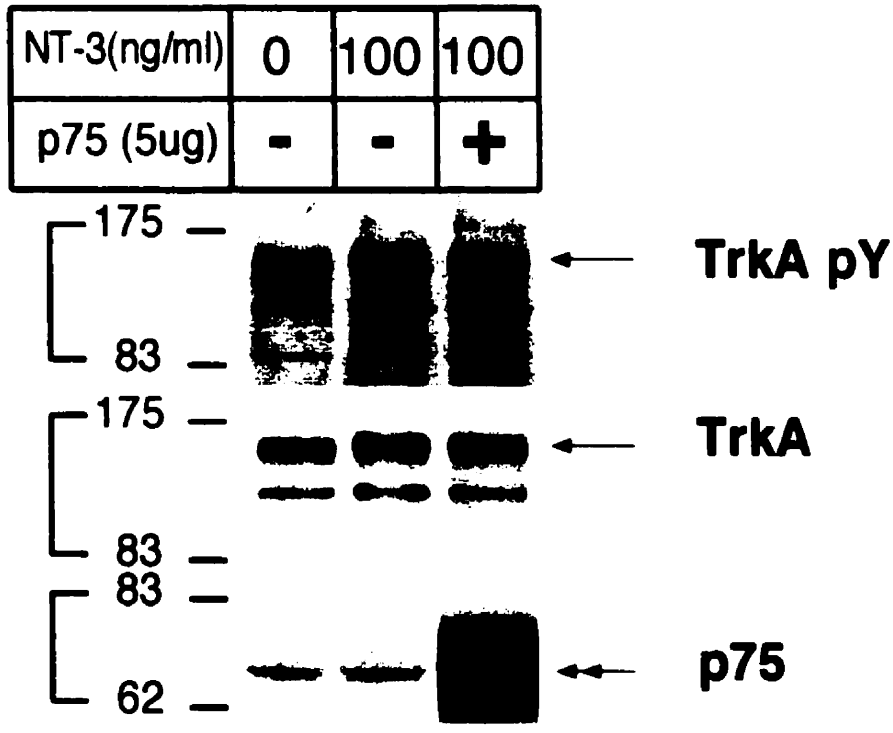

Figure 7

6. REFERENCES

Airaksinen, M. S., Koltzenburg, M., Lewin, G. R., Masu, Y., Helbig, C., Wolf, D. E., Brem, G., Toyka, K. V., Thoenen, H., and Meyer, M. (1996). Specific subtypes of cutaneous mechanoreceptors require neurotrophin-3 following peripheral target innervation. *Neuron* **16**, 287-295.

Angletti, R. H. and Bradshaw, R. A. (1971). Nerve growth factor from mouse submaxillary gland: Amino acid sequence. *Proc.Natl.Acad.Sci.USA* **68**, 2417-2420.

Angletti, R. H., Bradshaw, R. A., and Wade, R. D. (1971). Subunit structure and amino acid sequence of mouse submaxillary gland nerve growth factor. *Journal of Molecular Biology* **10**, 463-469.

Baldwin, A. M., Bitler, C. M., Welcher, A. W., and Shooter, E. M. (1992). Studies on the structure and binding properties of the cysteine-rich domain of rat low affinity nerve growth factor receptor (p75NGFR). *J.Biol.Chem.* **267**, 8352-8359.

Bamji, S. X., Majdan, M., Pozniak, C. D., Belliveau, D. J., Aloyz, R., Kohn, J., Causing, C. G., and Miller, F. D. (1998). The p75 Neurotrophin Receptor Mediates Neuronal Apoptosis and is Essential for Naturally Occurring Sympathetic Neuron Death. *J.Cell Biology* **140**, 911-923.

Bandtlow, C. E., Heumann, R., Schwab, M. E., and Thoenen, H. (1987). Cellular localization of nerve growth factor synthesis by in situ hybridization. *EMBO J.* **6**, 891-899.

Barde, Y. A., Edgar, D., and Thoenen, H. (1980). Sensory neurons in culture; Changing requirements for survival factors during embryonic development. *Proc.Natl.Acad.Sci.USA* **77**, 1199-1203.

Barde, Y. A., Lindsay, R. M., Monard, D., and Thoenen, H. (1978). New factor released by cultured glioma cells supporting survival and growth of sensory neurons. *Nature* **274**, 818-819.

Barker, P. A., Lomen-Hoerth, C., Gensch, E. M., Meakin, S. O., Glass, D. J., and Shooter, E. M. (1993). Tissue-specific Alternative Splicing Generates Two Isoforms of the *trkA* Receptor. *J.Biol.Chem.* **268**, 15150-15157.

Barker, P. A. and Shooter, E. M. (1994). Disruption of NGF Binding to the Low Affinity Neurotrophin Receptor p75NTR Reduces NGF Binding to TrkA on PC12 Cells. *Neuron* **13**, 203-215.

Battleman, D. S., Geller, A. I., and Chao, M. V. (1993). HSV-1 Vector-Mediated Gene Transfer of the Human, Nerve Growth Factor Receptor p75hNGFR Defines High-Affinity NGF Binding. *Journal of Neuroscience* **13**, 941-951.

Baxter, R. M., Cohen, P., Obermeier, A., Ulrich, A., Downes, C. P., and Doza, Y. N. (1995). Phosphotyrosine residues in the nerve-growth-factor receptor (trkA). Their role in the activation of inositol phospholipid metabolism and protein kinase cascades in pheochromocytoma (PC12) cells. *European Journal of Biochemistry* **234**, 84-91.

Beg, A. A. and Baltimore, D. (1996). An essential role for NFkB in preventing TNF-α-induced cell death. *Science* **274**, 782-784.

Belliveau, D. J., Krivko, I., Kohn, J., Lachance, C., Pozniak, C. D., Rusakov, D., Kaplan, D. R., and Miller, F. D. (1997). NGF and NT-3 both activate TrkA on sympathetic neurons but differentially regulate survival and neuritogenesis. *J.Cell Biology* **136**, 374-388.

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

Berger, E. A. and Shooter, E. M. (1977). Evidence for pro-beta-nerve growth factor, a biosynthetic precursor to beta-nerve growth factor. *Proc.Natl.Acad.Sci.USA* **74**, 3647-3651.

Berkemeier, L. R., Winslow, J. W., Kaplan, D. R., Nikolics, K., Goeddel, D. V., and Rosenthal, A. (1991). Neurotrophin-5: A novel neurotrophic factor that activates trk and trkB. *Neuron* 7, 857-866.

Bhakar, A. L., Roux, P. P., Lachance, C., Kryl, D., Zeindler, C., and Barker, P. A. (1999). The p75 neurotrophin receptor (p75NTR) alters tumor necrosis factor-mediated NF-kappaB activity under physiological conditions, but direct p75NTR-mediated NF-kappaB activation requires cell stress. *J. Biol. Chem.* **274**, 21443-21449

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

Birren, S. J., Lo, L., and Anderson, D. J. (1993). Sympathetic neuroblasts undergo a developmental switch in trophic dependence. *Development* **119**, 597-610.

Blochl, A. and Sirrenberg, C. (1996). Neurotrophins stimulate the release of dopamine from rat mesencephalic neurons via Trk and $p75^{LNTR}$ receptors. *J.Biol.Chem.* **271**, 21100-21107.

Canossa, M., Twiss, J. L., Verity, A. N., and Shooter, E. M. (1996). $p75^{NGFR}$ and TrkA receptors collaborate to rapidly activate a $p75^{NGFR}$-associated protein kinase. *EMBO J.* **15**, 3369-3376.

Carroll, S. L., Silos-Santiago, I., Frese, S. E., Ruit, K. G., Milbrandt, J., and Snider, W. D. (1992). Dorsal root ganglion neurons expressing *trk* are selectively sensitive to NGF deprivation in utero. *Neuron* **9**, 779-788.

Carter, B. D., Kaltschmidt, C., Kaltschmidt, B., Offenhauser, N., Bohm-Matthaei, R., and Baeuerle, P. A. (1996). Selective Activation of NF-kappa B by Nerve Growth Factor through the Neurotrophin Receptor p75. *Science* **272**, 542-545.

Casaccia-Bonnefil, P., Carter, B. D., Dobrowsky, R. T., and Chao, M. V. (1996). Death of oligodendrocytes mediated by the interaction of nerve growth factor with its receptor $p75^{NTR}$. *Nature* **383**, 716-719.

Chao, M. V. (1992). Neurotrophin receptors: a window into neuronal differentiation. *Neuron* **9**, 583-593.

Clary, D. O. and Reichardt, L. F. (1994). An alternatively spliced form of the nerve growth factor receptor TrkA confers an enhanced response to neurotrophin-3. *Proc.Natl.Acad.Sci.USA* **91**, 11133-11137.

Cohen, S. and Levi-Montalcini, R. (1956). A nerve growth stimulating factor isolated from snake venom. *Proc.Natl.Acad.Sci.USA* **42**, 571-574.

Conover, J. C., Erickson, J. T., Katz, D. M., Bianchi, L. M., Poueymirou, W. T., McClain, J., Pan, L., Helgren, M., Ip, N. Y., Boland, P., Friedman, B., Wiegand, S., Vejsada, R., Kato, A. C., DeChiara, T. M., and Yancopoulos, G. D. (1995). Neuronal deficits not involving motor neurons in mice lacking BDNF and/or NT-4. *Nature* **375**, 235-238.

Conover, J. C. and Yancopoulos, G. D. (1997). Neurotrophin Regulation of the Developing Nervous System: Analyses of Knockout Mice. *Reviews in the Neurosciences* **8**, 13-27.

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

Countaway, J. L., Northwood, I. C., and Davis, R. J. (1989). Mechanism of phosphorylation of the epidermal growth factor receptor at threonine 669. *J.Biol.Chem.* **264**, 10828-10835.

Crowley, C., Spencer, S. D., Nishimura, M. C., Chen, K. S., Pitts-Meek, S., Armanini, M. P., Ling, L. H., McMahon, S. B., Shelton, D. L., Levinson, A. D., and Phillips, H. S. (1994). Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. *Cell* **76**, 1001-1011.

Davies, A. M., Lee, K. F., and Jaenisch, R. (1993). p75NTR-Deficient Trigeminal Sensory Neurons Have an Altered Response to NGF but Not to Other Neurotrophins. *Neuron* **11**, 565-574.

Dealtry, G. B., Naylor, M. S., Fiers, W., and Balkwill, F. R. (1987). DNA fragmentation and cytotoxicity caused by tumor necrosis factor is enhanced by interferon-gamma. *European Journal of Immunology* **17**, 689-693.

Dechant, G. and Barde, Y. A. (1997). Signalling through the neurotrophin receptor p75NTR. *Current Opinion in Neurobiology* 7, 413-418.

Dechant, G., Biffo, S., Okazawa, H., Kolbeck, B., Pottgiesser, J., and Barde, Y. A. (1993). Expression and binding characteristics of the BDNF receptor chick TrkB. *Development* **119**, 545-558.

DiCicco-Bloom, E., Friedman, W. J., and Black, I. B. (1993). NT-3 stimulates sympathetic neuroblast proliferation by promoting precursor survival. *Neuron* **11**, 1101-1111.

Distefano, P. S. and Johnson, E. M. J. (1988). Identification of a truncated form of the nerve growth factor receptor. *Proc.Natl.Acad.Sci.USA* **85**, 270-274.

Dobrowsky, R. T., Jenkins, G. M., and Hannun, Y. A. (1995). Neurotrophins induce sphingomyelin hydrolysis. *J.Biol.Chem.* **270**, 22135-22142.

Dobrowsky, R. T., Werner, M. H., Castellino, A. M., Chao, M. V., and Hannun, Y. A. (1994). Activation of the sphingomyelin cycle through the low-affinity neurotrophin receptor. *Science* **265**, 1596-1599.

Dreetz, M., Tindrup, T., and Jakobsen, J. (1997). Personnal Communication found in: Bergmann, I., Priestley, J.V., McMahon, S.B., Brocker, E-B., Toyka, K.V., Koltzenburg, M. (1997) Analysis of Cutaneous Sensory Neurons in Transgenic Mice lacking the Low Affinity Neurotrophin Receptor p75. *European Journal of Neurosciences* **9**, 18-28.

Ebendal, T., Larhammar, D., and Persson, H. (1986). Structure and expression of the chicken beta nerve growth factor gene. *EMBO J.* **5**, 1483-1487.

Ehrhard, P. B., Erb, P., Graumann, U., and Otten, U. (1993a). Expression of nerve growth factor and nerve growth factor receptor tyrosine kinase Trk in activated CD4-positive T-cell clones. *Proc.Natl.Acad.Sci.USA* **90**, 10984-10988.

Ehrhard, P. B., Ganter, U., Bauer, J., and Otten, U. (1993b). Expression of functional *trk* protooncogene in human monocytes. *Proc.Natl.Acad.Sci.USA* **90**, 5423-5427.

Erickson, J. T., Conover, J. C., Borday, V., Champagnat, J., Barbacid, M., Yancopoulos, G. D., and Katz, D. M. (1996). Transgenic mice lacking brain-derived neurotrophic factor exhibit visceral sensory neuron losses distinct from mice lacking NT-4 and display a severe developmental deficit in control of breathing. *Journal of Neuroscience* **1617**, 5361-5371.

Erickson, S. L., de Sauvage, F. J., Kikly, K., Carver-Moore, K., Pitts-Meek, S., Gillett, N., Sheehan, K. C., Schreiber, R. D., Goeddel, D. V., and Moore, M. W. (1994). Decreased sensitivity to tumour-necrosis factor but normal T-cell development in TNF receptor-2-deficient mice. *Nature* **372**, 560-563.

Ernfors, P., Ibanez, C. F., Ebendal, T., Olson, L., and Persson, H. (1990). Molecular cloning and neurotrophic activities of a protein with structural similarities to nerve growth factor: Development and topographical expression in the brain. *Proc.Natl.Acad.Sci.USA* **87**, 5454-5458.

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

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

Ernfors, P., Merlio, J. P., and Persson, H. (1992). Cells expressing mRNA for neurotrophins and their receptors during embryonic rat development. *European Journal of Neurosciences* **4**, 1140-1158.

Ernfors, P., Van De Water, T., Loring, J., and Jaenisch, R. (1995). Complementary roles of BDNF and NT-3 in vestibular and auditory development. *Neuron* **14**, 1153-1164.

Fainzilber, M., Smit, A. B., Syed, N. I., Wildering, P. M., Hermann, R. C., Van der Schors, C., Jimenez, K. W., Li, J., Van Minnen, A. G., Bulloch, M., Ibanez, C. F., and Geraerts, W. P. M. (1996). CRNF, a molluscan neurotrophic factor that interacts with the p75 neurotrophin receptor. *Science* **274**, 1540-1543.

Farinas, I., Jones, K. R., Backus, C., Wang, X.-Y., and Reichardt, L. F. (1994). Severe sensory and sympathetic deficits in mice lacking neurotrophin-3. *Nature* **369**, 658-661.

Feinstein, D. L. and Larhammar, D. (1990). Identification of a conserved protein motif in a group of growth factor receptors. *FEBS Letters* **272**, 7-11.

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

Frade, M. J., Rodriguez-Tebar, A., and Barde, Y. A. (1996). Induction of cell death by endogenous nerve growth factor through its p75NTR receptor. *Nature* **383**, 166-168.

Frazier, W. A., Boyd, L. F., and Bradshaw, R. A. (1973). Interaction of nerve growth factor with surface membranes: Biological competence of insoluble nerve growth factor. *Proc.Natl.Acad.Sci.USA* **70**, 2931-2935.

Frisen, J., Verge, V. M., Fried, K., Risling, M., Persson, H., Trotter, J., Hokfelt, T., and Lindholm, D. (1993). Characterization of glial *trk*B receptors: differential response to injury in the central and peripheral nervous systems. *Proc.Natl.Acad.Sci.USA* **90**, 4971-4975.

Fritzsch, B., Silos-Santiago, I., Smeyne, R., Fagan, A. M., and Barbacid, M. (1995). Reduction and loss of inner ear innervation in trkB and trkC receptor knockout mice: A whole mount Dil and scanning electron microscopic analysis. *Auditory Neurosciences* **1**, 401-417.

Funabashim, T., Sasaki, H., and Kimura, F. (1988). Intraventricular injection of antiserum to nerve growth factor delays the development of amygdloid kindling. *Brain Research* **458**, 132-136.

Gall, C. M. and Isackson, P. J. (1989). Limbic seizures increase neuronal production of messenger RNA for nerve growth factor. *Science* **245**, 758-761.

Gargano, N., Levi, A., and Alema, S. (1997). Modulation of nerve growth factor internalization by direct interaction between p75NTR and TrkA receptors. *J.Neurosci.Res.* **50**, 1-12.

Gotz, R., Koster, R., Winkler, C., Raulf, F., Lottspeich, F., Schartl, M., and Thoenen, H. (1994). Neurotrophin-6 is a new member of the nerve growth factor family. *Nature* **372**, 266-269.

Greene, L. A. and Kaplan, D. R. (1995). Early events in neurotrophin signaling via Trk and p75 receptors. *Current Opinion in Neurobiology* **5**, 579-587.

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

Hamburger, V., Brunso-Bechtold, J. K., and Yip, J. W. (1981). Neuronal death in the spinal ganglia of the chick embryo and its reduction by nerve growth factor. *Journal of Neuroscience* **1**, 60-71.

Hantzopoulos, P. A., Suri, C., Glass, D. J., Goldfarb, M. P., and Yancopoulos, G. D. (1994). The Low Affinity NGF Receptor, p75NTR, can Collaborate with Each of the Trks to Potentiate Functional Responses to the Neurotrophins. *Neuron* **13**, 187-201.

Hartman, D. S., McCormack, M., Schnubenel, R., and Hertel, C. (1992). Multiple trkA proteins in PC12 cells bind NGF with a slow association rate. *J.Biol.Chem.* **267**, 24516-24522.

Hempstead, B. L., Martin-Zanca, D., Kaplan, D. R., Parada, L. F., and Chao, M. V. (1991). High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor. *Nature* **350**, 678-683.

Hempstead, B. L., Patil, N., Thiel, B., and Chao, M. V. (1990). Deletion of cytoplasmic sequences of the nerve growth factor receptor leads to loss of high affinity ligand binding. *J.Biol.Chem.* **265**, 9595-9598.

Heumann, R., Korshing, S., Scott, J., and Thoenen, H. (1984). Relationnship between levels of nerve growth factor (NGF) and its messenger RNA in sympathetic ganglia and peripheral target tissues. *EMBO J.* **3**, 3183-3189.

Higgins, G. A. and Mufson, E. J. (1989). NGF receptor expression is decreased in the nucleus basalis in Alzheimers disease. *Experimental Neurology* **106**, 222-236.

Hofer, M., Pagliusi, S., Hohn, A., Leibrock, J., and Barde, Y. A. (1990). Regional distribution of brain-derived neurotrophic factor mRNA in the adult mouse brain. *EMBO J.* **9**, 2459-2464.

Hohn, A., Leibrock, J., Bailey, K., and Barde, Y. A. (1990). Identification and characterisation of a member of the nerve growth gactor/ brain-derived neurotrophic factor family. *Nature* **344**, 339-341.

Holland, D. R., Cousens, L. S., Meng, W., and Matthews, B. W. (1994). Nerve growth factor in different crystal forms displays structural flexibility and reveals zinc binding sites. *Journal of Molecular Biology* **239**, 385-400.

Holtzman, D. M., Li, Y., Parada, L. F., Kinsman, S., Chen, C. K., Valleta, J. S., Zhou, J., Long, J. B., and Mobley, W. C. (1992). p140trk mRNA marks NGF-responsive forebrain neurons: evidence that *trk* gene expression is induced by NGF. *Neuron* **9**, 465-478.

Horigome, K., Pryor, J. C., Bullock, E. D., and Johnson, E. M. J. (1993). Mediator release from mast cells by nerve growth factor. Neurotrophin specificity and receptor mediation. *J.Biol.Chem.* **268**, 14881-14887.

Huber, L. J. and Chao, M. V. (1995). A potential interaction of p75NTR and trkA NGF receptors revealed by affinity cross-linking and immunoprecipitation. *J.Neurosci.Res.* **40**, 557-563.

Hyman, C., Hofer, M., Barde, Y. A., Juhasz, M., Yancopoulos, G. D., Squinto, S. P., and Lindsay, R. M. (1991). BDNF is a neurotrophic factor for dopaminergic neurons of the substantia nigra. *Nature* **350**, 230-232.

Ibanez, C. F., Ernfors, P., Timmusk, T., Ip, N. Y., Arenas, E., Yancopoulos, G. D., and Persson, H. (1993). Neurotrophin-4 is a target-derived neurotrophic factor for neurons of the trigeminal ganglion. *Development* **117**, 1345-1353.

Ip, N. Y., Ibanez, C. F., Nye, S. H., McClain, J., Jones, P. F., Gies, D. R., Belluscio, L., Le Beau, M. M., Expinosa, R., Squinto, S. P., Persson, H., and Yancopoulos, G. D. (1992). Mammalian neurotrophin-4: Structure, chromosomal location, tissue distribution , and receptor specificity. *Proc.Natl.Acad.Sci.USA* **89**, 3060-3064.

Ip, N. Y., Stitt, T. N., Tapley, P., Klein, R., Glass, D. J., Fandl, J., Greene, L. A., Barbacid, M., and Yancopoulos, G. D. (1993). Similarities and Differences in the Way Neurotrophins Interact with the Trk Receptors in Neuronal and Nonneuronal Cells. *Neuron* **10**, 137-149.

Isackson, P. J., Huntsman, M. M., Murray, K. D., and Gall, C. M. (1991). BDNF mRNA expression is increased in adult rat forebrain after limbic seizures: Temporal patterns of induction distinct from NGF. *Neuron* **6**, 937-948.

Itoh, N., Yonehara, S., Ishii, A., Yonehara, M., Mizushima, S., Sameshima, M., Hase, A., Seto, Y., and Nagata, S. (1991). The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell* **66**, 223-243.

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

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

Johnson, E. M. Jr., Gorin, P. D., Brandeis, L. D., and Pearson, J. (1980). Dorsal root ganglion neurons are destroyed by exposure in utero to maternal antibody to nerve growth factor. *Science* **210**, 916-918.

Jones, K. R., Farinas, I., Backus, C., and Reichardt, L. F. (1994). Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development. *Cell* **76**, 989-999.

Jones, K. R. and Reichardt, L. F. (1990). Molecular cloning of a human gene that is a member of the nerve growth factor family. *Proc.Natl.Acad.Sci.USA* **87**, 8060-8064.

Kanety, H., Feinstein, R., Papa, M. Z., Hemi, R., and Karasik, A. (1995). Tumor necrosis factor alpha-induced phosphorylation of insulin receptor substrate-1 (IRS-1). Possible mechanism for suppression of insulin-stimulated tyrosine phosphorylation of IRS-1. *J.Biol.Chem.* **270**, 23780-23784.

Kaplan, D. R., Hempstead, B. L., Martin-Zanca, D., Chao, M. V., and Parada, L. F. (1991b). The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. *Science* **252**, 554-558.

Kaplan, D. R., Martin-Zanca, D., and Parada, L. F. (1991a). Tyrosine phosphorylation and tyrosine kinase activity of the trk proto-oncogene product induced by NGF. *Nature* **350**, 158-160.

Kaplan, D. R. and Miller, F. D. (1997). Signal transduction by the neurotrophin receptors. *Current Opinion in Cell Biology* **9**, 213-221.

Kaplan, D. R. and Stephens, R. M. (1994). Neurotrophin signal transduction by the Trk receptor. *Journal of Neurobiology* **25**, 1404-1417.

Kim, M. Y., Linardic, C., Obeid, L., and Hannun, Y. (1991). Identification of sphingomyelin turnover as an effector mechanism for the action of tumor necrosis factor alpha and gamma-interferon. *J.Biol.Chem.* **266**, 484-489.

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

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

Klein, R., Lamballe, F., Bryant, S., and Barbacid, M. (1992). The trkB tyrosine protein kinase is a receptor for neurotrophin-4. *Neuron* **8**, 947-956.

Klein, R., Martin-Zanca, D., Barbacid, M., and Parada, L. F. (1990b). Expression of the tyrosine kinase receptor gene *trk*B is confined to the murine embryonic and adult nervous system. *Development* **109**, 845-850.

Klein, R., Nanduri, V., Jing, S. Q., Lamballe, F., Tapley, P., Bryant, S., Cordon-Cardo, C., Jones, K. R., Reichardt, L. F., and Barbacid, M. (1991b). The trkB tyrosine protein kinase is a receptor for brain-derived neurotrophic factor and neurotrophin-3. *Cell* **66**, 395-403.

Klein, R., Parada, L. F., Coulier, F., and Barbacid, M. (1989). trkB, a novel tyrosine protein kinase receptor expressed during mouse neural development. *EMBO J.* **8**, 3701-3709.

Klein, R., Smeyne, R. J., Wurst, W., Long, L. D., Auerbach, B. A., Joyner, A. L., and Barbacid, M. (1993). Targeted disruption of the trkB neurotrophin receptor gene results in nervous system lesions and neonatal death. *Cell* **75**, 113-122.

Korshing, S., Auberger, G., Scott, J., and Thoenen, H. (1985). Levels of nerve growth factor and its mRNA in the central nervous system of the rat correlate with cholinergic innervation. *EMBO J.* **4**, 1389-1393.

Korshing, S. and Thoenen, H. (1983). Nerve growth factor in sympathetic ganglia and corresponding target organs of the rat: Correlation with density of sympathetic innervation. *Proc.Natl.Acad.Sci.USA* **80**, 3513-3516.

Kucera, J., Fan, G., Jaenisch, R., Linnarrsson, S., and Ernfors, P. (1995). Dependence of developing 1a afferents on neurotrophin-3. *Journal of Comparative Neurology* **363**, 307-320.

Ladiwala, U., Lachance, C., Simoneau, S. J. J., Bhakar, A., Barker, P. A., and Antel, J. P. (1998). p75 neurotrophin receptor expression on adult human Oligodendrocytes: signaling without cell death in response to NGF. *Journal of Neuroscience* **18**, 1297-1304.

Lai, K. O., Fu, W. Y., Ip, F. C., and Ip, N. Y. (1998). Cloning and expression of a novel neurotrophin, NT-7, from carp. *Molecular and Cellular Neurosciences* **11**, 64-76.

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

Lamballe, F., Smeyne, R. J., and Barbacid, M. (1994). Developmental expression of *trk*C, the neurotrophin-3 receptor, in the mammalian nervous system. *Journal of Neuroscience* **14**, 14-28.

Lamballe, F., Tapley, P., and Barbacid, M. (1993). trkC encodes multiple neurotrophin-3 receptors with distinct biological properties and substrate specificities. *EMBO J.* **12**, 3083-3094.

Large, T. H., Weskamp, G., Helder, J. C., Radeke, M. J., Misko, T. P., and Shooter, E. M. (1989). Structure and developmental expression of the nerve growth factor receptor in the chicken central nervous system. *Neuron* **2**, 1123-1134.

Larrick, J. W. and Wright, S. C. (1990). Cytotoxic mechanism of tumor necrosis factor-α. *FASEB J.* **4**, 3215-3223.

Lee, K. F., Bachman, K., Landis, S. C., and Jaenisch, R. (1994b). Dependence on p75 for Innervation of Some Sympathetic Targets. *Science* **263**, 1447-1449.

Lee, K. F., Davies, A. M., and Jaenisch, R. (1994a). p75-deficient embryonic dorsal root sensory and neonatal sympathetic neurons display a decreased sensitivity to NGF. *Development* **120**, 1027-1033.

Lee, K. F., Li, E., Huber, L. J., Landis, S. C., Sharpe, A. H., Chao, M. V., and Jaenisch, R. (1992). Targeted Mutation of the Gene Encoding the Low Affinity NGF Receptor p75 Leads to Deficits in the Peripheral Sensory Nervous System. *Cell* **69**, 737-749.

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

Levi-Montalcini, R. (1987). The nerve growth factor: thirty-five years later. *EMBO J.* **6**, 1145-1154.

Levi-Montalcini, R. and Hamburger, V. (1953). A diffusible agent of mouse sarcoma producing hyperplasia of sympathetic ganglia and hyperneurotization of viscerain the chick embryo. *J.Exp.Zool.* **123**, 233-288.

Liu, X., Ernfors, P., Wu, H., and Jaenisch, R. (1995). Sensory but not motor neuron deficits in mice lacking NT-4 and BDNF. *Nature* **375**, 238-241.

Liu, Z., Hsu, H., Goeddel, D. V., and Karin, M. (1996). Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-kB activation prevents cell death. *Cell* **87**, 565-576.

MacPhee, I. J. and Barker, P. A. (1997). Brain-derived Neurotrophic Factor Binding to the p75 Neurotrophin Receptor Reduces TrkA Signaling While Increasing Serine Phosphorylation in the TrkA Intracellular Domain. *J.Biol.Chem.* **272**, 23547-23551.

Mahadeo, D., Kaplan, L., Chao, M. V., and Hempstead, B. L. (1994). High affinity nerve growth factor binding displays a faster rate of association than p140trk binding. *J.Biol.Chem.* **269**, 6884-6891.

Maisonpierre, P. C., Belliscio, L., Squinto, S., Ip, N. Y., Furth, M. E., Lindsay, R. M., and Yancopoulos, G. D. (1990). Neurotophin-3: A neurotrophic factor related to NGF and BDNF. *Science* **247**, 1446-1451.

Majdan, M., Lachance, C., Gloster, A., Aloyz, R., Zeindler, C., Bamji, S., Bhakar, A., Belliveau, D., Fawcett, J., Miller, F. D., and Barker, P. A. (1997). Transgenic Mice Expressing the Intracellular Domain of the p75 Neurotrophin Receptor Undergo Neuronal Apoptosis. *Journal of Neuroscience* **17**, 6988-6998.

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

Martin-Zanca, D., Hughes, S. H., and Barbacid, M. (1986). A human oncogene formed by the fusion of truncated tropomyosin and protein tyrosine kinase sequences. *Nature* **319**, 743-748.

Martin-Zanca, D., Oskam, R., Mitra, G., Copeland, T., and Barbacid, M. (1989). Molecular and biochemical characterization of the human trk proto-oncogene. *Mol.Cell.Biol.* **9**, 24-33.

McDonald, N. Q., Lapatto, R., Murray-Rust, J., Gunning, J., Wlodawer, A., and Blundell, T. L. (1991). New protein fold revealed by a 2.3-A resolution crystal structure of nerve growth factor. *Nature* **354**, 411-414.

McKean, D. J., Bell, M., Hunton, S., Rastogi, M., Van Norstrand, R., Podzorski, A., Nilson, A., and Paya, C. (1995). IL-1 receptor and TCR signals synergize to activate NF-kappa B-mediated gene transcription. *International Immunology* 7, 9-20.

Meakin, S. O., Suter, U., Drinkwater, C. C., Welcher, A. A., and Shooter, E. M. (1992). The rat trk proto-oncogene product exhibits properties characteristic of the slow nerve growth factor receptor. *Proc.Natl.Acad.Sci.USA* **89**, 2374-2378.

Meier, R., Becker-Andre, M., Gotz, R., Heumann, R., Shaw, A., and Thoenen, H. (1986). Molecular cloning of bovine and chick nerve growth factor (NGF): Delineation of conserved and unconserved domain and their relationship to the biological activity and antigenicity of NGF. *EMBO J.* **5**, 1489-1493.

Merlio, J. P., Ernfors, P., Jaber, M., and Persson, H. (1992). Molecular cloning of rat *trkC* and distribution of cells expressing messenger RNAs for members of the *trk* family in the rat central nervous system. *Neuroscience* **51**, 513-532.

Middlemas, D. S., Lindberg, R. A., and Hunter, T. (1991). trkB, a neural receptor protein-tyrosine kinase: evidence for a full-length and two truncated receptors. *Mol.Cell.Biol.* **11**, 143-153.

Minichiello, L., Piehl, F., Vazques, E., Schimmang, T., Hokfelt, T., Repressa, J., and Klein, R. (1995). Differential effects of combined trk receptor mutations on dorsal root ganglion and inner ear sensory neurons. *Development* **121**, 4067-4075.

Mufson, E. J., Bothwell, M., and Kordower, J. H. (1989). Loss of nerve growth factor-containing neurons in Alzheimers disease: A quantitative analysis across subregions of the basal forebrain. *Experimental Neurology* **105**, 221-232.

Nakamura, T., Sanokawa, R., Sasaki, Y., Ayusawa, D., Oishi, M., and Mori, N. (1996). N-shc: a neuronal-specific adapter molecule that mediates signaling from neurotrophin/trk to ras/MAPK pathway. *Oncogene* **13**, 1111-1121.

Nilsson, A. S., Fainzilber, M., Falck, P., and Ibanez, C. F. (1998). Neurotrophin-7: a novel member of the neurotrophin family from the zebrafish. *FEBS Letters* **424** , 285-290.

Northwood, I. C. and Davis, R. J. (1990). Signal transduction by the epidermal growth factor receptor after functional desensitization of the receptor tyrosine protein kinase activity. *Proc.Natl.Acad.Sci.USA* **87**, 6107-6111.

Obermeier, A., Bradshaw, R. A., Seedorf, V., Choidos, A., Schlessinger, J., and Ulrich, A. (1994). Neuronal differentiation signals are controlled by nerve growth factor receptor/Trk binding sites for SHC and PLC-γ. *EMBO J.* **13**, 1585-1590.

Ochmichi, M., Decker, S., and Saltiel, A. R. (1991). Nerve growth factor induces the association of a 130 Kd phosphoprotein with its receptor in PC12 pheochromocytoma cells. *Cell Reg.* **2**, 691-697.

Oishi, I., Sugiyama, S., Liu, Z. J., Yamamura, H., Nishida, Y., and Minami, Y. (1997). A novel Drosophila receptor tyrosine kinase expressed specifically in the nervous system. Unique structural features and implication in developmental signaling. *J.Biol.Chem.* **272**, 11916-11923.

Olson, J. E. and Pledger, W. J. (1990). Transmodulation of epidermal growth factor binding by platelet-derived growth factor and 12-O-tetradecanoylphorbol-13-acetate is not sodium-dependent in Balb/c/3t3 cells. *J.Biol.Chem.* **265**, 1847-1851.

Oppenheim, R. W. (1991). Cell death during development of the nervous system. *Annual Review in Neurosciences* **14**, 453-501.

Pearson, J., Johnson, E. M., and Brandeis, L. D. (1983). Effects of antibodies to nerve growth factor on intrauterine development of derivatives of cranial neural crest and placode in the guinea pig. *Developmental biology* **96**, 32-36.

Peng, X., Greene, L. A., Kaplan, D. R., and Stephens, R. M. (1995). Deletion of a conserved juxtamembrane sequence in Trk abolishes NGF-promoted neuritogenesis. *Neuron* **15**, 395-406.

Phillips, H. S., Hains, J. M., Armanini, M., Laramee, G. R., Johnson, S. A., and Winslow, J. W. (1991). BDNF mRNA is decreased in the hippocampus of individuals with Alzheimers disease. *Neuron* 7, 695-702.

Purves, D. (1988). 'A trophic theory of neural connections.' (Harvard University Press: Cambridge.)

Pushkareva, M. Y. and Hannun, Y. A. (1994). Modulation of cytosolic protein phosphorylation by sphingosylphosphorylcholine. *Biochimica et Biophysica acta* **1221**, 54-60.

Rabizadeh, S., Oh, J., Zhong, L., Yang, J., Bitler, C. M., Butcher, L. L., and Bredesen, D. E. (1993). Induction of Apoptosis by the Low-Affinity NGF Receptor. *Science* **261**, 345-348.

Radeke, M. J., Misko, T. P., Hsu, C., Herzenberg, L. A., and Shooter, E. M. (1987). Gene transfer and molecular cloning of the rat nerve growth factor receptor. *Nature* **325**, 593-597.

Rodriguez-Tebar, A., Dechant, G., and Barde, Y. A. (1990). Binding of brain-derived neurotrophic factor to the nerve growth factor receptor. *Neuron* **4**, 487-492.

Rodriguez-Tebar, A., Dechant, G., Gotz, R., and Barde, Y. A. (1992). Binding of neurotrophin-3 to its neuronal receptors and interactions with nerve growth factor and brain-derived neurotrophic factor. *EMBO J.* **11**, 917-922.

Rosenthal, A., Goeddel, D., Nguyen, T., Lewis, M., Shih, A., Laramee, G. R., Nikolics, K., and Winslow, J. W. (1990). Primary structure and biological activity of a novel human neurotrophic factor. *Neuron* **4**, 767-773.

Rosenthal, A., Goeddel, D., Nguyen, T., Martin, E., Burton, L. E., Shih, A., Laramee, G. R., Wurm, F., Mason, A., Nikolics, K., and Winslow, J. W. (1991). Primary structure and biological activity of human brain-derived neurotrophic factor. *Endocrinology* **129**, 1289-1294.

Ross, A. H., Daou, M. C., McKinnon, C. A., Condon, P. J., Lachyankar, M. B., Stephens, R. M., Kaplan, D. R., and Wolf, D. E. (1996). The Neurotrophin Receptor, gp75, Forms a Comple with the Receptor Tyrosine Kinase TrkA. *J.Cell Biology* **132**, 945-953.

Schneider, R. and Schweiger, M. (1991) A novel modular mosaic of cell adhesion motifs in the extracellular domains of the neurogenic *trk* and *trkB* tyrosine kinase receptors. *Oncogene* **6**, 1807-1811.

Scott, J., Selby, M., Urdea, M., Quiroga, M., Bell, G. I., and Rutter, W. J. (1983). Isolation and nucleotide sequence of a cDNA encoding the precursor of mouse nerve growth factor. *Nature* **302**, 538-540.

Shelton, D. L. and Reichardt, L. F. (1984). Expression of beta-nerve growth factor gene correlates with the density of sympathetic innervation in effector organs. *Proc.Natl.Acad.Sci.USA* **81**, 7951-7955.

Shelton, D. L. and Reichardt, L. F. (1986). Studies on the expression of the nerve growth factor (NGF) gene in the central nervous system: Level and regional distribution of NGF mRNA suggest that NGF functions as a trophic factor for several distinct populations of neurons. *Proc.Natl.Acad.Sci.USA* **83**, 2714-2718.

Sherrington, C. S. (1905). 'The integrative action of the nervous system.' (Cambride University Press: Cambride.)

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

Smith, C. A., Farrah, T., and Goodwin, R. G. (1994). The TNF receptor superfamily of cellular and viral proteins: Activation, co-stimulation, and death. *Cell* **76**, 959-962.

Soppet, D., Escandon, E., Maragos, J., Middlemas, D. S., Reid, S. W., Blair, J., Burton, L. E., Stanton, B. R., Kaplan, D. R., Hunter, T., Nikolics, K., and Parada, L. F. (1991). The neurotrophic factors brain-derived neurotrophic factor and neurotrophin-3 are ligands for the trkB tyrosine kinase receptor. *Cell* **65**, 895-903.

Spina, M. B., Squinto, S., Miller, J., Lindsay, R. M., and Hyman, C. (1992). Brain-derived neurotrophic factor protects dopamine neurons against 6-hydroxydopamine and N-methyl-phenylpyridinium ion toxicity; Involvement of the glutathione system. *Journal of Neurochemistry* **59**, 99-106.

Squinto, S. P., Stitt, T. N., Aldrich, T. H., Davis, S., Bianco, S. M., Radziejewski, C., Glass, D. J., Masiakowski, P., Furth, M. E., Valenzuela, D. M., Distefano, P. S., and Yancopoulos, G. D. (1991). trkB encodes a functional receptor for brain-derived neurotrophic factor and neurotrophin-3 but not nerve growth factor. *Cell* **65**, 885-893.

Steininger, T. L., Wainer, B. H., Klein, R., Barbacid, M., and Palfrey, H. C. (1993). High-affinity nerve growth factor receptor (Trk) immunoreactivity is localized in cholinergic neurons of the basal forebrain and striatum in the adult rat brain. *Brain Research* **612**, 330-335.

Stephens, R. M., Loeb, D. M., Copeland, T. D., Pawson, T., Greene, L. A., and Kaplan, D. R. (1994). Trk receptors use redundant signal transduction pathways involving SHC and PLC-γ1 to mediate NGF responses. *Neuron* **12**, 691-705.

Strada, O., Hirsch, E. C., Javoy-Agid, F., Lehericy, S., Ruberg, M., Hauw, J. J., and Agid, Y. (1992). Does losss of nerve growth factor receptors precede loss of cholinergic neurons in Alzheimers disease? An autoradiographic study in the human striatum and basal forebrain. *Journal of Neuroscience* **12**, 4766-4774.

Sutter, A. (1979). Nerve growth factor receptors. Characterization of two distinct classes of binding sites on chick embryo sensory ganglia. *J.Biol.Chem.* **254**, 5972-5982.

Tartaglia, L. A., Pennica, C., and Goeddel, D. V. (1993). Ligand passing: the 75-kDa tumor necrosis factor (TNF) receptor recruits TNF for signaling by the 55-kDa TNF receptor. *J.Biol.Chem.* **268**, 18542-18548.

Tessarollo, L., Tsoulfas, P., Martin-Zanca, D., Gilbert, D. J., Jenkins, N. A., Copeland, N. G., and Parada, L. F. (1993). trkC, a receptor for neurotrophin-3, is widely expressed in the developing nervous system and in non-neuronal tissues. *Development* **118**, 463-475.

Tessarollo, L., Vogel, K. S., Palko, M. E., Reid, S. W., and Parada, L. F. (1994). Targeted mutation in the neurotrophin-3 gene results in loss of muscle sensory neurons. *Proc.Natl.Acad.Sci.USA* **19**, 11844-11848.

Theroux, S. J., Latour, D. A., Stanley, K., Raden, D. L., and Davis, R. J. (1992). Signal transduction by the epidermal growth factor receptor is attenuated by a COOH-terminal domain serine phosphorylation site. *J.Biol.Chem.* **267**, 16620-16626.

Thoenen, H. (1991). The changing scene of neurotrophic factors. *Trends Neurosci.* **14**, 165-170.

Thoenen, H. and Barde, Y. A. (1980). Physiology of nerve growth factor. *Physiological Reviews* **60**, 1284-1335.

Timmusk, T., Belluardo, N., Metsis, M., and Persson, H. (1993). Widespread and developmentally regulated expression of neurotrophin-4 mRNA in rat brain and peripheral tissues. *European Journal of Neurosciences* **5**, 605-613.

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

Ulrich, A., Gray, A., Berman, C., and Dull, T. (1983). Human beta-nerve growth factor gene sequence highly homologous to that of mouse. *Nature* **303**, 821-825.

Valenzuela, D. M., Maisonpierre, P. C., Glass, D. J., Rojas, E., Nunez, L., Kong, Y., Gies, D. R., Stitt, T. N., Ip, N. Y., and Yancopoulos, G. D. (1993). Alternative forms of rat TrkC with different functional capabilities. *Neuron* **10**, 963-974.

Van Antwerp, D. J., Martin, S. J., Kafri, T., Green, D. R., and Verma, I. M. (1996). Suppression of TNF-α-induced apoptosis by NF-kB. *Science* **274**, 787-789.

Van der Zee, C. E. E. M., Ross, G. M., Riopelle, R. J., and Hagg, T. (1996). Survival of Cholinergic Forebrain Neurons in Developing $p75^{NTR}$-Deficient Mice. *Science* **274**, 1729-1732.

Vazquez, M. E. and Ebendal, T. (1991). Messenger RNAs for trk and the low-affinity NGF receptor in rat basal forebrain. *Neuroreport* **2**, 593-596.

Venero, J. L. and Hefti, F. (1993). TrkA NGF receptor expression by non-cholinergic thalamic neurons. *Neuroreport* **4**, 959-962.

Verdi, J. M., Birren, S. J., Ibanez, C. F., Persson, H., Kaplan, D. R., Benedetti, M., Chao, M. V., and Anderson, D. J. (1994). $p75^{NTR}$ Regulates Trk Signal Transduction and NGF-Induced Neuronal Differentiation in MAH Cells. *Neuron* **12**, 733-745.

Volonte, C., Angelastro, J. M., and Greene, L. A. (1993). Association of protein kinases ERK1 and ERK2 with p75 nerve growth factor receptors. *J.Biol.Chem.* **268**, 21410-21415.

Welcher, A. A., Bitler, C. M., Radeke, M. J., and Shooter, E. M. (1991). Nerve growth factor binding domain of the nerve growth factor receptor. *Proc.Natl.Acad.Sci.USA* **88**, 159-163.

Wolf, D. E., McKinnon, C. A., Daou, M. C., Stephens, R. M., Kaplan, D. R., and Ross, A. H. (1995). Interaction with TrkA Immobilizes gp75 in the High Affinity Nerve Growth Factor Receptor Complex. *J.Biol.Chem.* **270**, 2133-2138.

Wyatt, S. and Davies, A. M. (1993). Regulation of expression of mRNAs encoding the nerve growth factor receptors p75 and TrkA in developing sensory neurons. *Development* **119**, 635-647

Wyatt, S. and Davies, A. M. (1995). Regulation of nerve growth factor gene expression in sympathetic neurons during development. *J.Cell Biology* **130**, 1435-1446.

Yan, H. and Chao, M. V. (1991). Disruption of cysteine-rich repeats of the p75 nerve growth factor receptor leads to loss of ligand binding. *J.Biol.Chem.* **266**, 12099-12104.

Yan, H., Schlessinger, J., and Chao, M. V. (1991). Chimeric NGF-EGF receptors define domains responsible for neuronal differentiation. *Science* **252**, 561-563.

Yeo, T. T., Chua-Couzens, J., Butcher, L. L., Bredesen, D. E., Cooper, J. D., Valleta, J. S., Mobley, W. C., and Longo, F. M. (1996). Absence of $p75^{NTR}$ causes increased basal forebrain cholinergic neuron size, choline acetyltransferase activity, and target innervation. *Journal of Neuroscience* **17**, 7594-7605.